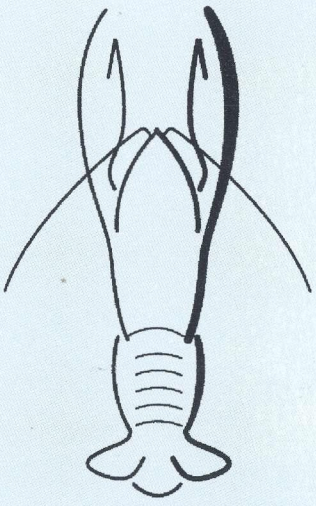
ADVANCES IN CHITIN SCIENCE

Volume IV



EUCHIS´ 99

Edited by
Martin G. Peter
Alain Domard
Riccardo A.A. Muzzarelli

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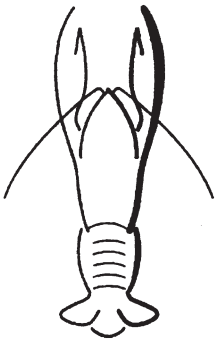

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Edited by M. G. Peter, A. Domard, R. A. A. Muzzarelli

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EUCHIS´99

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Preface

International Conferences of the European Chitin Society, EUCHIS, are held every two years. Following the Inauguration of EUCHIS in Lyon, March 1992, the first meeting took place in Brest, France, in 1995, and the second was organized together with the 7th International Conference on Chitin and Chitosan in 1997 in Lyon, France. Both meetings were chaired by Professor Dr. Alain Domard, who was at that time President of the European Chitin Society. During the last Conference in Lyon, the Board of EUCHIS has chosen the University of Potsdam, Germany, as the place for the third International EUCHIS Meeting which took place from August 31 to September 3, 1999.

The University of Potsdam was a good choice, as it has an outstanding reputation in Polymer Science, interacting closely in co-operations with *inter alia* the *Max-Planck*-Institute for Colloid and Interface Research, and the *Fraunhofer*-Institute for Applied Polymer Research. Investigation of Biopolymers, including chitin and chitosan, is pursued in a number of groups, and the foundation of the Interdisciplinary Research Center for Biopolymers (IFZB) at the University of Potsdam in 1994 was an important event in our scientific history. The most recent highlight in the activities of the Potsdam Polymer Scientists is seen in the establishement of the "Interdisciplinary Master's Course in Polymer Science, Berlin-Brandenburg" to which also the three Universities of our neighbouring Capital city Berlin contribute essential expertise.

EUCHIS'99 was a successful meeting. Despite the fact that the number and frequency of Conferences on chitin and chitosan is increasing worldwide, more than 240 participants, 45 of them coming from industry, representing 34 countries attended the meeting. This book contains the Proceedings of EUCHIS'99 with 102 of the 124 scientific papers presented at the Conference, documenting the rapid progress and the wide interest in chitin and chitosan. A special workshop on "Oral Administration of Chitosan" was organized by Prof. Muzzarelli, and another book entitled "Chitosan *per* os" emerged from this session. Nevertheless, the editors decided to include shorter versions of the papers presented at the workshop in the Proceedings of EUCHIS'99.

The editors are grateful to all authors for observing the guidelines with respect to the length and format of the manuscripts. This faciltitated the editing work enormously though is was not possible in all cases to adhere to an uniform style. In particular, I am grateful to Dr. Dirk Schanzenbach and to Marlis Patzelt for their help in preparing transcripts of some of the more difficult manuscripts. We hope that no errors were introduced during retyping of texts and arrangements of figures and that all authors are satisfied with the presentation of their papers in this book.

Last not least, I would like to express my thanks again to those supporting EUCHIS'99. Wella AG provided financial support and Henkel KGaA donated conference materials. I gratefully acknowledge the generous support from the University of Potsdam who provided rooms and facilities for all sessions and social events taking place on campus, and I thank Karin Baumann for invaluable assistance in publishing this book.

Potsdam, April 2000 Martin G. Peter

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Krill as a promising raw material for the production of chitin in Europe

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Introduction

The natural abundance of chitin makes it possible to produce it out of shrimp, crab, krill, fungi [1,2], silk reelings [3], freshwater crayfish [4] and other naturally occurring matters. The yield of chitin in the process does not exceed, as a rule, 2% of the original mass of the raw material. Due to the fact only those objects of raw material have primary importance the resources of which on the one hand are assessed in hundreds of thousand tonnes and on the other hand are not spread at random but are accumulated in one place making it possible to harvest them industrially. Krill represents a very good example of industrially caught natural resources.

Discussion

The resources of krill in the Antarctic Regions according to some estimations make 50 mln tonnes and yield of chitin after processing makes appr. 1%. Allowable catch of krill for Antarctic Regions is assessed in 15-16 mln tonnes annually. It is possible to catch up to 2.0 - 3.5 mln tonnes annually in the recommended regions where krill show a strong build-up. At present, the actual world catch of krill makes appr. 100 thousand tonnes. As for the European countries only Poland and Ukraine use to catch krill taking 21000 and 13000 tonnes respectively. Such situation is conditioned by two factors:

- firstly, the environmental conditions of the region, great distance between fishing grounds and ports of refuge require the use of large vessels only. Not every country has such vessels;
- secondly, it is necessary to use modern krill processing technologies which will make catching of this resource efficient.

Russia has all the necessary vessels and technologies. In the beginning of nineties the USSR used to catch more than 300 thousand tonnes annually. With the yield of chitosan being 0.7% of the total mass of krill the country was able to produce 2100 tonnes of chitosan annually.

Previously krill was intended, first of all, for the use in food purposes and as a component of fodder mixtures. In our opinion, today it is possible to consider it primarily as the basic raw material to produce chitin and, secondary as a source of food and fodder raw material. In the latter case it is possible to obtain not only chitin but a number of by-products such as: protein isolate used to produce food products imitating fillets of delicacy fish and food fibres imitating meat and salmon caviar; krill meat; mince, fodder for animals, poultry, farmed fish; enzymes, carotinoids. Methods for choosing optimal variants of catching and processing krill have been already developed [5].

Table 1. Krill catches (according to STATLANT data), tonnes

Country	1988	1989	1990	1991	1992	1993	1994	1995	1996
Chile	5938	5329	4500	3679	6065	3261	3634		
Germany			396						
Japan	73112	78928	62187	67582	74325	59272	62322	60303	60546
Latvia							71		
Republic of Korea	1525	1779	4039	1210	519				
Panama								141	495
Poland	5215	6997	1275	9571	8607	15909	7915	9384	20610
USSR	284873	301498	302376	275495					
Russia					157725	4249	965		
South Africa							2		
Ukraine					61719	6083	8852	48884	13388
Total	370663	394531	374773	357537	302960	88774	83961	118712	95039

Russian industry developed two alternatives for the complex use of krill. The first one which make the main emphasis on the processing of krill at sea is shown in the Table 2.

Table 2. Complex use of krill - first alternative

Type of product		SEA	SHORE
Food products	Krill paste	Frozen	Cookery
	Blanched meat	Canned Frozen	Canned
	Boiled meat	Frozen	Cookery
	Minced meat	Frozen, Canned	Cookery
Feed and technical products	Feed flour	In bulk Granulated	As a fodder for poultry, cattle, animal and fish farming
	Whole raw krill	Frozen	
	Crustaceous wastes	Frozen, dry, chemically preserved	Chitin, chitosan, feed protein
	Enzymatic concentrate	Frozen	Enzymatic preparations
	Waste fraction containing heads	Frozen	Caratinoid preparations

The second alternative is basing upon the principle of processing krill mostly at shore-based factories. Pre-treatment of raw material is carried out onboard fishing vessels where it is usually frozen but the main production process takes place at shore. The details are given in Table 3 .

Table 3 . Complex use of krill - second alternative

	SEA		SHORE	
Pressed frozen krill	Semi-finished product for further complex processing into food, fodder and technical products	I	Protein isolate Crustaceous wastes	Analogues of traditional products Chitin, Chitosan Feed protein
Whole frozen raw krill	Semi-finished product for further complex processing into food, fodder and technical products	II	Feed protein Crustaceous wastes	Feed paste Feed flower Chitin, chitosan
		III	Food hydrolysate Crustaceous wastes	Sauces Chitin, chitosan Food protein

Taking into account the existing situation when practically all European countries are interested in using chitin and its derivatives, but many of them have no raw material for its industrial production, the Giprorybflot (State Research and Design Institute for Fishing Fleet) would like to offer to the parties interested to start the development of international project on supplying European countries with chitin or raw material for its production.

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CHITEX: Containerized plant for producing chitin

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Summary
The GIPRORYBFLOT Institute (Russia) has developed a technology for producing chitin by an electrochemical method. Some advantages of this method in comparison with the traditional ones - enzymatic and alkali-acid have been revealed. On the basis of this technology, normative documentation, initial requirements for chitinous raw material processing a specialised containerised plant named CHITEX has been developed .

Capacity of the containerised plant, its technical-and-economical characteristics have been assessed. Critical matters connected with utilisation of waste gases and water have been settled as well. Small size of the plant, possibility of its easy transportation inside a container by means of automobile vehicles makes it possible to produce chitin in any region and especially on the spots where shellfish processing is taking place. The technology and equipment for electrochemical production of chitin are protected by the Russian Federation patents.

Materials and methods
Fresh water shrimp *Gammarus* (fresh, frozen and dried) , krill and shrimp shell were used as raw material for producing chitin. Chitin is the basic product obtained after the treatment of crustaceous raw material; its by-products are represented by protein hydrolysate, lipids, bactericidal anolytic solution.

Crustateous raw material was electrochemically treated in tandem by means of its processing inside the cathode chamber (deproteinisation) and anode chamber (demineralisation) of specially designed electrolysers [1].

Results and discussion
The process of producing chitin by electrochemical method involves the following technological operations : raw material preparation - cutting - mixing with electrolytic solution - treatment of mixture inside the electrolyser's cathode chamber, heat and thermostatic treatment inside the reactor (deproteinisation) - separation of the mixture - rinsing of deproteinised shell with water - mixing with electrolytic solution - treatment inside the electrolyser anode chamber and settling inside the accumulating tank (demineralisation) - extraction of chitin from analytic solution - rinsing with water - drying and packing of chitin.

The specialised containerised modular type plant of unique design has been developed to carry out all these processes [2,3].

A plant module consists of two membrane electrolysers, a power supply and a control panel, blenders, reaction vessels with actuators, pumps, filters, tanks for electrolytic solution, raw material and final products.

The membrane electrolysers have a form of flat-parallel constructions with cathode and anode made of specially chosen for this purpose materials. The separating membrane is made of ionoselective, chemically and heat resistant polymer which is sanitary treatable. The design of the electrolysers makes it very easy to replace electrodes and membranes and to change distance between electrodes by increasing and decreasing cathode and anode space.

Operation of the plant is organised in such way that deproteinisation in the cathode chamber and denitrogination in the anode chamber take place simultaneously in countercurrent flow mode.

Duration of separate operations, temperature and pH, electrolyte solution concentration and hydromodulus, current density, other electric and technological parameters can be varied within a wide range depending upon the type and state of raw material being processes and requirements towards quality of final products .

To attain these ends the electrolysers are equipped with all the necessary instrumentation, flowmeters, final control elements and actuators regulating supply of suspension into the electrolysers. All the control systems are terminated at the control board.

Capacity of the modular unit, its technical-and-economical characteristics have been defined. The problems connected with utilisation of waste gases and water have been settled with the use of a scrubber, plenum-exhaust ventilation and neutralisation of spent liquids by electrolytic solutions.

Overall dimensions of the plant modular :

- length	4 m (the unit is not solid)
- width	2.2 m
- height	2.5 m
Square occupied	20 m^2
Power consumption (220/380 V, 50 Hz)	20 kW per hour
Capacity	1.5 t of chitin per year

Electrochemical treatment of dispersed crustacea raw material inside the cathode and anode chambers of electrolysers combined with concurrent influence upon the raw material of active ions H^+ and OH^- (i.e. pH media), redox-potential, stream of charged particles and electric current itself make it possible to provide the maximum rate of protein and mineral substances extraction and to produce chitin with better functional capacities in more mild conditions. This electrochemically extracted chitin named "CHITINEL" complies with technical conditions and with the sanitary certificate issued for this type of products.

"CHITINEL" is intended to be used as a sorbent and bioactive substance in ecoreabilitating situations, agriculture, in food, cosmetics, pulp-and-paper and other industries, as well as a semi-finished item for producing chitosan.

Conclusions

Use of the plant in the service mode of operation showed stable functioning of all the units and equipment ensuring achievement of optimum parameters in accordance with the technological instructions , technological schedule and maintenance instructions.

Production of chitin by electrochemical method has the following advantages :

- maximum use of such valuable components of the processed raw material as protein, lipids, shell;
- less units of equipment are used and technological processes take less time than usual;
- better wear resistance properties of the technological equipment and less factor of ecological risk due to the fact that such aggressive media as acids and alkali are not used and pH in neutral effluents is equal to 7.0;
- consumption of water and other auxiliary materials is reduced;
- it is possible to change capacity of the plant very quickly by configuring it from the different number of production modules having regard to the availability of raw material and needs in final products;
- small size of the plant made it possible to manufacture its containerised version which is able to produce chitin in any region and especially on the spots where shellfish processing is taking place.

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Preparation and characterization of chitosan from Mucorales

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Summary
From a screening of 24 fungi belonging to the Mucorales the best chitosan producers were identified as *Absidia coerulea* and *Absidia spinosa* with a maximum chitosan yield of 1,3 g/l when grown in shake flasks. Addition of azole compounds, digitonin, chitosan oligomers or cobalt did not positively influence the yield of chitosan. An increase in chitosan yield was observed with additional ammonia. The fungal chitosans had a molecular weight of 700 kDa and a degree of deacetylation of 90 %. The viscosity of the fungal chitosans was lower than that of crab chitosan of comparable size. NMR-analysis of chitosan oligomers from enzymatically cleaved fungal chitosan showed a structure very similar to oligomers of crab chitosan. From a fermentation of *Absidia coerulea* 2,7 g/l chitosan were recovered. Using an enhanced chitosan isolation method clearly soluble chitosan free of colour and odour was obtained.

Introduction
The cationic biopolymer chitosan, industrially manufactured from crustacean shells rich in chitin, finds diverse applications in industry e.g. in the treatment of waste water, in agriculture, in the pharmaceutical industry and in cosmetics [1,2].

A critical process step towards chitosan is the deacetylation of chitin which utilizes hot concentrated caustic [3,4]. Alternatives to the chemical deacetylation could be the use of chitin deacetylases for an enzymatic process [5,6] or the utilization of chitosan producing microorganisms as raw material source.

It is known for over 30 years that fungi of the order Mucorales incorporate chitosan into their cell walls [7]. These fungi possess a chitin deacetylase, which acts in tandem with chitin synthetase on nascent polymer strains to produce chitosan [8,9]. White et al. [10] were the first to isolate chitosan from *Mucor rouxii*. Since then a variety of strains have been screened for chitosan productivity [11, 12] and methods for the fermentation of Mucorales and extraction of chitosan have been elaborated [12-18]. Recently chitosan was also isolated from solid-state fermentations of the fungi *Lentinus edodes* [19].

As chitosan is not excreted into the medium but a constituent of the fungal cell wall, methods to increase the chitosan yield are limited. Either the medium and culture conditions have to be optimized in a way that the growth of the fungi reaches a maximum and that optimum conditions for the chitin deacetylase are maintained or the cell wall of the fungi have to be manipulated during growth. Azole compounds are known to thicken fungal cell walls [20,21] having a positive effect on chitin and chitosan content [21]. Another possibility to enhance chitin deacetylase activity and chitosan production in vitro is the addition of

digitonin, which causes dissociation of chitosomes [22, 23]. Other methods reported are the addition of Co^{2+} or chitin [12, 23].

The intention of our study was to screen for the best chitosan producers among the Mucorales and to determine the chances of improving the chitosan yield of the best producers by modifications of the medium composition. We were also interested in physical parameters of the fungal chitosans including structure, degree of acetylation, viscosity and size of the polymer [2]. Other important criteria for the use of chitosan in high price applications are colour, odour and clear solubility of the product, so we took a closer look on the quality of the fungal chitosan.

Materials and Methods

Strains: The following 24 strains were used for screening: *Absidia coerulea* DSM 1143, *Absidia pseudocylindrospera* DSM 2254, *Absidia spinosa* DSM 3192, *Absidia glauca* DSM 63295, *Absidia repens* DSM 812, *Absidia coerulea* DSM 3018, *Absidia glauca* DSM 811, *Absidia californica* ATCC 24167, *Absidia corymbifera* ATCC 22742, *Absidia spinosa* ATCC 38188, *Rhizopus oryzae* ATCC 34104, *Rhizopus oryzae* ATCC 34102, *Rhizopus oryzae* ATCC 4858, *Rhizopus oryzae* ATCC 10404, *Rhizopus oryzae* ATCC 24563, *Rhizopus oryzae* ATCC 9374, *Rhizopus oryzae* ATCC 12883, *Rhizopus rhizopodiformis* D 0084, *Rhizopus rhizopodiformis* A 0001, *Rhizopus pusillus* ATCC 46883, *Rhizopus delemar* ATCC 34612, *Mucor miehei* NRRC 5283, *Mucor miehei* NRRC 5284, *Mucor javanicus* CMI 25330

Culture conditions and standard chitosan extraction protocol: The standard medium used for cultivation of the fungi consisted of 20 g/l glucose, 10 g/l peptone, 1 g/l yeast extract, 5 g/l $(NH_4)_2SO_4$, 1 g/l K_2HPO_4, 0,5 g/l $MgSO_4$ and 0,1 g/l $CaCl_2$ adjusted to pH 4.5. 200 ml cultures were inocculated with 10 ml of a fresh overnight culture and shaken at 26 °C and 150 rpm for 48 h or 72 h respectively. Fungi were recovered by filtration, washed, disrupted with a turrax and then autoclaved in 80 ml of a 2 % NaOH solution. The caustic solution was cooled under stirring, cell wall polymers were centrifuged and washed with water. Acid extraction of chitosan was performed twice, at 30 °C overnight and at 60 °C for additional 3 h with 40 ml of a 2 % HAc each time. The chitosan was precipitated with a pH-shift to 9.0, separated by centrifugation, washed with water and lyophilized.

Medium compositions: All changes to the medium as varying ammonium or phosphate content and varying pH are based on the standard medium as described above. Bifonazole, etridiazole and digitonin were added to the culture after 24 h. Chitosan oligomers and $CoCl_2$ were added prior to inocculation of the culture. 20 g/l glucosamine and N-acetylglucosamine were added to the medium in exchange for glucose.

Fermentation of *A.coerulea*: Fermentation was performed in a 10 l Braun Biostat E fermenter equipped with a rushton-turbine, pH-, temperature- and oxygen-control. 4 l of standard medium were inocculated with a fresh 200 ml culture and stirred at 26 °C and pH 5.0. After 24 h 1.0 g/l*h glucose and 0.125 g/l*h $(NH_4)_2SO_4$ was added continuosly. After 96 h the fermenter was heated to 80 °C for 0.5 h, cooled and the cells were recovered by filtration.

NMR-analysis of chitosan polymers and oligomers: NMR-analysis was carried out on a DPX 400 Bruker with a QNP probe head. ^1H-spectra were taken for the determination of the degree of deacetylation. Polymers were dissolved in diluted formic acid/D_2O and the degree of acetylation was determined from the proportion of sugar to acetate signals. For structure comparison between fungal and crab chitosan a higher resolution was obtained by using the respective chitosan-oligomers dissolved in DCl/D_2O. ^{13}C-spectra and 2-dimensional TOCSY (total correlation spectroscopy) spectra were taken for structure elucidation.

Oligomers of chitosan were synthesized by enzymatic cleavage with chitosanase (Seikagaku) for 48 h at 25 °C under stirring. The oligomers were separated by filtration over a 10 kDa cutoff membrane and dried.

HPLC-analysis: Relative molecular weight measurements were performed on a Waters HPLC with a Shimadzu RI-detector at 30 °C and a flow rate of 0.5 ml/min with a TSK-Gel aequous GPC-column set and 0.5 M formic acid/ 0.15 M $NaNO_3$ as eluent. PEG and PEO standards were used for calibration.

Viscosity-measurements: Viscosity of the polymer-solutions was determined with a RS 150 viscosimeter from Haake at 25 °C. Chitosan was dissolved either as 0.5 % solution in 0.5 % glycolic acid or as 1 % solution in 1 % HAc.

Results and Discussion

Screening for chitosan producers: Chitosan could be isolated in varying amounts from all of the 24 fungal strains in the screening approach. As observed before [11, 12] *Absidia* strains were the best chitosan producers with a chitosan content of up to 15 % of dry cell weight. All *Rhizopus* strains had chitosan yields under 100 mg/200 ml with a maximum of 9 % chitosan of dry cell weight, while the *Mucor* strains tested had chitosan contents below 2 %.

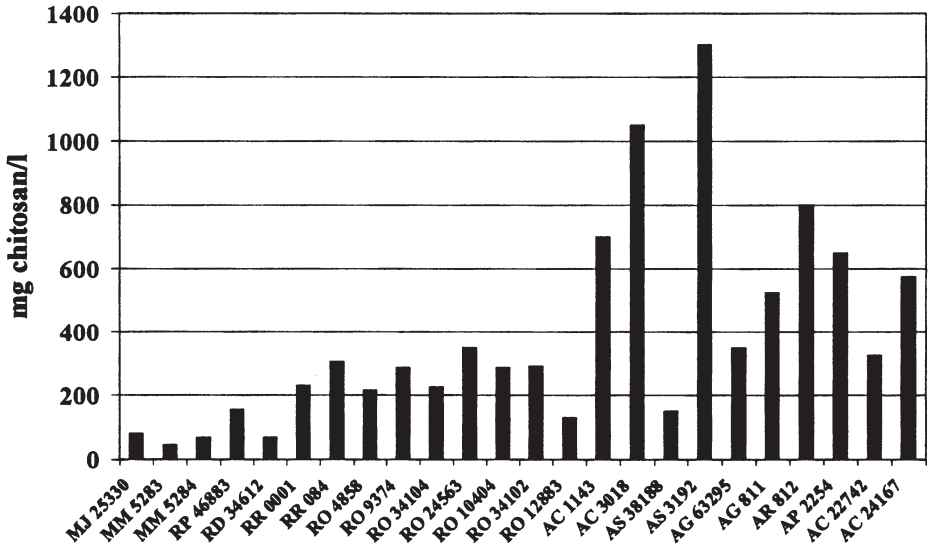

Figure 1: Chitosan productivity of 24 fungal strains of the order Mucorales. Values are in mg chitosan per liter of standard medium obtained from shake flask experiments.

Absidia spinosa DSM 3192 was identified as the best chitosan producer with yields of up to 260 mg/200 ml chitosan, however, this strain was difficult to grow to high cell densities repeatedly. Therefore we chose the second best chitosan producer, *Absidia coerulea* DSM 3018 (210 mg/200 ml), for further experiments. Fermentation with continuous dosage of ammonia and glucose resulted in a fungal dry cell weight of 11 g/l after heating to 80 °C and a chitosan yield of 2,7 g/l. The growth of the fungi in the fermenter was probably limited due

to too high shear stresses, thus under optimized conditions 2-4 times higher yields should be possible.

Influence of medium and additives on chitosan yield: All experiments were carried out with *Absidia coerulea* DSM 3018. The fungi produces similar amounts of chitosan in a broad pH-range between 3.5 and 7.5 with an optimum around pH 5.0. At pH-values below 3.0 the overall yield of the fungi decreases significantly and the percentage of chitosan compared to dry cell weight is also reduced to 1 %.

Phosphate in the standard medium might be reduced three times without any loss of chitosan yield. An increase in chitosan yield is obtained when the amount of ammonia is increased by 50 %, however, this effect is only visible at incubation times of >/= 72 h.

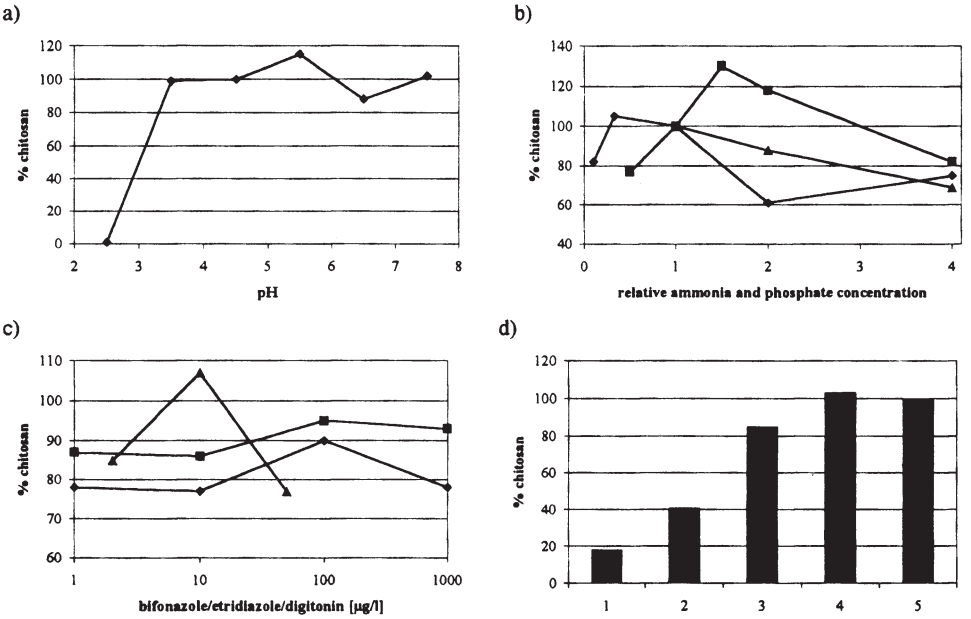

Figure 2: 100 % corresponds to chitosan yield in standard medium. **a)** pH-dependency of chitosan yield **b)** influence of different phosphate and ammonia concentrations on the chitosan yield, 1 corresponds to 100 % = standard medium, ▲= ammonia (48 h incubation) , ■= ammonia (72 h incubation), ◆= phosphate. **c)** influence of bifonazole (◆), etridiazole (■) and digitonin (▲) on the chitosan yield. **d)** 1 = exchange of glucose against glucosamine, 2 = exchange of glucose against N-acetylglucosamine, 3 = addition of 80 mg/l chitosan oligomer, 4 = addition of 800 mg/l chitosan oligomer, 5 = addition of 5,0 mg/l $CoCl_2$.

Neither additions of the azole containing fungizides etridiazole and bifonazole in sublethal doses, known to cause fungal cell wall thickening, increased the amount of extractable chitosan, nor influenced digitonin the chitosan yield from *A.coerulea* significantly. Thus the previously observed rise in chitin deacetylase activity and chitosan synthesis in vitro in the presence of digitonin does not reflect the in vivo situation at least in *A. coerulea*. The effect of an increased chitosan productivity in the presence of Co^{2+} could not be detected with *A. coerulea* either.

We exchanged glucose against glucosamine and N-acetylglucosamine in the medium to evaluate wether the fungi possesses metabolic pathways to phosphorylate the aminated sugars to incorporate them directly into chitin and chitosan. Our results of a decreased chitosan yield at comparable dry cell weights ruled out the hypothesis of an alternative metabolic route. With the addition of chitosan oligomers to the medium our intention was to find out wether the fungi can utilize the oligomers as starting points for the synthesis of new chitosan strains, however, no positive effects could be detected either.

Summarizing our results we did not find additives which enhanced the in vivo chitosan productivity significantly. Thus screening for the best chitosan producers and optimizing the medium composition and fermentation conditions are to our opinion the methods of choice to maximize fungal chitosan production.

Physical properties of fungal chitosan: For a characterization of the fungal chitosans the molecular weight, viscosity, structure and degree of deacetylation were compared to chitosan from crustacean origin. The relative molecular weights of *A.coerulea* and *A.spinosa* chitosans were in the range of 700 kDa, lower than high molecular weight chitosan from crustacean origin (e.g. Hydagen® CMF). The viscosity of *A.coerulea* chitosan is clearly lower when compared to crustacean chitosan of similar size (e.g. Hydagen® DCMF). The low viscosity of the fungal chitosan may be an advantage regarding handling of solutions and ease of formulation. The degree of deacetylation determined by NMR was around 90 % for the fungal chitosans, which corresponds to or even exceeds values found for high quality chitosans from crustacean origin.

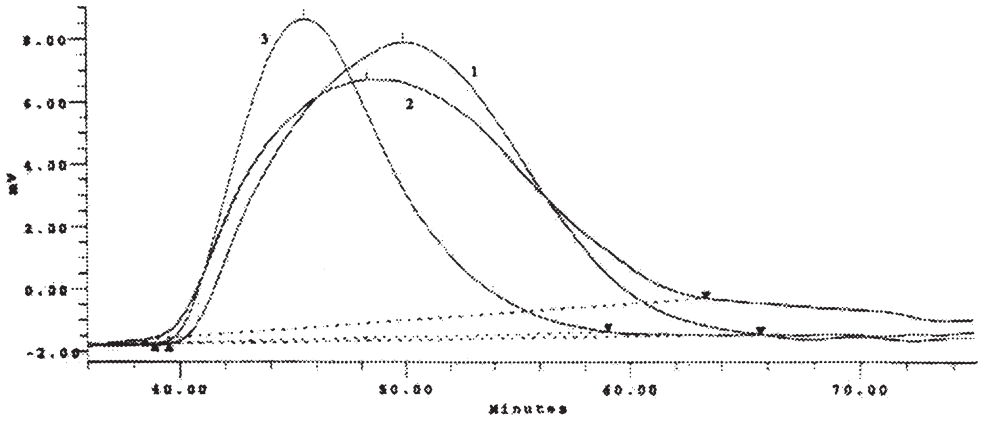

Figure 3: GPC-diagram of chitosan from *A.coerulea* (1), *A.spinosa* (2) and Hydagen® CMF (3).

Table 1: a) Viscosity of *A.coerulea* chitosan in comparison to Hydagen® DCMF and Hydagen® CMF at a chitosan concentration of 0,5 % and 1,0 % in solution; b) degree of deacetylation after NMR; c) molecular weight after GPC.

		A.coerulea	*A.spinosa*	Hydagen® DCMF	Hydagen® CMF
a)	0,5 % chitosan	20 mPa*s	n.d.	n.d.	200 mPa*s
	1,0 % chitosan	20 mPa*s	n.d.	200 mPa*s	1200 mPa*s
b)	degree of deacetylation	91 %	86 %	> 80 %	> 80 %
c)	MW	670 kDa	770 kDa	>/= 700 kDa	> 1000 kDa
	MW at peak maximum	450 kDa	670 kDa		> 1000 kDa

It was reported that the structure of the fungal chitosans differs from chitosan of crustacean origin, though these differences have not been characterized so far [16,24]. We investigated the structure *of A.coerulea* chitosan in comparison to crustacean chitosan by NMR-analysis. When the chitosans were oligomerized to improve the resolution of the spectra, we could not detect any significant differences between the fungal and the crustacean chitosan oligomers.

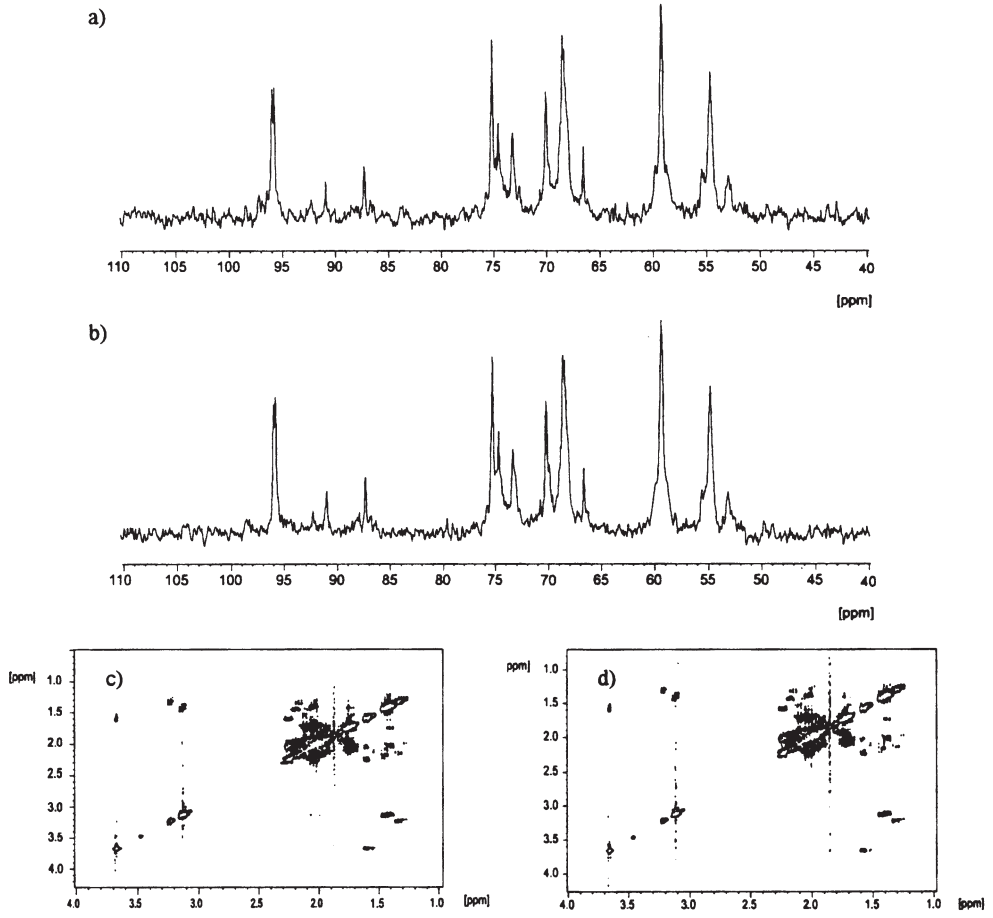

Figure 4: Comparison of NMR-spectra of chitosan oligomers from *A.coerulea* and Hydagen; a) 13C-spectrum of *A.coerulea* oligomers; b) 13C-spectrum of Hydagen® oligomers; c) TOCSY-spectrum of *A.coerulea* oligomers; d) TOCSY-spectrum of Hydagen® oligomers.

Quality of fungal chitosan: With the standard chitosan isolation procedure colloidal parts remained unsoluble in dilute acids. With a flocculation agent these colloids could be removed from the solution completely. Colloloidal chitin prepared from crab chitin was used succesfully for the removal of colloidal particles. From 2 day old cultures yellow-white chitosan free of odour was isolated. With a short H_2O_2 bleaching step white chitosan was obtained. Cultures grown for a longer period of time, however, resulted in more brownish chitosan which was more difficult to bleach.

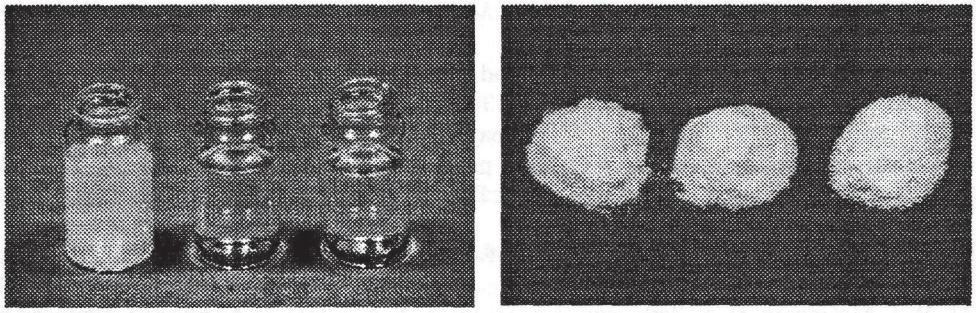

Figure 5: **a)** left: 0,5 % solution of fungal chitosan in 1 % HAc before flocculation, middle: 0,5 % solution of fungal chitosan in 1 % HAc after flocculation, right: 0,5 % solution of Hydagen® DCMF in 1 % HAc; **b)** left: dried fungal chitosan before bleach, middle: dried fungal chitosan after bleach, right: Hydagen® DCMF;

Summarizing our results two additional purification steps, flocculation of colloids and a short bleach, are neccessary for the isolation of high quality chitosan clearly soluble in dilute acids and free of colour and odour. As the fungi are cultivated under controlled conditions the quality of the chitosan is not dependent on environmental conditions, so impurities e.g. heavy metal ions, in the chitosan can easily be avoided.

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Chitosan from *Absidia orchidis*

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Summary
The influence of ferrous and manganic ions on *Absidia orchidis* growth and chitosan production is presented. It was found that both ions increase the fungal growth but inhibit the chitosan production. The addition of Fe^{2+} ions increases the chitosan yield from a volume unit of culture medium from 0.7 g/L to 1.8 g/L while addition of Mn^{2+} increases the yield only to 1.05 g/L. Acetylation degrees of chitosans from fungi cultivated with addition of ferrous and manganic ions were higher (even above 30%) than acetylation degree of chitosan from the basic medium (15%).

1. Introduction

Chitosan is a natural component of fungi (*Mucoraceae*) cell walls. Chitosan separation from fungi can be an alternate way to produce this important biopolymer.

The method of chitosan extraction from fungi cell walls was proposed by White, Farina, Fulton in 1979 [1]. Although many investigations focused on this subject were done, there is no a technology, which would be worth from the economical point of view. The chitosan production from fungi cell walls is still an open problem.

The yield of chitosan from fungi mass or from a unit of culture medium depends on several factors as: strain of fungi used, cultivation method (shaking culture, bath culture, continuous culture, solid state culture), cultivation parameters (pH, temperature, mixing rate, time of cultivation).

The increase of chitosan yield can be obtained either by increasing of biomass yield or by increase of chitosan content in cell wall

The presence of chitosan in the fungi cell wall is a result of complex action of two enzymes: chitin synthase, responsible for chitin chain building and chitin deacetylase, responsible for chitin deacetylation and transformation of chitin into chitosan.

Some compounds influencing activity of these enzymes (Co, Mn, Fe, Ca, Zn, EDTA, trypsine) have been reported in literature [2, 3] but there are no data concerning the effect of them on chitosan production by alive microorganisms.

The aim of presented work was to determined the influence of selected compounds on chitosan production by *Absidia orchidis* fungi. In the preliminary investigations the addition of ferrous, manganic, cobalt ions as well as addition of trypsin and chitin to nutrient medium were investigated [4]. On the base of these preliminary investigations the Fe^{+2} and Mn^{+2} ions were chosen to experiments.

2. Materials and Methods

2.1. Fungi

The *Absidia orchidis* NCAIM F 00642 (Budapest, Hungary) strain was used. The 2-days old shaking culture were used as an inoculum.

2.2. Cultivation medium

The YPG medium enriched with following salts was used: 0,5g $(NH_4)_2SO_4$, 0,1 g K_2HPO_4, 0,1 g NaCl, 0,5 g $MgSO_4 \times 7H_2O$, 0,1 g $CaCl_2$, 100 ml H_2O [5]. pH of the medium was 6,3. $FeSO_4 \times 7H_2O$ and $MnSO_4 \times 5H_2O$ were used for Fe^{+2} and Mn^{+2} ions experiments. All reagents were analytical grade or higher

2.3. Culture conditions

The bath cultures were carried out in bioreactor BIOFLO III (New Brunswick, USA). The culture medium (4.5 dm^3) was added to the bioreactor, sterilized (121^{0}C, 20 min) and inoculated with 500 cm^3 of 2-day old shaken culture (total liquid volume was 5 dm^3). The fungi were incubated at 26^{0}C, pH = 5.5, aerated and mixed. The time of cultivation was 48 h. The 1N NaOH and 1N HCl solutions were used to stabilize the pH value.

2.4. Biopolymer separation

The biopolymer separation method consisted of the following steps:

A. Biomass separation Fungi biomass was homogenized in the bioreactor (900 rpm, 30 min). The biomass was then centrifuge (6 000 rpm, 20 min) and washed with deionized water.

B. Cell wall separation The fungi biomass was treated with 1 N NaOH solution at 121^{0}C, (10 min). The alkali insoluble fraction (cell walls) was then centrifuged (6 000 rpm, 20 min), washed with deionized water and dried 24 h at 60^{0}C. Finally dry cell walls (d.cw) were ground.

C. Biopolymer extraction The fungi cell walls were treated with 1% CH_3COOH solution. The solution was centrifuged and an acid soluble fraction was collected. The liquid was mixed and alkalized to pH 10.0 with 1 N NaOH - biopolymer was precipitated. Biopolymer was separated from the liquid by centrifugation (20 000 rpm, 20 min), washed with deionized water and dried at 50^{0}C. The biopolymer was stored at room temperature in an air-tight vessel.

2.5. Analytical methods

A. Acetylation degree Degree of acetylation was calculated on the base of infrared spectra (IR) according to the direct method of Shigemasa et al [6]. IR spectra were measured on Perkin-Elmer System 2000 spectrometer. The resolution was 4 cm^{-1}. The average of 32 scans for each spectrum were used. Samples of chitosans were used as KBr disc (2mg / 250mg). The calculation method used the amide II band at 1560 cm-1 and the band at 1070 cm-1 as an internal standard.

B. Mean viscosimetric molecular weight The mean molecular weight of the biopolimers was determined on the base of viscosity measurement of chitosan solution in acetate buffer (0,3 M CH_3COOH + 0,2 M CH_3COONa) at 25^{0}C, using Mark-Houwink equation with constants evaluated by Roberts and Wang [7]: K = 0,075 cm^3/g and a = 0,76.

3. Results

Literature data report influence of Fe^{2+} and Mn^{2+} on pure chitin deacetylase [**3**, **4**], enzyme responsible for transformation of chitin into chitosan. There are no literature data showing influence of these ions on chitin synthase, enzyme responsible for building of chitin chains. Presented experiments were focused on determination of the influence of ferrous and manganic

ions on the chitosan formation in fungal cell walls during their growth in bath culture. Investigations were performed on typical YPG culture medium enriched in several mineral salts (p. 2.2.). The medium is sufficient to support the growth of fungi.

Biomass yield was calculated as a mass of dry biomass per volume of culture medium [g d.bm/L], **Cell Walls** yield wad calculated as a mass of dried cell walls per volume of culture medium [gd.cw/ L],
Chitosan yields were calculated as:
- a mass of chitosan per volume of culture medium [g / L],
- a mass of chitosan per mass of dry biomass [g / g d.bm],
- a mass of chitosan per mass of dry cell walls [g / g d.cw].

The mass of chitosan corresponds to dry form (not a gel form).

3.1. Influence of Fe^{2+} ions

Figure 1. presents the yields of dry biomass, cell walls and chitosan from a volume unit of culture medium.
It can be observed that increase of Fe^{2+} ions causes the increase of:
- biomass yield from 7.4 g d.bm/L to 45.3 g d.bm/L,
- cell wall yield from 2.5 g/ L to 12-13 g/ L and
- chitosan yield from 0,7 g/ L to 1.8 g/L.

Addition of ferrous ions caused decrease of chitosan yields from a unit of biomass and a unit of cell walls what can be observed at Fig. 2.
For Fe^{2+} concentration equal 9 mM the chitosan yield from a mass unit of biomass was 3 times smaller and for the chitosan yield from a mass unit of cell walls was 4 times smaller than the yields obtained from the basic medium.

It was observed that acetylation degree was lower in

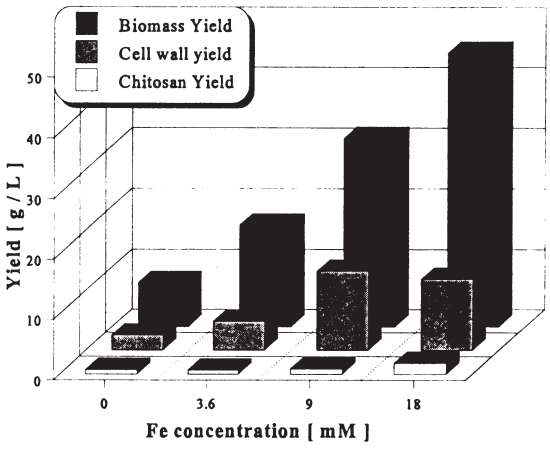

Figure 1. The influence of ferrous ion concentration on the yield of biomass, yield of cell walls and yield of chitosan.

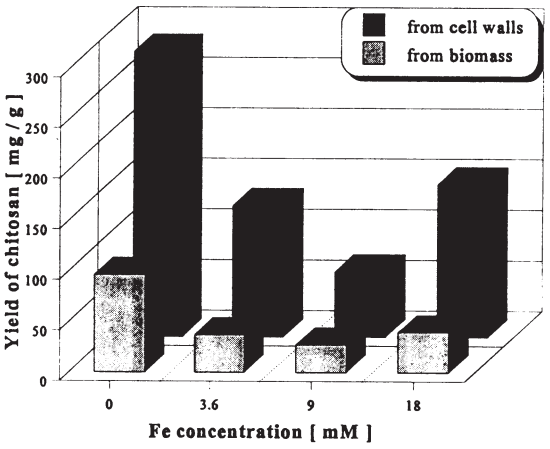

Figure 2. The influence of ferrous ion on the chitosan yield from a unit of dry biomass and from a unit of dry cell walls.

the culture without Fe^{2+} ions (15%) than in the cultures with addition of ferrous ions (26 - 30%). Also mean molecular weight was higher in the standard culture (750 kDa) than in modified ones (88 - 300 kDa).

3.2. Influence of Mn^{2+} ions

Figure 3 presents the yields of dry biomass, cell walls and chitosan from the volume unit of culture medium. The highest yields were obtained in the culture containing 20 mM Mn^{2+}:

- biomass - 15 g d.bm/L,
- cell walls - 4.5 g d.cw/L,
- chitosan - 1,05 g/L.

Addition of Mn^{2+} doesn't influence significantly the chitosan yields from a mass unit of dry biomass (69 - 96m g/ g d.bm) or a mass unit of cell walls (232 - 314 mg/ g d.cw), Fig. 4.

The acetylation degree of chitosan obtained from the culture containing 10.4 mM Mn^{2+} was similar to chitosan obtained from the basic medium (18,8% and 15% respectively). Further increase of manganic ions concentration caused significant increase in acetylation degree - above 30 %.

The mean molecular weight changes from 98 kDa at Mn^{2+} concentration equal 10.4 mM to 1156 kDa for 20.7 mM while for the basic medium chitosan mean molecular weight was 750 kDa.

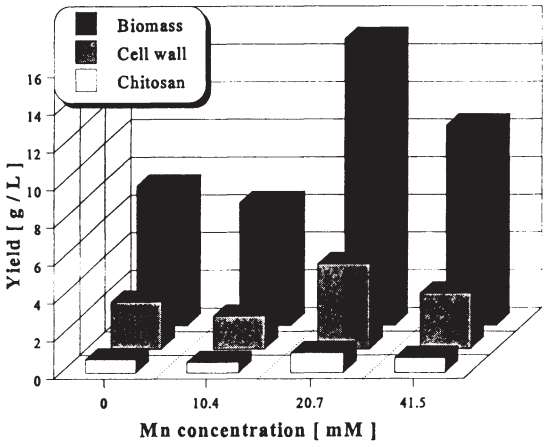

Figure 3. Influence of manganic ions concentration on the yields of biomass, yield of cell walls and yield of chitosan from a unit of culture medium.

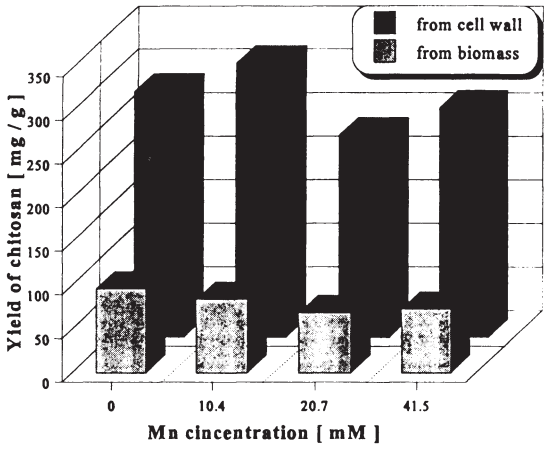

Figure 4. Influence of manganic ions concentration on the chitosan yield from a mass unit of dry biomass and a mass unit of cell walls.

4. Discussion

According to presented data it can be stated that ferrous ions increase the biomass yield from a volume unit of culture medium (up to 45 g d.bm./L),

the cell wall yield (up to 13 g d.cw/L) and the chitosan yield (up to 1.8 g/L). But they also decrease chitosan content in biomass (even 3 times) and in cell walls (even 4 times). The increase of chitosan amount is due to increase of biomass yields than increase of chitosan content in fungi cell walls. For Fe^{2+} concentration equal 18 mM biomass yield from a volume unit of culture medium was 6 times higher and chitosan yield from a volume unit of nutrient was 2.5 times higher than these obtained in the basic medium. In the same time the chitosan yield from a mass unit of dry biomass was 2-3 times smaller than in standard medium.

Ferrous ions are reported as substance which decreases activity of chitin deacetylase [2]. This action was confirmed in our experiments. Chitosan from fungi cultivated in culture medium containing Fe^{2+} ions has higher acetylation degrees (26 - 30%) than chitosan from the basic medium (15%).

Ferrous ions decreased also activity of chitin synthase. Mean molecular weights of chitosan from modified cultures have lower values (88 - 300 kDa) than that from standard one (750 kDa).

Manganic ions act in similar way as ferrous ions. Above concentration equal 10.4 mM they increase the biomass yield (up to 15 g d.bm/L), the cell wall yield (up to 4.5 g d.cw/L) and the chitosan yield (up to 1.05 g/L) from a volume unit of culture medium. Similarly to Fe^{2+}, the increase of chitosan production was caused by the increase of biomass yield. In contrary to ferrous ions, manganic ions doesn't influence the chitosan yield from a mass unit of dry biomass and the chitosan yield from a mass unit of cell walls.

Manganic ions are reported in literature as an activator for chitin deacetylase [2]. This activation was not observed in our experiments. Acetylation degrees of chitosan produced from fungi cultivated in culture containing Mn^{2+} ions were much higher (above 30%) than that from the basic medium (15%) what was an effect of chitin deacetylation inhibition by Mn^{2+}.

There was no simple relationship between mean molecular weight of chitosan and concentration of manganic ions in medium. At lower concentration (10.7 mM) we produced chitosan with lower mean molecular weight (96 kDa) while for higher concentration (20.7 mM) we produced chitosan with high mean molecular weight (1156 kDa) - mean molecular weight for the basic chitosan was 750 kDa. There are no literature data presenting influence of Mn^{2+} on property of chitosan produced from fungi.

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Scaling up of lactic acid fermentation of prawn wastes in packed-bed column reactor for chitin recovery

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Abstract

Several carbohydrate sources, such as sugar cane, lactose and whey powder at 10 and 20% (w/w wet basis) were added to prawn head wastes; 5 and 10% (v.w. wet basis) of *Lactobacillus* spp. B2 were used for selection of fermentation conditions. Sugar cane 10% and 5% of inoculum were chosen due that the acid production and homofermentative pattern was also observed.

Further the lactic acid fermentation was scaled-up in a packed-bed column reactor, pH was kept lower than 5.0, allowing the preservation of the wastes until 3 months. About 90 and 80 % of deproteinisation and demineralisation respectively were observed at the sixth day of fermentation. The raw chitin from silage was treated with acid and alkali for completion of minerals and proteins removal.

Introduction

Prawn production and catching has grown as an important income for Mexico, the total production of 1998 was 80,000 tones. Approximately 45% of the total weight of the animal are waste, such as heads and exoskeleton, the majority of wastes are disposed offshore or inland causing pollution. Waste can be sun-dried or cooked for prawn meal production however the waste supply is irregular and seasonal being a limitation. The above mentioned might become worse considering the perishability of the prawn wastes that difficulty the processing.

Lactic acid ensilation has been applied for many years in the elaboration of fermented sauces and pastes found in South East Asia. The fermentation route has shown to be a good mean of preservation and potentially might generate product lines, considering that wastes are composed of valuable products such as chitin, protein and pigments (astaxanthin), which have an important market [1,2,3].

Chitin is the second most abundant polysaccharide in the nature after cellulose, has many applications in food, medicine, water waste treatment, textiles and cosmetics.

Traditionally chitin is extracted by alkali and acid treatments, which might cause damage to the polymer quality.

Lactic acid fermentation combined with chemical treatments has been studied as alternative method of chitin recovery that reduce the amount of alkali and acid. The removal of protein and calcium from shells are due to the enzymatic activity and mineral solubilisation by organic acids produced by bacteria growth [2,4].

Earlier studies have been done using dynamic bioreactors, the main qualities of these types of fermenters where the agitation maintained a growth-promoting environment and the ready separation of the waste into the solid and liquid fractions [2]. However even that operation cost of these rotational reactors can be low many prawn producers countries have not available infrastructure. The application of a solid state reactor reported in this work allow to ferment the prawn waste with low energy consumption, do not produce aqueous wastes, it can be scaled-up and separation of solid fraction was also observed.

The purpose of this paper was to compare the suitability for prawn fermented silage prepared with various type and amounts of unexpensive carbohydrate sources and inocula levels. Later on, the set conditions were scaled-up in solid state column reactor and the alkali-acid treatments were established for purification of raw chitin from silage.

Materials and Methods

Materials

Prawn waste

Prawn (*Penaeus* spp.) waste was obtained from seafood market of Mexico City and consisted of heads (thorax). The waste was minced with a meat mincer (Sanitary, Chicago U.S.A.) and stored at –20°C until required.

Sugars

Refined sugar cane (Azucar, Mexico), lactose (J.T. Baker, Mexico) food grade and spray dried cheese whey (KemFuds SA, Mexico) were used.

Inoculum preparation

Lactic acid bacteria used was *Lactobacillus* spp. strain B2, isolated from shellfish waste in Dr. Hall laboratory and selected for its ability of fast acidification and homofermentative pattern. *Lactobacillus* spp. was maintained with MRS broth and 15% glycerol as cryoprotectant at –20°C. MRS agar slopes were prepared and stored at 4°C. The inoculum preparation was done using APT broth with a loopful of cells from a slope of MRS agar and incubated at 30°C for 24 h. Starter had a cell concentration of 10^8cfu/ml.

Packed bed column reactor

Construction

The column reactor consisted of two modules of stainless steel. The modules were joined one above the other by eight projecting screws from the bottom side of the flange to the corresponding holes in the upper side of the flange of the lower module. The modules had an acrylic mesh plate and two "O" rings between them.

Flask fermentations

Prawn wastes were added with sugar cane, lactose and whey powder at 10 and 20% (w/w), inoculated with starter at levels of 0, 5 and 10% (v/w). 50 g of the mixture prawn, sugar and inoculum were placed in flasks and incubated at 30°C. The samples were taken every 24 hours for 4 days.

Reactor

Once that type and quantity of carbohydrate source and starter level were determined, the scale-up of the fermentation was carried out in the bioreactor. The prawn waste mixture was transferred in the reactor, and placed in temperature controlled room at 30°C.

Sample Analyses

The pH of samples of the liquor produced during the fermentation was measured using a electrode (Conductronic pH 20, U.S.A.). The sample was diluted (1:10) and total titratable acidity (TTA) determined with NaOH 0.1 M to a final pH of 8.4, and expressed as % lactic acid [3]. Total soluble sugars were measured from an aqueous extract by the method of phenol-sulphuric acid [5].

The concentration of lactic and acetic acids were determined by HPLC system (Perkin Elmer, Norwalk, U.S.A.) which consisted of a column Phenomenex Rezex for sugars and organic acids at 50°C and detector refractometer 30mM of sulphuric acid as mobile phase.

Elemental Analyser (Perkin Elmer 2400 Norwalk, U.S.A.) determined the total nitrogen (TN). After the sample was purified of minerals and proteins by acid and alkali treatments, respectively, Elemental Analyser also determined chitin nitrogen. Corrected protein was obtained by subtracting chitin nitrogen from total nitrogen and multiplying by 6.25, the Kjeldahl conversion factor for meat protein assuming that protein has 16% nitrogen [6].

Moisture, lipids, ashes contents were determined by standard methods [7].

Calcium was determined from ashes with an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, U.S.A.) [6].

Chitin isolation

After the fermentation the prawn waste silage was separated into solid and liquid fractions, solid (raw chitin) was used for elemental analysis, calcium determination and acid-alkali treatments.

Pigments from raw chitin were extracted by a solvent system of chloroform-methanol-water. Uncoloured solid was used for demineralisation with several concentrations of HCl. Subsequently, samples were taken in order to measure the calcium content and compare with the initial samples.

After demineralisation, various NaOH concentrations were used for protein removal. Nitrogen content was determined in the samples by Elemental Analysis.

Results and Discussion

Flask fermentations: carbohydrate source and inoculation level

For previous studies reported that showed the amount of carbohydrate was a critical factor for the performance of fermentation. It was observed at lower sugar concentration than 5%, prawn and fish were not well preserved silage, in fact at low concentration, 3.8% and 5%, the fish and prawn respectively putrefied [8,9]. The amount of sugar in this work was established out minimum 10% of concentration.

A comparison between lactose, sugar cane and whey powder as additives for prawn waste silage was done. Statistical analyses showed that there were significantly differences between carbohydrates sources, the additive with highest mean was sugar cane at 10% of concentration and starter level of 5%. These conditions carried out highest final acidification from an initial pH of 7.5 to 4.4 and TTA of 0.5 mmol/g after 96 hours of fermentation (Fig. 1A). Neither increment of sugar cane (20%) nor inoculation improved the acid production, in fact 5 and 10% presented the same TTA and pH were just slightly different.

Fermentation added with lactose presented a similar behaviour although that took longer time, 72 h, for reaching pH 4.5 and TTA was lower than 0.5 mmol/g. Whey powder contained lactose, proteins and fat at 750 g/kg, 120 g/kg and 20 g/kg respectively [10]. Lactose is a highly fermentable sugar, nevertheless fermentations made with whey powder displayed the lowest acid concentration, pH of 5.5 and TTA of 0.28 mmol/g at 96 h (Figure 1B), as a consequence of that, after 4 days at 30°C prawn waste displayed signal of putrefaction. In spite of the increase of whey powder concentration, 20%, the results were almost the same, and not likeness fermentations mixed with lactose. Fish silage added with 7.7 % of whey powder appeared to be an acceptable carbohydrate source, however the rather slow initial decline in pH was seen as limiting and a risk [9].

Fermentation behaviours were rather similar and just in terms of amounts of acid produced was possible to establish the sugar for the next stage of this work. Mexico is sugar cane producer and besides the acidification, economical reason made to take this carbohydrate as the suitable additive. According with the above mentioned sugarcane at 10% (w/w) and 5% (v/w) of *Lactobacillus* spp. were used for fermentation scaling-up.

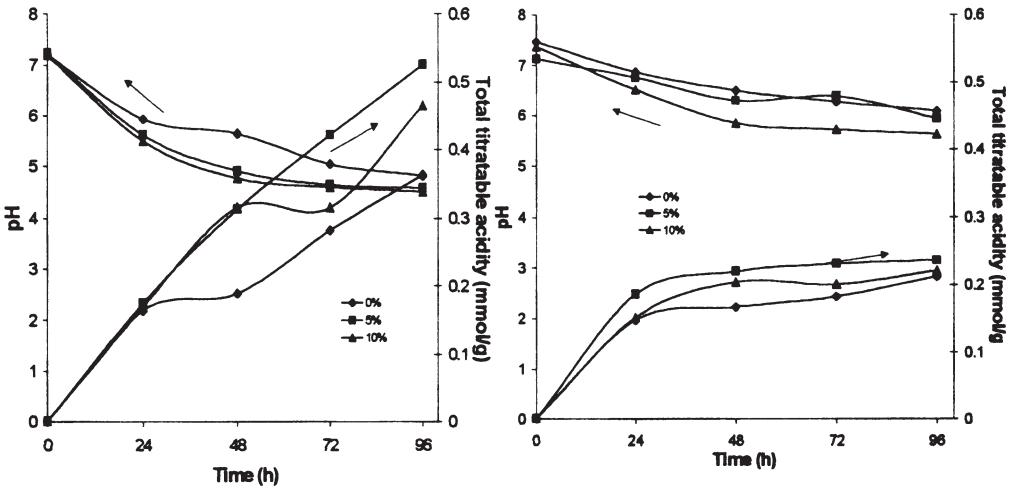

Figure 1. Evolution of pH and acid production (total titratable acidity) of prawn waste fermentation added with various inocula levels of *Lactobacillus spp.* and (A) 10% (w/w) sugar cane; (B) 10% (w/w) whey powder.

Scaling-up

The prawn waste silage was scaled up to 2 kg reactor size, fermentation monitored during 83 days, measuring TTA, pH, protein and calcium in solid. Approximately 41%, 52%, and 7% were solid, liquid fractions and loss matter respectively, the latter due to the manipulation, gas production and moisture loss. After the second day the fermentation reached pH 4.6 and TTA of 0.34 mmol/g, remaining constant for 83 days, the acid especially lactic acid, has been reported as a very effective inhibitor for spoilage organisms growth, hence the preservation was established.

Deproteination observed during the fermentation was 90% at sixth day and perhaps was by means of enzymes that acted on the proteins associated to chitin and minerals (Figure 2). Shirai *et al.* [4] reported protein hydrolysis during lactic acid ensilation of prawn wastes, mainly due to trypsin-like proteases, which liquefied the waste. The percentage of protein removal achieved in this study improved the results of Shirai [8] in flask fermentation that just obtained 76%, and Zakaria *et al.* [2] had similar results in scampi waste fermentation in rotating horizontal reactor. It was also observed the maximum percentage of demineralisation at sixth day of fermentation, 80% (Figure 2). Lactic acid production might react on calcium carbonate attached to chitin and proteins, causing partial solubilisation of minerals to liquor, actually at the end of 83 days of fermentation was possible to observe mineral granules composed mainly of calcium.

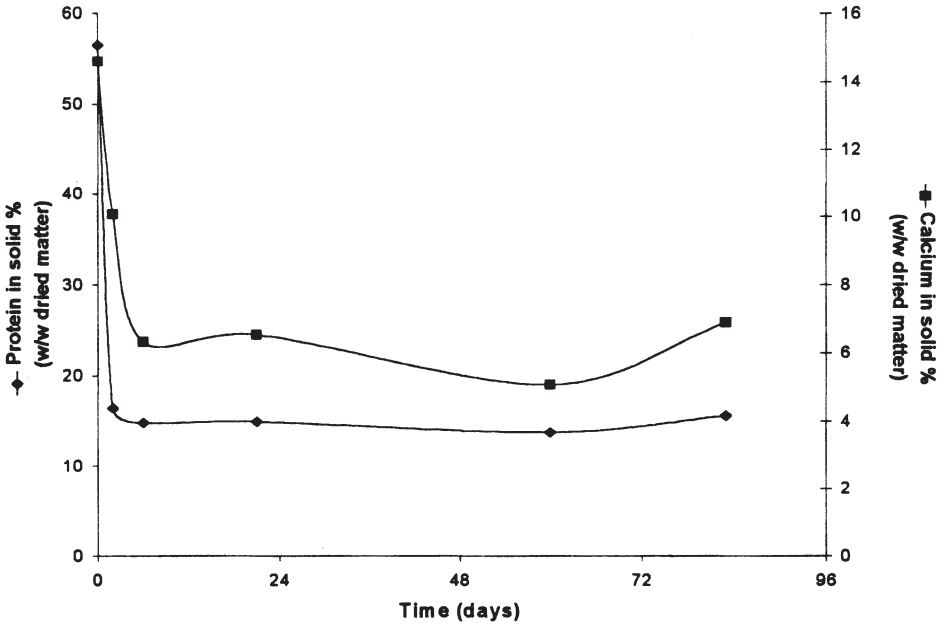

Figure 2. Changes in protein and calcium during lactic acid fermentation of prawn waste fermentation added with 10% (w/w) sugar cane and 5% (v/w) *Lactobacillus spp.* in column reactor (2 kg size).

The scale-up criterion employed was trial and error techniques, which involved the maintenance of pH reduction and acid production [11]. Capacity was 2 kg and it was further increased to 30 kg maintaining geometric similarity, changing the height and diameter. The reactors of 2 and 30 kg sizes were of equal magnitude of pH and acid evolution (Figure 3).

The pH decrement was observed for the sugar consumption and acid production, the organic acids produced were determined by HPLC and identified as lactic acid that means homofermentative trend (Figure 3). An inconvenient of this kind of reactor was the distribution of nutrients due to the sugar cane went out to the reactor being a potential limitation for lactic acid bacterium growth (Fig. 3).

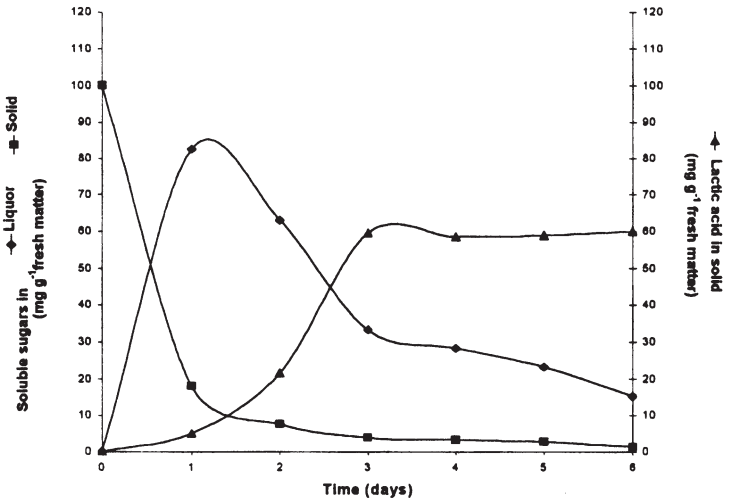

Figure 3. Sugar consumption and lactic acid production during lactic acid fermentation of prawn waste fermentation added with 10% (w/w) sugar cane and 5% (v/w) *Lactobacillus spp.* in column reactor (30 kg size).

Chitin isolation by chemical method after fermentation

Raw chitin (solid fraction) obtained from prawn silage was employed for further purification by means of several amounts of acid and alkali (Fig. 4).

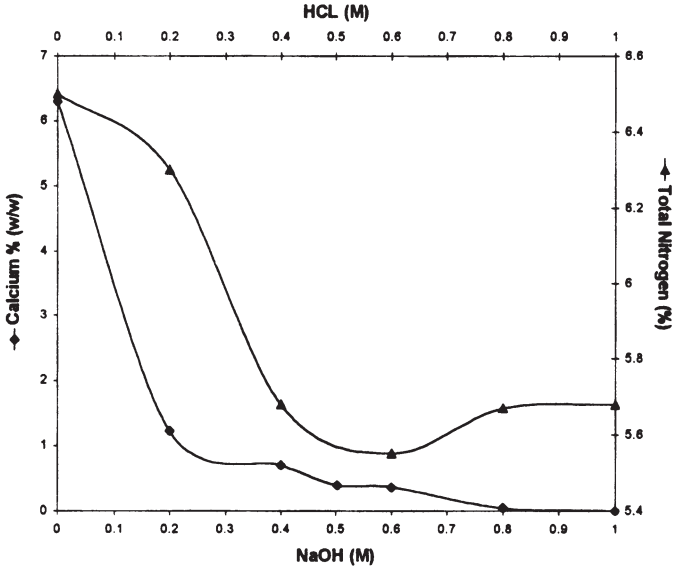

Figure 4. Calcium and protein contents after various acid and alkali treatments for chitin purification after fermentation.

The lowest decrements of calcium content and total nitrogen detected were approximately at 0.4M of HCl and NaOH. The above results confirmed that lactic acid fermentation reduces the amount of chemicals for chitin purification, also feasible preserved the waste and recover more compounds, since fermentation is a mild process.

Conclusions

Several inexpensive carbon sources were tested for lactic acid fermentation of prawn wastes in order to scaling-up in suitable conditions for catching and farming areas. Column reactor design was efficient for prawn waste ensilation observing high protein and mineral removal which makes easier the chitin extraction, as consequence of that alkali and acid savings were observed.

Chitin purification from scaled-up prawn waste silage has been done and will be necessary to select the best inoculation procedure for semi-industrial scale, so might be back slopping, i.e. taking a proportion of previous batch of silage.

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Preparation of chitin by acetic acid fermentation

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Summary
Fed-batch production of acetic acid from deproteinised whey by co-immobilised cells of *Lactococcus lactis* and *Clostridium formicoaceticum* in a spirally wound fibrous sheet packed column reactor has been investigated. Different industrial-grade nitrogen sources (corn steep liquor, casein hydrolysate and sunflower protein hydrolysate) have been tested to improve acetic acid production, showing that 2 g/L sunflower protein hydrolysate was adequated for an effective fermentation. This cocultured fermentation process has been studied to produce low-cost acetic acid "in situ" and used directly for the demineralisation of crawfish chitinaceous fraction (exoskeleton). Chitin obtained by this process is chemically and spectrometrically (FT-IR and ^{13}C-NMR) characterised.

Introduction
The potential use of chitin and chitosan is widely recognised, and an important increase on the development of new applications has been observed. Actually, chitin, chitosan and their derivatives have a large range of applications in chemistry, medicine, pharmacy, cosmetic, food technology, water treatment, etc. However, industrial use of chitin is limited because of the high price for technical grade chitin and chitosan [1], and its use is restricted to specific applications. Therefore an extended use of chitin in industrial use need from cheap fabrication processes, or from the development of profitable processes based on the recovery of chitin and by-products such as protein-pigments or carotenoproteins, if crustacean processing waste products are used as starting material.

Traditional methods for commercial preparation of chitin from crustacean shell (exoskeleton) involves processes that alternate hydrochloric acid and alkali treatment stages to remove calcium carbonate and protein, respectively, followed by a bleaching stage with chemical reagents to obtain a white product. Processing costs are elevated because, wherever environmental controls are enforced, disposal costs must be added [2]. If a process could be devised for maximising the recovery of useful products cost-effectively, then it may facilitate the way for great commercial exploitation of chitin. For such a process to be successful the cost of the reagents used in the process must be of low cost.

Demineralisation is the step that need the greater amount of reagents, and therefore one of the costly steps. It can be carried out with different acids (hydrochloric [3], lactic [2], sulphuric [4], and acetic [5]). The production "in situ" of acetic acid at low cost from

byproducts such as whey [6], low quality wine [7], ethanol [8], lignocellulose [9], might offer a commercial route for the recovery of chitin from crawfish exoeskeleton, or chitinaceous fraction.

Materials and Methods

Acetic acid production was carried out as shown schematically in Figure-1, following, basically, the procedure described by Huang and Yang [6].

Cultures and media: The homolactic acid bacterium, *Lactococcus lactis* and the homoacetogenic bacterium, *Clostridium formicoaceticum* were used in this study. The stock culture of these bacteria were maintained in synthetic media containing either lactose or lactate as the carbon source. The composition of these media are that used by Tang and coworker [10].

Fresh sweet whey permeate (WP) and concentrated acid whey (AW) used in this study were obtained from Puleva S.A (Sevilla, Spain). They were stored at 4°C until use, usually less than 1 week. Both, WP and diluted AW, were filter-sterilised using a 0.2-μm membrane filter. To promote the fermentation, WP and AW were supplemented with some industrial-grade nitrogen sources, including yeast extract, casein hydrolysate (CH), sunflower protein hydrolysate (SFPH) [11], and corn steep liquor (CSL).

Bioreactor construction and operation: The immobilised-cell bioreactor was made of a glass column packed with spiral-wound terry cloth as described by Yang et al [12]. The column reactor itself had a working volume of 0.5 L, and was connected to a 15 L stirred-tank loaded with the chitinaceous material. A positive pressure of ≈ 3 psig of N_2/CO_2 gas mixture was applied to the stirred tank head space to maintain anaerobic conditions in the system. The entire reactor system contained 10.5 L of fermentation medium and 500 g of chitinaceous material.

Sample treatment: Crawfish (*Progambarum clarkii*) meal was fractionated by sedimentation/flotation [5] in two fractions, a proteinaceous fraction (PF) and a chitinaceous fraction (CF). The CF was used as starting material for chitin preparation.

Analyses: The pH of samples and fermentation broth was measured using a Gelplas electrode. Protein concentration was determined by HPLC amino acid analysis after hydrolysis with 6 N HCl, at 105° during 18 hours [13]. Total nitrogen (TN) was estimated by the Kjeldahl method [14]. Chitin nitrogen (CN) was also estimated by the Kjeldahl method, after the sample (2-3 g, dry weight) had been purified of its calcium carbonate and protein by boiling it with acid and alkali, respectively [15]. Chitin was calculated by multiplying CN by 14.5, assuming that the pure chitin contains 6.9% nitrogen [16]. Acetic acid, lactic acid and lactose were determined by HPLC as described elsewhere [10].

Solid state ^{13}C-NMR: CP/MAS ^{13}C-NMR spectra were recorded on a Chemagnetics CMX 360 NMR spectrometer (Chemagnetics Co. Ltd., Fort Collins) at room temperature according to the procedure described by Struscczyk et al., [17]. FT-IR spectroscopy: FT-IR spectra were recorded on a Jasco FT-IR 5300 spectrometer (Jasco Co. Ltd., Tokyo). Resolution was 4 cm^{-1}, and scanning number was 15, [18].

Results and Discussion

Crawfish meal was fractionated by sedimentation/flotation, according to the procedure described by Bautista et al., [5], in two fractions, a proteinaceous fraction (PF) and a

chitinaceous fraction (CF). The PF was sedimented at the bottom of the tank, while the CF floated at the top. The CF was used as starting material for chitin preparation.

In an attempt to find an alternative to the use of HCl in the demineralisation of chitinousous materials (crustacean exoskeleton), some investigation groups are studying different fermentation processes, such as lactic [2,] and acetic acid production. In particular we are interested in acetic acid production using low cost fermentation substrates, such as whey [6], low quality wine [7], ethanol [8] or lignocellulose, and its direct use for demineralisation of CF, integrating both processes, fermentation and demineralisation, in one integrating system (see Figure-1).

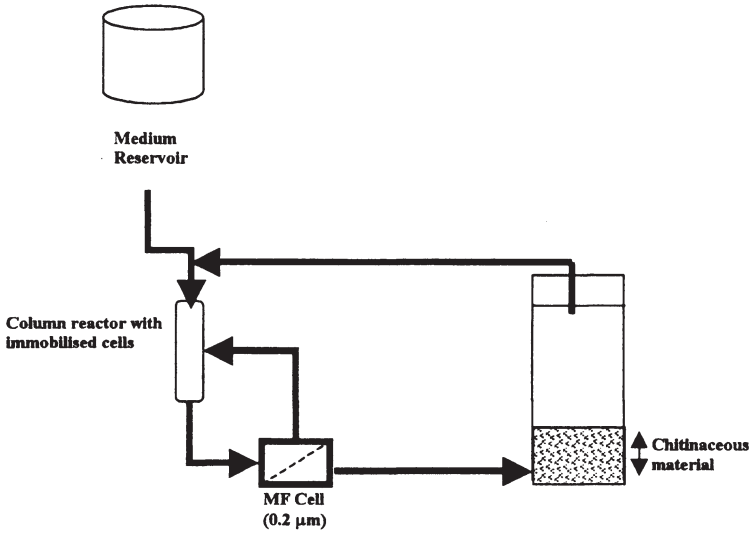

Figure-1 Schematic representation of the system used for demineralisation of CF by acetic acid fermentation.

In this study acetic acid was produced from whey lactose in batch and fed-batch fermentation using co-immobilised cells of *Clostridium formicoaceticum* and *Lactococcus lactis*, according to the procedure described by Huang and Yang [6]. The cells were immobilised in a spiral wound fibrous sheet packed in a 0.5 L column reactor, with liquid recirculation through a 15 L stirred-tank, containing the chitinousous material, CF, (see Figure-1). Industrial grade nitrogen sources, including, CSL, CH, and SFPH, were studied as nutrient supplements to WP and AW. Supplementation with either 2 g/L SFPH or CH or 1,5% CSL were adequated for the cocultured fermentation (results non shown). In all cases supplementation with industrial-grade nitrogen sources (2 g/L CH or SFP, or 1.5% CSL) were sufficient to achieve fast fermentation and high conversion yield. For availability reasons we use SFPH in all the following fermentations. The optimal supplement level must be determined with more experiments and based on cost analysis. However, these experiments showed that efficient acetate production from WP and AW, supplemented with small amount of industrial-grades nitrogen sources, can be achieved by fermentation with co-immobilised homolactic and homoacetic cells. Studies carried out in batch fermentation at different lactose concentration shows that when the lactate concentration was lower than 20 g/L, the specific growth rate of the homoacetogen increased by increasing the lactate concentration in the

medium. However, at higher lactate concentrations (> 20 g/L), lactate inhibited the cell growth. Consequently this high concentration of accumulated lactate negatively affected acetate production in the cocultured fermentation (results non shown), then lactate is the substrate for *C. formicoaceticum* [12] . The acetate production rate decrease can be interpreted as a substrate inhibition to *C. formicoaceticum*. Therefore, it is important to keep the lactate concentration lower than 20 g/L to allow good acetate production rate. To attain higher acetate concentration, feed-batch fermentation was used. Figure-2 shows a fed-batch fermentation of WP supplemented with 2 g/L SFPH. As shown in this figure, steady acetic acetate production to reach a high acetate concentration was achieved in fed-batch fermentation. Lactate accumulation and inhibition were reduced to a minimal by periodic addition of lactose. The highest acetate concentration reached in the fermentation was 58 g/L when the fermentation stopped, probably because of product (acetate) inhibition.

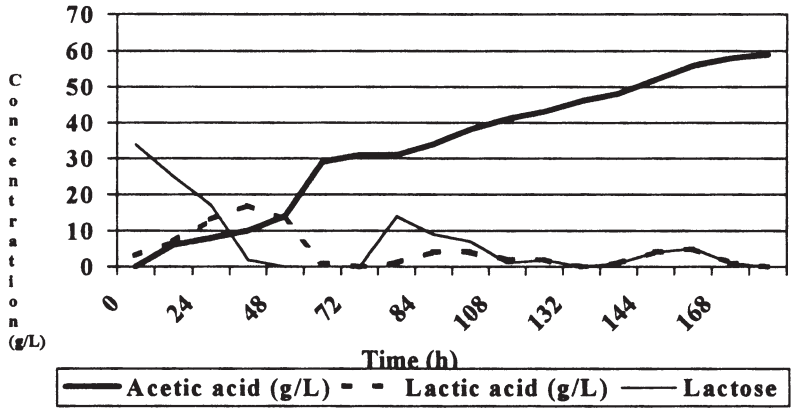

Figure-2 Fed-batch fermentation of whey permeate supplemented with 2 g/L SFPH.

Demineralisation of the chitinousous material is initially associated to the production of both lactic and acetic acid, but after the first 36-40 hours of fermentation the process is basically dependent of acetic acid production, then at this stage lactic acid is rapidly consumed by *C. formicoaceticum* (J. Blanco, personal communication). As Figure-2 shows lactose is rapidly transformed into lactate and this is transformed into acetate, mainly, as it is shown by the constant increase of the acetate production curve. The pH drops along the fermentation process, showing a rapid drop at the beginning of the process (from 7.8 to 6.5). After this point the decreasing of the pH occurs at a slower rate, until it reaches a minimum of 5.2 ± 0.1. After this point the pH is stable, and the fermentation is stopped and harvest 6 hours after. Results obtaining in the demineralisation of crawfish CF is shown in Table 1.

The treatment of the chitin obtained by acetic acid fermentation with 0.25 M NaOH and a 1:5 dilution of hypochlorite, at room temperature during 6 hours, lead to a good quality chitin (purified chitin) as shown by its FT-IR and ^{13}C-NMR spectra (see Fig. 3).

Table 1. Demineralisation of crawfish CF by acetic acid fermentation.

	CF	Raw Chitin[a]	Purified Chitin[b]
Protein	17.8 ± 0.6 %	3.3 ± 0.2 %	0.8 ± 0.2 %
Fat	0.7 ± 0.1 %	n.d.	n.d.
Chitin	25.7 ± 1.3 %	88.2 ± 0.8 %	94.8 ± 0.5 %
Ash	51.8 ± 3.5 %	4.1 ± 0.5 %	2.0 ± 0.4 %

-Results are expressed (dry basis) as the mean ± S.D. of three experiments.
[a] Chitin directly obtained by acetic acid fermentation.
[b] Chitin obtained by acetic acid fermentation followed by deproteinisation with 0.25 M NaOH and decoloration with
hypochlorite.
n. d.: non determined.

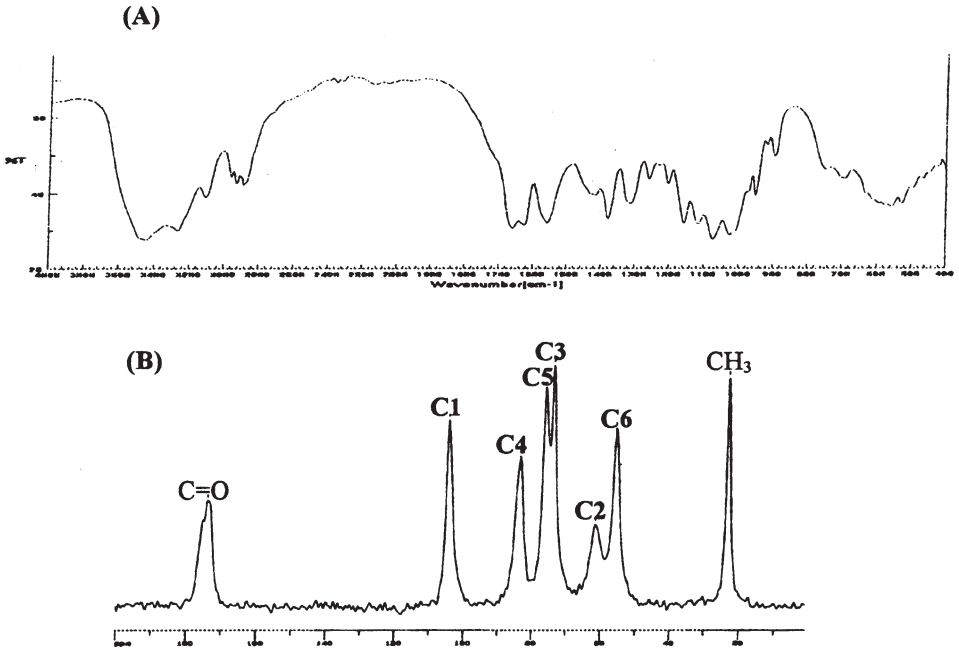

Figure-3: FT-IR (A) and ^{13}C-NMR (B) spectra of chitin demineralised by acetic acid fermentation followed by deproteinisation with 0.25 M NaOH and decoloration with hypochlorite.

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Inter-source reproducibility of the chitin deacetylation process

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Summary
Chitin samples from five different species were deacetylated and the rate constants for the reaction determined. Although those for crab, lobster and scampi were typical of those expected for a pseudo-first order reaction, those for squid and prawn were found to increase with increase in time of reaction. This was attributed to swelling of the substrate. Activation energies were found to be roughly similar for all samples. These chitins, together with langoustine and shrimp chitin, were deacetylated for varying lengths of time under carefully controlled conditions and the chitosans analysed. The results show that in a plot of '% solubility *versus* % deacetylation' all the chitosans fall on a single curve. This suggests that a consistent product may be obtained from a variety of chitin feedstocks provided proper control is maintained over the processing conditions, and that the correct reaction time is selected for each different chitin to ensure the same level of deacetylation is achieved.

Introduction

One crucial requirement if chitosan is to become an important commercial material is its guaranteed production on a regular basis to a consistent standard. Since the amount available from any particular species is limited [1], large scale production of chitosan is likely to require deacetylation of chitins obtained from several species, possibly depending on the time of year. It is therefore of interest to determine whether it is possible, through careful control of the processing conditions, to produce chitosans of similar properties from a range of chitins obtained from different species.

There are occasional statements in the literature regarding the alleged superiority of chitosan from a given species relative to that from another species. Thus Ramachandran Nair and Madhavan [2] compared the metal binding capacities of chitosan from crab (*Scylla senata*), prawn (*Penaeus indicus*), squid (*Loligo sp.*) and squilla (*Oratosquilla nepa*). Kurita *et al.* have compared the physical structures and hygroscopic characteristics of chitosans prepared from shrimp chitin and squid chitin [3] and also their chemical reactivity [4]. Additionally Peter and co-workers [5] have compared the analysis of the degree of acetylation (DA) for chitosans from Northern Atlantic shrimp (*Pandalus borealis*) and Antarctic krill (*Eupausin superba*). Finally Arredendo *et al.* [6,7] have compared the effects of chitosans from the Japanese fan-lobster (*Ibacus ciliatus*), spear squid (*Doritauthis blekeri*) and Japanese swimming crab (*Portinus trituberculatus*) on the state of water and denaturation of myofibriliar protein during the freeze drying and frozen storage of fish meat.

However these have been application-specific investigations and there has been no systematic study of the comparability of chitosans prepared from different chitins. In the current work chitin samples from a variety of sources – crab, langoustine, lobster, prawn,

shrimp, scampi and squid – have been deacetylated under carefully controlled conditions to determine the feasibility of producing a consistent chitosan material regardless of the source of the chitin feed stock.

Materials and Methods

Materials

Samples of chitin, identified only as crab, langoustine and shrimp, were obtained from industrial companies. Other chitin samples, from scampi (*Nephrops novegicus*), prawns (Thai Pink) and lobster (*Homarus gammarus*), together with β-chitin from squid (*Loligo sp.*), were prepared in the laboratory from the crude waste material using standard procedures for the deproteinisation and demineralisation steps. All the purified chitin samples were ground in a hammer mill and sieved, the fraction between 500-2000μm being used in the experiments.

The dye, C.I. Acid Orange 7 (Orange II), was a commercial sample that was purified by recrystallisation from aqueous ethanol.

Methods

Deacetylation was carried out using 45% (wt/wt) NaOH in all cases. During the deacetylation process the temperature, NaOH:chitin ratio, and level of agitation were carefully controlled at predetermined values.

The % deacetylation was determined by dye adsorption [8] using C.I. Acid Orange 7 (Orange II).

The solution viscosities were determined on 1% (wt/v) solutions of chitosan in 1% (v/v) acetic acid using a Brookfield Rotating Viscometer.

The % solubility was determined by dissolving 2g of chitosan in 200 mL 0.1M acetic acid, stirring for 24h, then filtering the solution and determining the dry weight of insoluble material retained by the filter.

Results and Discussion

Rate of deacetylation

The rates of initial deacetylation in 45% (wt/wt) NaOH at 41.5°C, 51°C and 61°c were determined for five chitin samples (crab, lobster, prawn, scampi, squid). The plots of 'extent of deacetylation *versus* time' were of two types. In the first type, which was found in the case of the crab, lobster and scampi chitin, the plot is rectilinear, indicating a constant rate of deacetylation. In the second type, found with the prawn and squid chitin, the slope of the plot increases with increase in time of deacetylation (Figure 1).

The most obvious explanation for this increase in reactivity is that the substrate is gradually swelling, opening up the chitin structure and making it more accessible to the NaOH. That the swelling effect should be pronounced with the squid chitin is readily understandable since β-chitin is less highly H-bonded, having inter-chain H-bonds between adjacent chains in the same 'stack', as has α-chitin, but being without the additional inter-chain H-bonds between adjacent stacks that are present in α-chitin [9]. This makes β-chitin more hygroscopic, allowing it to swell more in aqueous systems [10]. However why the α-chitin from the prawns should show behaviour similar to that of β-chitin, while the other samples of α-chitin do not, is not immediately obvious. Another peculiarity is that the swelling effect induced by NaOH might, by analogy with the cellulose/aqueous NaOH system [11], be expected to decrease with increase in temperature. However this does not appear to be the case (Fig. 1).

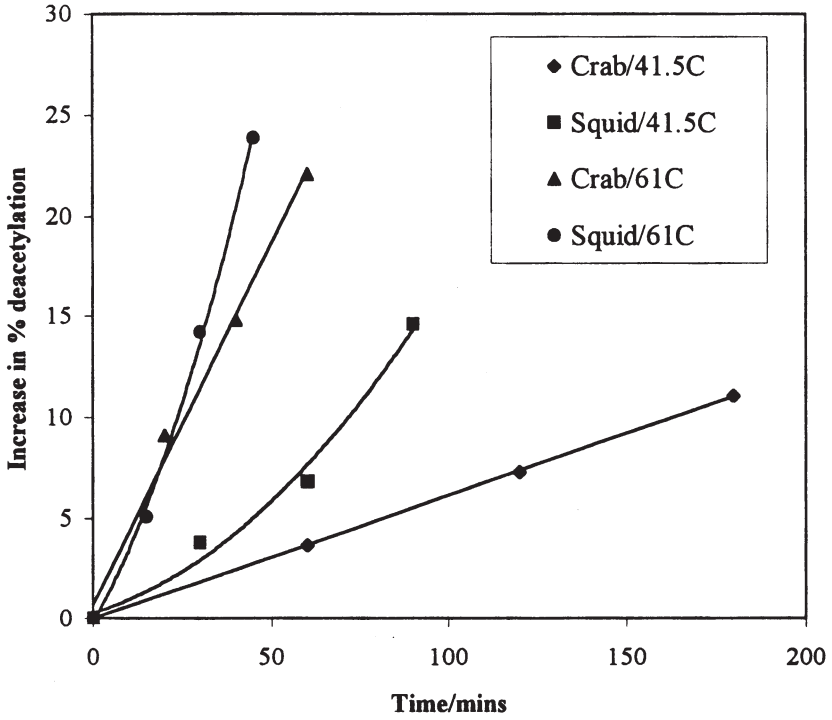

Figure 1. Increase in deacetylation of crab chitin and squid chitin at 41.5°C and 61°C.

Another swelling effect was observed during the washing off of these samples after deacetylation. In nearly all cases the particles became more swollen during the washing process, causing a change in the physical appearance. In the case of the squid chitin a number of samples formed highly swollen gels containing 80-100 g liquid/ g chitin, requiring a solvent exchange treatment - steeping in methanol – before proper filtration could be achieved. Again by analogy with the cellulose/NaOH system [11] it can be argued that during the washing process the NaOH solution in the particles is diluted down through the region of maximum swelling power. This effect could be enhanced by the disruption to the structural regularity of the chain caused by the limited deacetylation.

Determination of rate constants

The rate constants (k) were calculated for each of these samples at the three temperatures. The results for crab, lobster and scampi chitin show that the reaction is pseudo-first order with respect to the amide group concentration (Table 1). This agrees with the conclusions of Castelli *et al.* [12] who studied the deacetylation of crab chitin at 150°C using 30% (wt/wt) NaOH under pressure.

Table 1. Typical values of rate constants for the deacetylation of chitin by 45% (wt/wt) NaOH.

Temp/°C	Chitin source	Reaction time/min	k/s^{-1}	Chitin source	Reaction time/min	k/s^{-1}
41.5	Crab	60	9.27×10^{-6}	Squid	30	7.49×10^{-6}
41.5	"	120	9.24×10^{-6}	"	60	10.77×10^{-6}
41.5	"	180	9.51×10^{-6}	"	90	21.36×10^{-6}
61	Crab	20	7.76×10^{-5}	Squid	15	2.05×10^{-5}
61	"	40	5.85×10^{-5}	"	30	5.88×10^{-5}
61	"	60	6.02×10^{-5}	"	45	7.74×10^{-5}

In the case of the squid and prawn chitins the rate constants, calculated assuming a first order reaction, were found to increase with time of reaction in line with the increases shown for squid in Figure 1. However a similar trend is found on calculating them assuming a second order reaction hence it is not possible to determine the reaction order. However it may be assumed, in view of the very much greater molar concentration of NaOH relative to that of $-NHCOCH_3$ groups and the behaviour of the other chitins, that a pseudo-first order reaction is the more likely, with the increasing values attributed to the swelling effect as described above. The initial rate constants are in the order

lobster < scampi < prawn < crab < squid

and range from $3.53 \times 10^{-6} s^{-1}$ to $10.77 \times 10^{-6} s^{-1}$ at 41.5°C.

The activation energies of deacetylation were calculated using the initial rate constants at each temperature. The values were all quite similar, falling between 81.2 – 90.2 kJ mol^{-1}, and were in the order

lobster < crab < scampi < prawn < squid

The values are high when compared to the values of 35.7 – 57.9 kJ mol^{-1} reported previously [12] but this may reflect the differences in alkali concentration and reaction conditions.

Product consistency

Seventeen samples of chitin, covering all the source types listed above, were deacetylated for varying lengths of time under carefully controlled conditions, and the products analysed for % deacetylation, % solubility, and viscosity. In a plot of '% solubility *versus* % deacetylation' all the samples, regardless of the chitin source, fell on a single curve (Fig. 2, curve [a]). The adherence to a single curve means that a consistent product may be produced, regardless of the origin of the chitin feed stock, by controlling the % deacetylation through control of the time of reaction. However the importance of close control of the other process parameters is demonstrated by curve [b] of Fig. 2. This is data from a series of chitosans produced in a deacetylation process in which the temperature, NaOH:chitin ratio and extent of agitation differed from those used for the curve [a] samples. It is clear that products having the same level of deacetylation, but produced under different reaction conditions, may have totally different solubility characteristics.

Another important solution characteristic is that of viscosity. In the current work no attempt was made to exclude oxygen from the deacetylation reaction by working under a N_2 blanket. Hence not too much reliance can be placed on the solution viscosity results. However it is evident that the preliminary processes of deproteinisation and of demineralisation are as

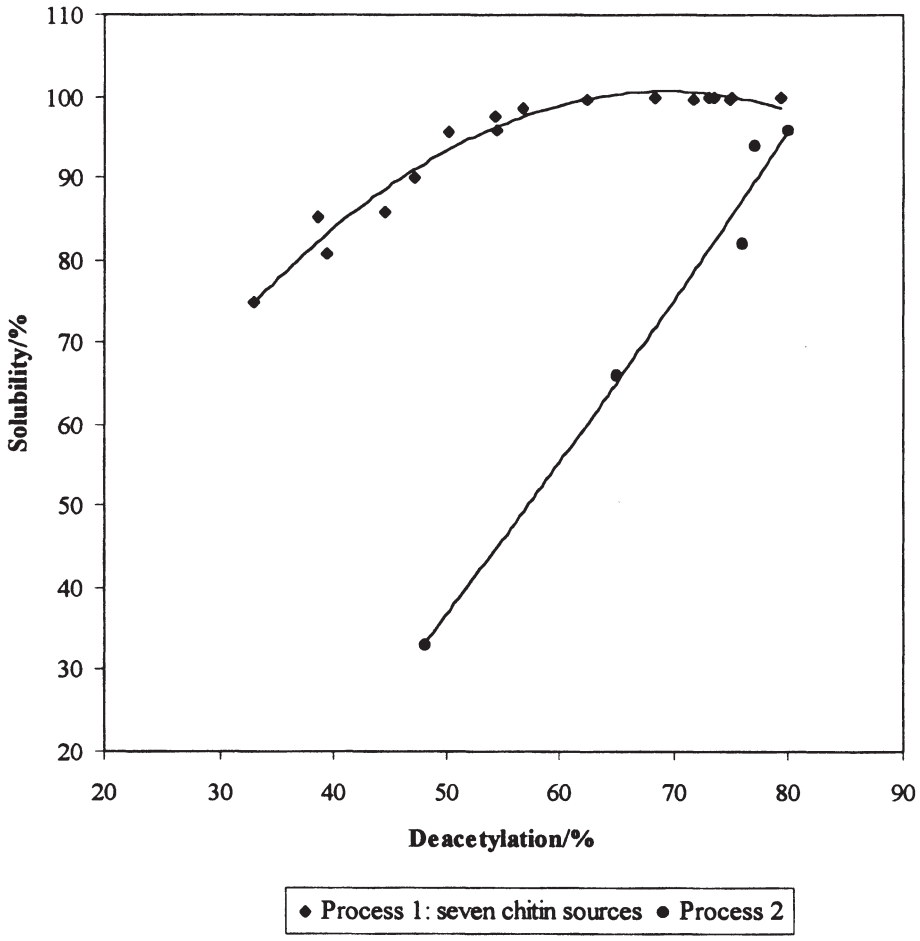

Figure 2. Solubilities of chitosans produced from various chitin sources.

critical to the quality of the final product as is oxygen exclusion from the deacetylation process. The 1% solution viscosities of the chitosans prepared from the three chitins obtained from commercial sources all lie in the range 50-160 cps. Compared to this, those produced from chitin prepared in our laboratory had, with one exception, viscosities > 1000 cps. Particularly outstanding in this respect were the chitosan samples produced from squid chitin, which had viscosities in the range 2500-3500 cps.

Conclusions

Uniformity of treatment, through rigorous control of the conditions of the deacetylation process, is essential for the production of a consistent chitosan material from a variety of chitin sources, or indeed from chitin from a single source. If this control is achieved then chitosans having the same % deacetylation will have similar solution properties regardless of

the chitin source. The viscosity results suggest that careful control of the initial processes of deproteinisation and demineralisation are at least as important as exclusion of oxygen from the deacetylation step if high molecular weight chitosan is to be produced.

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Comparative analysis of chitosans from Insects and Crustacea

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Summary
The properties of chitosans produced from larvae of the fly, *Calliphora erythrocephala*, are compared with crustacean chitosans that were prepared under various conditions. Chitosan was obtained from cuticles of *C. erythrocephala* generally under milder conditions than crustacean chitosan. When, for example, a DD of ca. 90% is required, *P. borealis* chitin must be heated in 50% NaOH for 8 h at 120°C which leads do a decline of Mv by ca. 81 %. *E. superba* chitin requires at least 3 h at 120°C and the Mv decreases by ca. 86%, whereas *C. erythrocephala* chitin requires 3 h at 100°C with a decrease in average molecular weigh by only approx. 67%. Thus, insect chitin seems to be a superior starting material when high Mv, high DD chitosan is required.

Introduction
The main source for chitosan production is crustacean chitin. The process of chitin deacetylation usually proceeds under drastic conditions: high-concentrated alkali, high temperature, and often pressure. As a final product, chitosans with various ranges of average molecular weights (Mv; from 60 to 500 kDa) and degree of deacetylation (DD; from 60% to 99%) are obtained. The aim of present work was to compare the properties of chitosan obtained from larva of *C. erythrocephala* with those of crustacean chitosans.

Material and Methods
Preparation of chitin cuticles: *Cuticles I and III:* Frozen larvae (-20°C) of *Calliphora erythrocephala* were washed with distilled water and homogenized for 15 s at 80 rpm, the slurry was filtered over a Büchner funnel and washed repeatedly. Homogenization and washing were repeated twice. The primary deproteinization process was carried out in 1450 cm^3 of a 2.5 wt% aqueous solution of NaOH for 4 h, at room temperature with stirring (400 rpm). The deproteinized cuticles were washed with distilled water until neutrality, and homogenized twice (for 30 s, at 80 rpm). A second deproteinization followed at 50 °C under otherwise unchanged conditions. Finally, the crude cuticles were washed with copious amounts of water, followed by washing with ethanol, and air-drying. The product contained some contaminants of the breeding medium for larvae (saw dust) which could be removed partially by sedimentation. In a typical example, 6.670 g (wet weight) frozen larvae yielded 867 g (dry weight; 13%) crude cuticles and 30.6 g (dry weight; 3.5%) chitin of white to light grey colour.

Cuticles II: The procedure of the purification was similar as described above, except that the temp. for deproteinization was 50 °C in both steps.

Preparation of chitosan: *Chitosan II-x/100*: Cuticles (8.1 g) were treated with a 40 wt% or 50 wt% aqueous solution of sodium hydroxide (250 cm^3) for 1, 2, 3 or 4 h at temperature of 100 °C or 120 °C with vigorous agitation. The product was washed with distilled water until neutrality and air-dried.

Sequential deacetylation of chitin: *Chitosan II-3/1/100*: Cuticles containing partially deacetylated chitosan (obtained after 3h deacetylation with 50 wt% aqueous solution of sodium hydroxide at 100 °C; 6,63 g; 82%) were treated in a second step with 50wt% NaOH at 100 °C for 1 h. Yield: 4.74 g (70.3%).

Preparation of microcrystalline chitosan (MCCh) [1]: *MCCh II-3/1/100* Chitosan cuticles 3/1/100-I (ca. 5g) were dissolved in 1 wt% aqueous hydrochloric acid (250 cm^3) for 12 h. Insoluble particles were removed by filtration through a glass sintered filter (Schott No. 2). Chitosan was precipitated from the filtrate by addition of a 2 wt% aqueous solution of sodium hydroxide to give pH 9.0. The MCCh gel-like dispersion was adjusted immediately to pH 8.0 with 1 wt% HCl (aq.). The slurry was filtered through a cheese cloth, and the MCCh was washed with distilled water to neutrality. Dehydration was achieved by treatment with ethanol (2 x 150 cm^3 of ethanol with vigorous agitation), and the MCCh was dried at 60°C and finally milled to give a powder.

The effect of the acidity of the solvent on M_V of MCCh was studied, using 1 wt% aqueous solution of acetic acid instead of HCl.

FTIR, ^{13}C-NMR and X-ray, determination of M_V, water retention value (WRV) and swelling coefficient were carried out as described in references [2,3,4,5,6,7,8,9].

Results

Comparison of chitins from various sources: Chitin samples prepared from *Calliphora erythrocephala* were compared with samples from the Northern Atlantic shrimp (*Pandalus borealis*) and from Antarctic krill (*Euphausia superba*). When the deproteinization is carried out at higher temperatures, the chitins show generally a higher DD. There is little difference in Mv (Table 1).

Table 1. Properties of chitin samples from various sources.

Type of chitin	Moisture content (%)	Mv (Da)	DD (by FT-IR) (%)	WRV (%)	Wc (%)	Cs (%)
C. erythrocephala cuticle - I	7.19	1.017.000	24.7	99.2	49.8	115.5
Cuticle - II	7.43	1.192.000	30.1	82.2	45.1	103.4
Cuticle - III	7.30	1.067.000	25.1	79.6	44.3	108.2
E. superba - I	2.78	994.000	38.7	126.4	55.8	124.1
E. superba - II	3.33	904.000	42.7	101.7	50.8	136.7
P. borealis	3.41	1.365.000	7.4	59.4	37.3	63.5

However, the chitosans prepared from chitins that were deproteinized at higher temperatures, showed a lower Mv and higher DD. The WRV values of deproteinized cuticles (chitin) were similar to those of chitins obtained from P. *borealis* shells. The Cs values of

insect chitins were considerably higher than those of P. *borealis* chitin and approached the values obtained for powdered chitin from Antarctic krill.

FT-IR spectroscopy: The IR spectra of chitin and chitosan show the following features: NH stretching at 3450 cm^{-1} which is superimposed with the OH band; absorption at 2950 cm^{-1} – 2880 cm^{-1} corresponding to aliphatic C-H stretching; Amide I band at 1660 and at 1630 cm^{-1} (singly and doubly hydrogen-bonded carbonyl groups, respectively); Amide II at 1550 cm^{-1}; superimposed C-N and N-H stretches in amide groups at 1310 cm^{-1}; and O-C-O stretching at 1150 cm^{-1} (asymmetric) and 1100 cm^{-1} – 1020 cm^{-1} (symmetric). The well resolved intensities at 1100 cm^{-1} are characteristic to highly *N*-acetylated chitin.

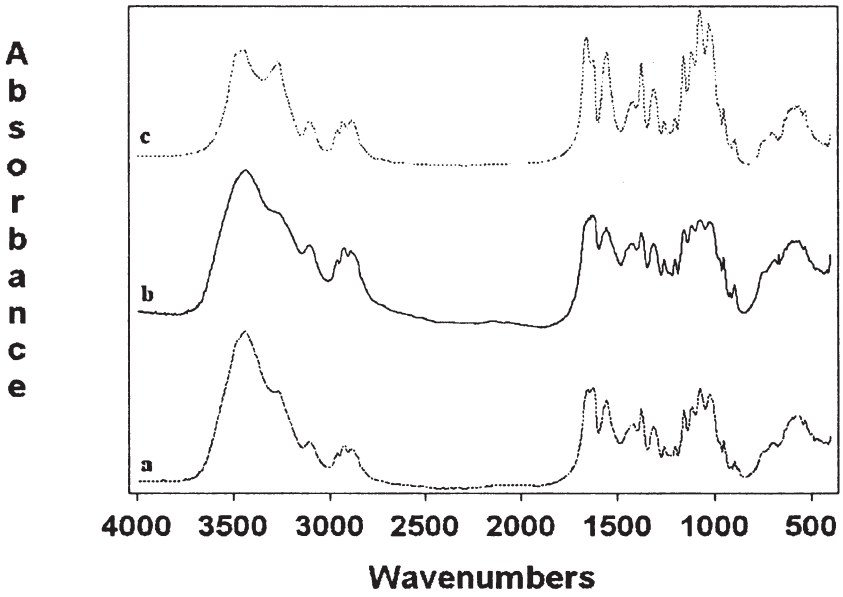

Figure 1. FTIR spectra of chitin from *E. superba* (a), chitin from *C. erythrocephala* (cuticle I), (b), chitin from *P. borealis* (c)

The IR spectra of some chitin samples are shown in (Fig. 1). The low DD (< 30%) is particularly evident in the spectrum of *P. borealis* chitin which shows well resolved hydrogen-bonded NH stretching bands of the amide group at 3120 (symmetric) and 3310 cm^{-1} (asymmetric).

Solid state ^{13}C-NMR spectra: The NMR spectra of chitins from *C. erythrocephala* (cuticle I) *P. borealis* and *E. superba* are shown in Fig. 2.

Demineralization seems to be effective, as revealed by the presence of only one carbonyl signal at *ca.* 174 ppm (i.e. absence of carbonate). The chitin samples from *C. erythrocephala* are contaminated with low amounts of protein, as indicated by the low intensity peaks ca. 30 ppm. These are also present in the NMR spectrum of chitin from

P. borealis but absent in the *E. superba* sample. The comparison of chemical shifts shown in Fig. 2 confirms that all samples are α-chitins.

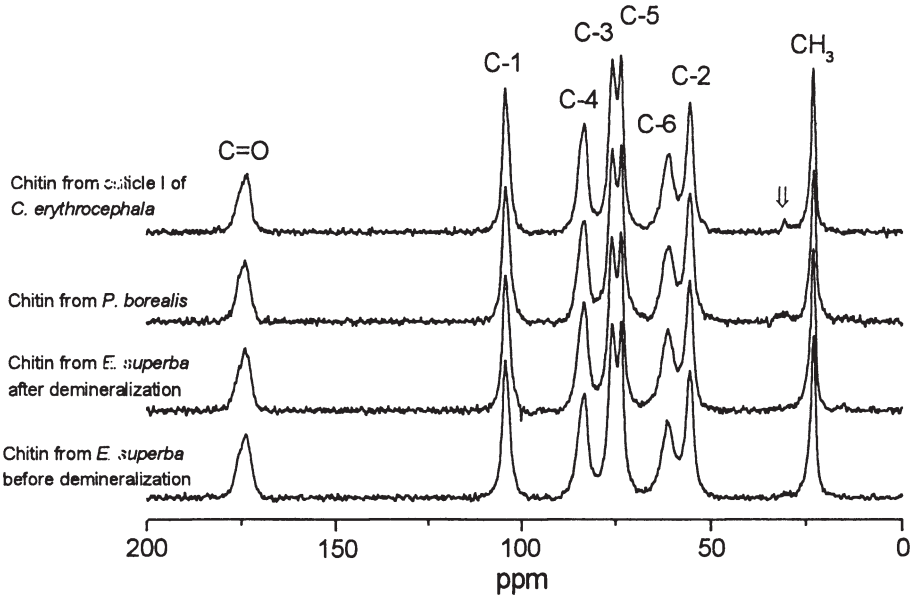

Figure 2. ^{13}C-NMR spectra of chitins from various sources; ⇓ indicates the presence of contaminants (possibly lipids).

Comparison of chitosans from various sources: Chitins obtained from larvae of *C. erythrocephala* are deacetylated generally under milder conditions as compared to chitins from Crustacea as suggested by the comparably lower degree of depolymerization during reactions under similar conditions (see Tables 1, 2, and 3). When, for example, a DD of ca. 90% is required, *P. borealis* chitin must be heated in 50% NaOH for 8 h at 120 °C which leads to a decrease of Mv by ca. 81 %, *E. superba* chitin requires at least 3 h at 120 °C and the Mv decreases by ca. 86%, whereas *C. erythrocephala* chitin requires 3 h at 100 °C with a decrease in Mv by only ca. 67%. Thus, insect chitin seems to be a superior starting material when high Mv, high DD is required. The data shown in Table 3 are represented graphically in Figs. 3-6. Fig. 7 shows the effects of the reaction conditions on WRV and Wc. The coefficients suggest that the internal surface of the biopolymers decrease with decreasing Mv after longer reaction times (< 1 h). Preparation of MCCh has little effect on Mv, but rather large increases of WRV and Wc are observed.

Table 2. Properties of chitosans from larvae of *Calliphora erythrocephala*.

Chitosan sample[a]	DD (by titration) (%)	Mv (Da)	WRV (%)	Cs (%)	Wc (%)	Moisture content (%)
Concentration of sodium hydroxide - 50 wt% NaOH aq.						
I - 3/A	78.7	352 000	141.9	170.3	58.7	8.71
I - 3/1/A	95.7	250 000	63.5	96.1	38.8	9.12
I - 3/B	96.1	83 000	104.8	128.3	51.2	9.91
II - 3/A	83.1	221 000	130.7	156.3	56.7	7.81
II - 3/1/A	96.3	189 000	27.4	100	21.5	8.81
III - 1/A	64.1[b]	775 000	133.5	170.9	57.2	6.22
III - 2/A	68.3	490 000	108.8	145.0	52.1	5.21
III - 3/A	77.3	386 000	99.8	152.2	50.0	8.74
III - 4/A	88.1	329 000	90.4	151.4	47.2	8.51
III - 1/B	68.6	384 000	117.5	147.9	54.0	8.60
III- 2/B	76.8	231 000	106.4	131.1	51.5	5.86
III - 3/B	96.7	86 000	86.2	128.7	46.3	8.87
MCCh I -3/1/A	97.6	237 000	74.5	80.6	42.7	8.83
MCCh II - 3/1/A	97.4	191 000	116.4	139.5	53.8	7.81
MCCh I - 3/B	94.5	83 000	119.1	121.7	54.4	6.69
Concentration of sodium hydroxide - 40 wt% NaOH aq.						
III - 3/A	57.0[b]	651 000	101.6	122.3	50.4	8.60
III - 4/A	70.3	599 000	94.7	125.5	48.7	8.15
III- 2/B	76.7	453 000	89.0	220.5	47.1	8.49
III - 3/B	85.1	177 000	75.2	118.1	42.9	9.04

[a] Key for sample codes: I, I, III: identifier for cuticle sample; roman figures: deacetylation time; A: deacetylation at 100 °C, B: deacetylation at 120 °C. [b] the sample was only partially soluble; MCCh – powdered microcrystalline chitosan.

Table 3. Variations of DD, Mv, and WRV of chitosans from Crustacean chitins under various conditions of deacetylation. [9]

Sample[a)]	Reaction time (h) and temp. (°C)	DD (%) (by potentiometry)	M_V	WRV (%)
PA10	7 + 3 / 100	83.3	543 000	63.2
PB8	7 + 1 / 120	89.9	248 000	65.2
PB9	5 + 4 / 120	92.5	261 000	62.8
PB10	7 + 3 / 120	98.4	227 000	56.8
M1A	1 / 100	68.2	336 000	204.5
M3A	3 / 100	73.5	187 000	171.1
M3A	1 + 1 + 1 / 100	66.3	264 000	153.6
M1B	1 / 120	67.8	273 000	162.7
M3B	3 / 120	84.2	155 000	98.5
M3B	1 + 1 + 1 / 120	86.8	135 000	99.7

[a)] Sample codes: P: chitin from *P. borealis* (DD 5%); M: chitin from krill (DD ca. 48.5%). Digits indicate reaction time, which may be two or three sequential reactions, as indicated.

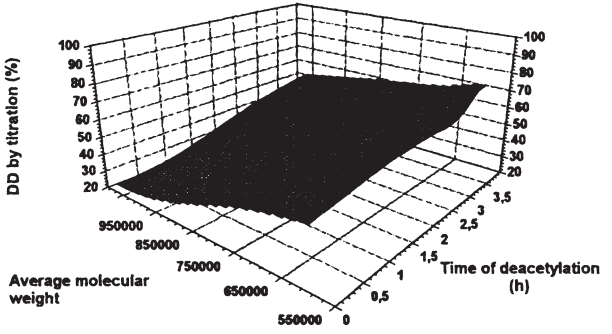

Figure 3. Effects of time of deacetylation on the reduction of Mv and increase of DD during deacetylation of insect chitin (cuticle I) at 100°C in 40 wt% NaOH.

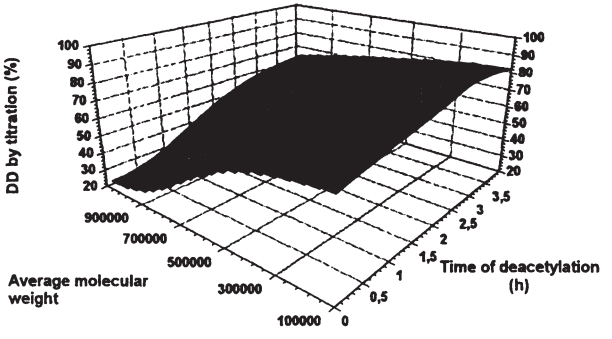

Figure 4. Effects of time of deacetylation on the reduction of Mv and increase of DD during deacetylation of insect chitin (cuticle I) at 120°C in 40 wt% NaOH.

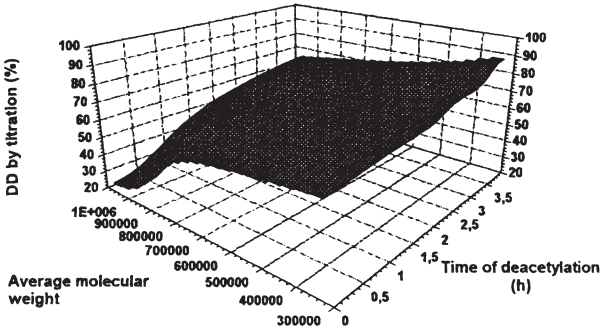

Figure 5. Effects of time of deacetylation on the reduction of Mv and increase of DD during deacetylation of insect chitin (cuticle I) at 100°C in 50 wt% NaOH

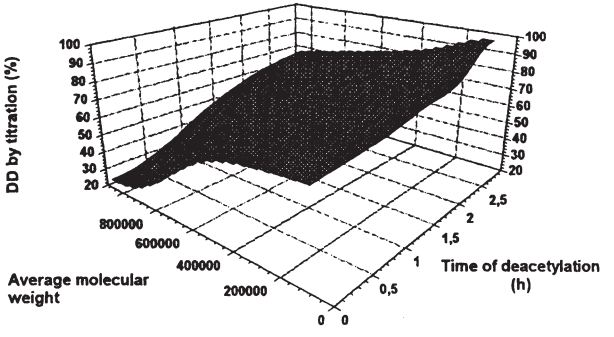

Figure 6. Effects of time of deacetylation on the reduction of Mv and increase of DD during deacetylation of insect chitin (cuticle I) at 120°C in 40 wt% NaOH.

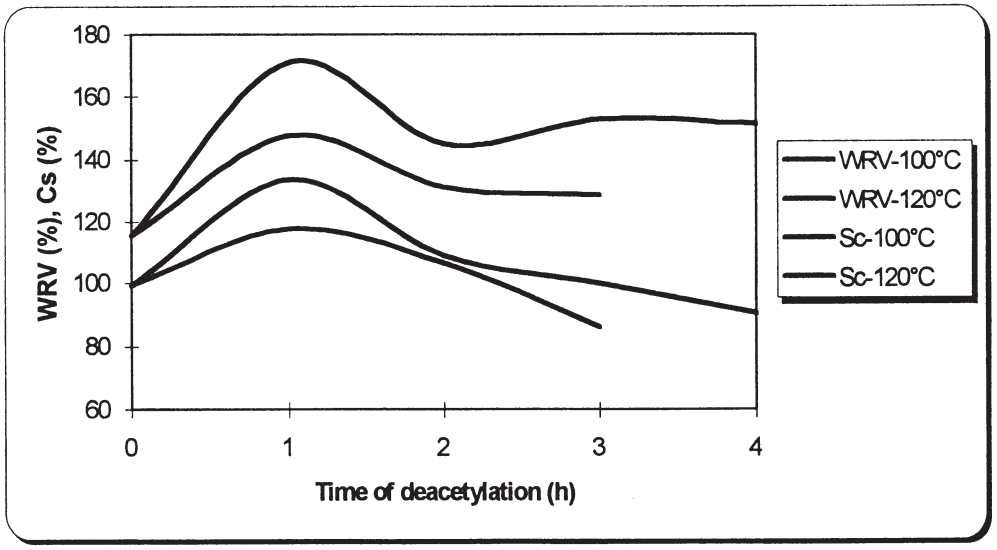

Figure 7. Change of WRV and Cs during deacetylation of insect chitins at various temperatures; 1) WRV for samples deacetylated at 120°C, 2) WRV at 100°C, 3) Cs at 120°C, 4) Cs at 100°C.

Solid-state ^{13}C-NMR spectroscopy: There are no significant differences in the appearance of the spectra of chitosans and chitins obtained from crustaceae or from insects. The NMR spectra of chitin and chitosan from insect cuticles are illustrated in Fig. 8.

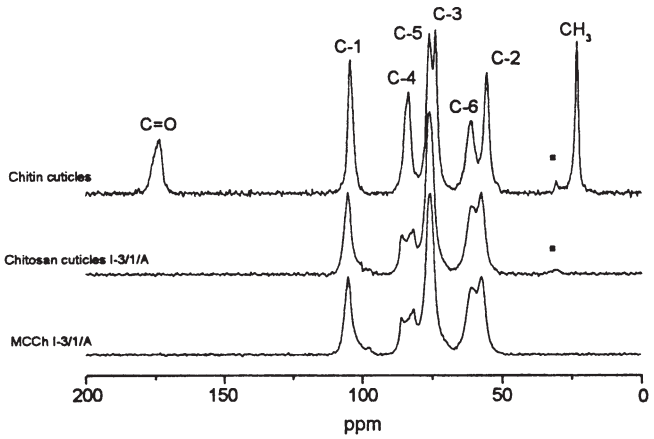

Figure 8. ^{13}C-NMR chemical shifts of chitin and chitosan from *C. erythrocephala* and of MCCh. ■: signals derived from contaminants (protein).

The high DD (89.5% and 99%, respectively) is evident from the absence of methyl (23 ppm) and carbonyl (174 ppm) signals in the chitosan samples. However, the cuticle chitosan still contains a small amount of contaminants (protein) which is absent in the MCCh. Thus, preparation of MCCh also means a higher degree of purity of chitosan.

FTIR spectroscopy: During deacetylation of crustacean chitin, the Amide I band decreases in intensity and Amide II is shifted to lower frequencies. The generation of NH_2 groups is reflected by the appearance of a new band at 1590 cm^{-1}. The NH-stretching at higher wave numbers is not resolved in highly deacetylated chitosans (Fig. 9). Similar results were observed upon deacetylation of chitin from *C. erythrocephala*. The high DD is confirmed by the shape of the NH-stretching, though carbonyl band is present, indicating the presence of protein contaminants. They are absent in MCCh.

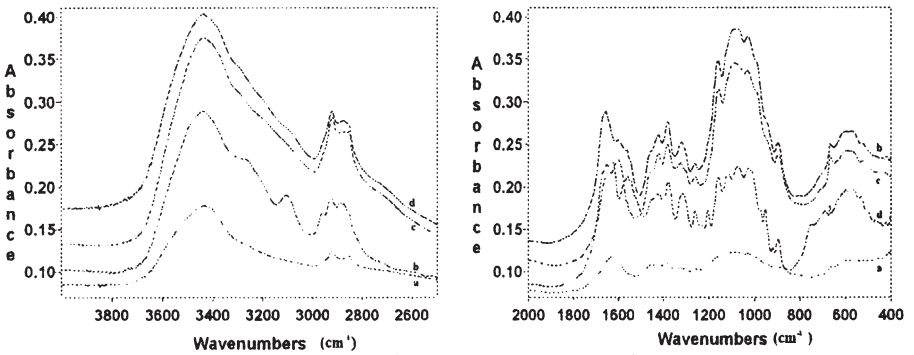

Figure 9. IR spectra (4000-2400 cm^{-1} and 2000-400 cm^{-1}) of (a) MCCh I – 3/1/A (DD=97,6%), (b) chitin from *Calliphora erythrocephala* (DD=24,7%), (c) chitosan cuticles I – 3/1/A (DD=95,7%), and (d) chitosan cuticles I – 3/A (DD=78,8%).

X-ray diffraction: The crystallinity of chitin has a strong influence on its properties, including susceptibility to hydrolysis and yield of deacetylation. Table 4 shows the CrI values calculated for various samples of chitin. The chitin cuticles from *C. erythrocephala* show the lowest CrI, which is consistent with the facility of deacetylation under relatively mild conditions. The X-ray diffraction patters reveal also some protein contaminations as has been observed by NMR and FT-IT spectroscopy (Fig. 10).

Table 4. Index of crystallinity of chitins

Sample	CrI (%)
Chitin M	73,3
Chitin Mm	67,1
Chitin P	63,5
Chitin cuticles I	39,4

Chitosan samples with DD lower than 75% are crystalline, however the crystallinity decreased gradually with higher DD. Long deacetylation times, giving chitosans with DD > 92%, resulted again in higher crystallinity, though the peak positions had shifted from 2Θ = 9,8° and 19,4° (chitin Mm) to 2Θ = 11,0° and 20,5° (chitosan M6mC). This fact suggests that the crystallinity of those chitosans increases for chain segments that already exist in the more ordered region of the chitin which are therefore susceptible to deacetylation at longer reaction times [.10,11].

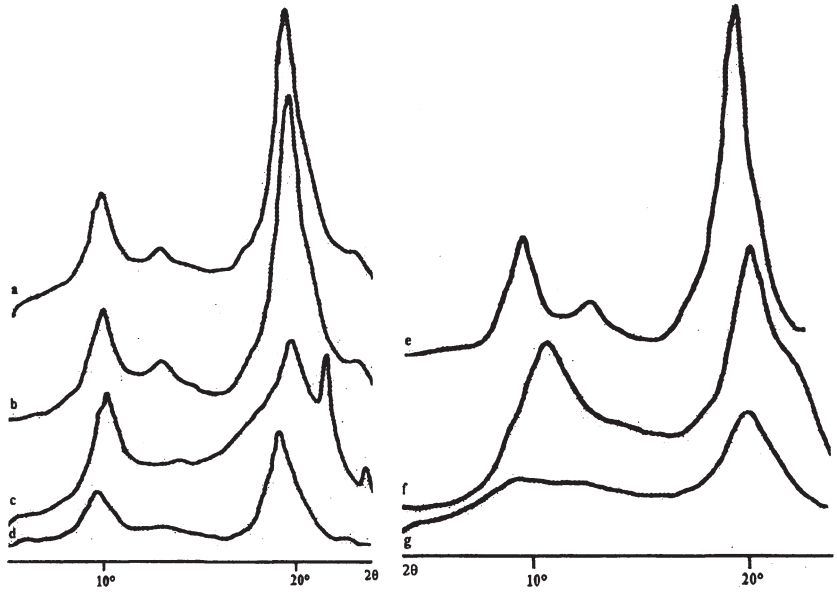

Figure 10. X-diagrams of chitins from various sources and heterogeneous deacetylated chitosan: a) - chitin from Antarctic krill, b) and e) - chitin from Antarctic krill after demineralization, c) – chitin cuticles from *Calliphora erythrocephala*, d) – chitin from *Pandalus borealis*, f) – chitosan M6mC (DD – 92.6%), g) chitosan M3A (DD – 73.5%)

Summary and Conclusions

1. The M_w values of crustacean chitosan decreases drastically with increasing reaction temperature and time, though the DD values are relatively much less affected.
2. *Pandalus borealis* is a good source for high M_w, low DD chitosan, whereas medium to high DD chitosan of medium to low M_w can ideally be prepared from krill chitin.
3. Insect chitosan is prepared under milder condition than crustacean chitosan of similar Mv and DD.
4. The crystallinity of the chitin determines the reactions conditions: low crystallinity insect chitin gives a chitosan with high DD and high Mv.
5. The WRV decreases strongly with deacetylation reaction times, especially with low crystallinity chitins.

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Effect of the rate of deacetylation on the physico-chemical properties of cuttlefish chitin

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Abstract
The chemical deacetylation of cuttlefish chitin has been carried out at temperatures between 30°C and 90° and the concentration of NaOH used was between 30~50% (w/v). The rate of deacetylation was determined by plotting the natural log of time against the natural log of the %DD. The slope is the deacetylation rate and it is termed deacetylation strength (DaS). Various deacetylation conditions, which lead to same DaS value, were studied. It is found that with the same DaS value, a higher temperature treatment will produce chitosan with higher insolubility and lower molecular weight. The distribution of the N-acetamide groups was also observed different when the deacetylation condition changed. At high temperature, the chromatogram is seen in 'L' shape, while at low temperature, the chromatograms are in asymmetrical 'V' shape, indicating that the modes of deacetylation are different.

Introduction

In the process of chemical deacetylation, chitin loses its acetyl group and is converted into chitosan. There are several factors that affect the process [1,2], including the alkali concentration, the reaction temperature, the reaction time and the method for preparation of chitin These factors together determine the outcome of the deacetylation process. For this concern, the term 'deacetylation strength' is proposed. The stronger the strength, the higher the rate of the deacetylation process. But other parameters will be affected as well, like the crystallinity [3], the distribution of the *N*-acetyl-D-glucosamine and the D-glucosamine, the chain linearity and other physico-chemical characteristics [4] when the deacetylation strength is different. Therefore, to understand the deacetylation process, it is important to study these parameters and their combined effects.

In this paper, a mathematical combination of deacetylation conditions allows a quantitative comparison of one set of condition with another. In this way, interesting physico-chemical changes in the system can be co-related and a clearer view of their inter-relationship can be obtained.

It is the objective of this paper to report the effect of the deacetylation conditions on the course of deacetylation behavior and its effect on the distribution of acetamide group along the polymeric chain, the molecular weight and its polydispersity.

Methods

The degree of deacetylation was analyzed by acid hydrolysis-HPLC [5,6], the distribution of *N*-acetamide groups by nitrous acid deamination [7] and the analysis of molecular weight was carried out by GPC.

Results and Discussion

The Effect of Treatment Conditions on the Deacetylation Behaviors of Cuttlefish Chitin

The deacetylation conditions and the deacetylation strength (DaS). The temperature and the alkali concentration are the two most critical factors that affect the deacetylation process of cuttlefish chitin. Different combinations of temperature and alkali concentration determine different deacetylation rate and influence directly the physico-chemical properties of cuttlefish chitosan. Therefore, the study of the effect of these combinations is essential, so that the deacetylation process can be handled precisely and producing cuttlefish chitosan with superior and consistent quality.

However, comparison among different combinations of temperature and alkali concentration could be more meaningful only if the factor of temperature and alkali concentration can be combined and be simplified into one value. Then the relationship among combinations can be drawn more easily and more directly.

One of the practical and precise methods for relating the temperature and the alkali concentration factors is the rate of deacetylation. As observed frequently, cuttlefish chitin deacetylated quickly to form chitosan under circumstances of high temperature and high alkali concentration; while the deacetylation process is observed much slower if the temperature and the alkali concentration is reduced. A plot of the reaction time against the degree of deacetylation (%DD) will find that high temperature and high alkaline concentration give steep slope, a mild condition will show flat slope. Since, the rate of deacetylation determines the extend of deacetylation that can be reached, it is termed the deacetylation strength (DaS).

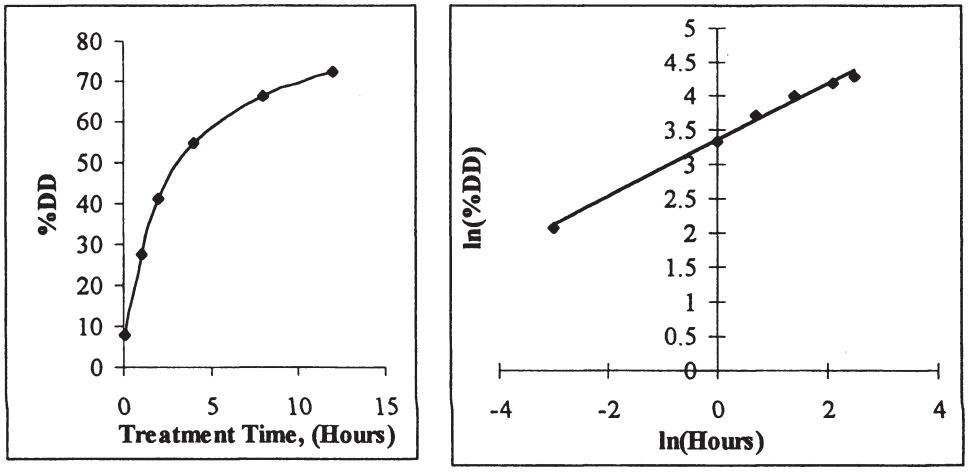

Figure 1. The determination of the deacetylation strength. The deacetylation strength is obtained by plotting the ln(Time, Hours) against the ln(%DD), its slope is DaS.

Generally, the deacetylation strength determines the deacetylation behavior of cuttlefish chitin. It is usually observed that similar DaS demonstrate close to similar deacetylation behavior throughout the range from chitin to chitosan of 70% deacetylation. A comparison has been made between temperature-alkali concentration combinations of 80°C-30% NaOH (abbreviated as 80(x)30, the 'x' being the deacetylation time), 30°C-40% NaOH (as 30(x)40) and 30°C-50% NaOH (as 30(x)50) (Figure 2). Their deacetylation strength are 0.302, 0.307 and 0.309, respectively.

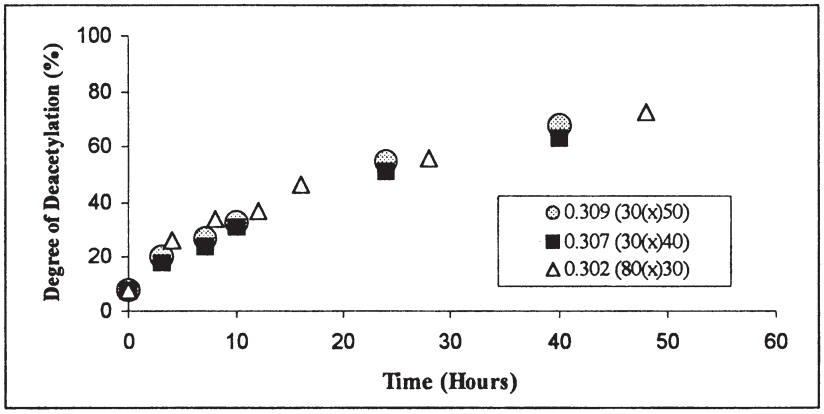

Figure 2. Plotting of the deacetylation conditions with similar DaS.

The Effect of the Deacetylation Conditions to the Distribution of N-Acetamide Groups in the Partially Deacetylated Chitin and Chitosan Samples

In this study, nitrous acid was used to cut the chains between Glu-Glu and Glu-NAG linkages through the deamination of the glucosamine moiety. The NAG-NAG and NAG-Glu linkages are remaining untouched [8,9,10]. The glucosamine moiety converts to form 2,5-D-mannose and the NAG oligomer is always attached by one molecule of 2,5-D-mannose. The expression of the oligomer after cutting is always in form of $(NAG)_{n-1}$-2,5-D-mannose, where n is the peak number appeared in the chromatogram.

The distribution of *N*-acetamide groups for various deacetylation conditions, with close DaS values are shown in Figure 3. When chitin is deacetylated by 50% NaOH, the 2,5-D-mannose peak is usually small in the sample of less than 50%DD. Therefore, the chromatogram resembles an inverted smooth asymmetrical 'V' shape. While deacetylation conducted in 30% NaOH showed a different *N*-acetamide group distribution pattern, in which an irregular distribution of *N*-acetamide groups was observed. The possible explanation for this consequence could be that the deacetylation of treatment 30(48)50 was proceeded in more random form, so that a shorter continuing unit of Glu-N was produced. When subjected to the deamination by nitrous acid, the 2,5-D-mannose was observed to have only a small peak on the GPC chromatogram, since most of them were bound to the *N*-acetamide groups. While for the treatment of 80(x)30, the deacetylation process may have proceeded in more random form, therefore, a large continuing unit of Glu-N formed. This is evidenced by the large peak of the 2,5-D-mannose.

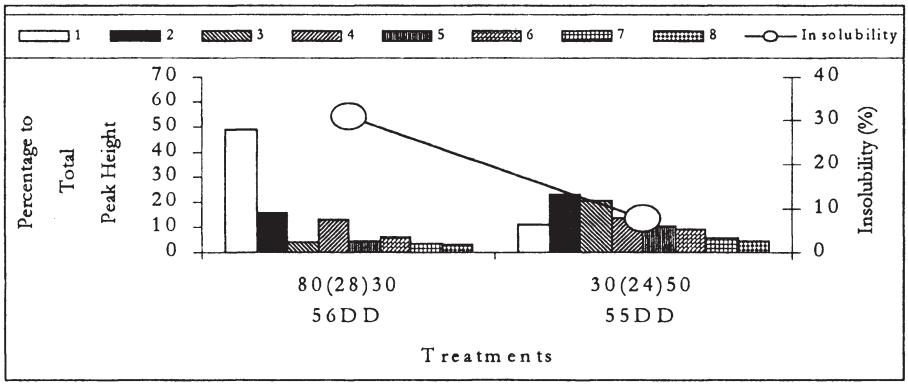

Figure 3. The effect of deacetylation conditions on the distribution of N-acetamide group. All selected deacetylation conditions are having the similar DaS value. The chromatogram usually contains 8 peaks that can be distinguished. The 2,5-D-mannose is marked by 1 and the peak containing one molecule of NAG and one molecule or 2,5-D-mannose is marked as 2, and so on.

Effects of the Deacetylation Conditions to the Molecular Weight of Chitosan

As shown by the figure, all treatments of deacetylated at 30°C are having peak molecular weight (Mp) of around 2 MDalton. The rate of chain degradation, i.e. the reduction of molecular weight over time (hour), for treatments at these range of temperatures are 0.0024 and 0.0013 Mdalton/hour for treatments 30(x)50 and 30(x)40, respectively. For treatment 80(x)30, the peak molecular weight at 48 and 96 hours of deacetylation are 1.78 and 1.63 Mdalton, respectively. Additionally, the treatment conducted at this temperature, even though at much lower alkali concentration, has demonstrated a faster rate of chain degradation, i.e. 0.0031 Mdalton/hour. This showed that the temperature factor is the determining factor for the chain degradation.

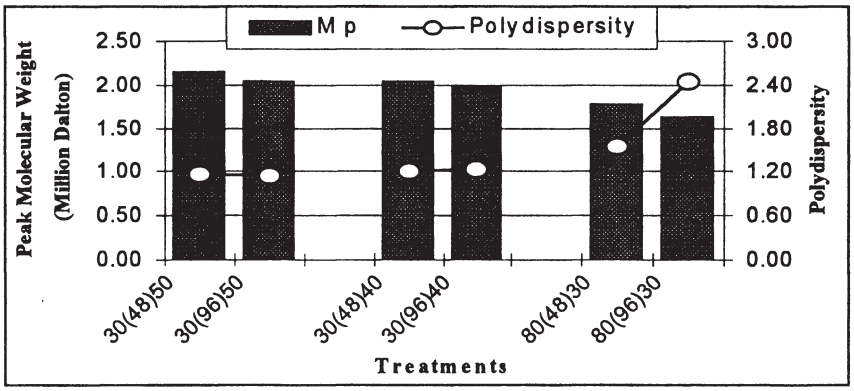

Figure 4. The effects of deacetylation conditions on the peak molecular weight and polydispersity of chitosan

Another important feature that can be extracted from the graph is the polydispersity. It is observed that the values of the polydispersity increase as the temperature increase. For treatments at 30°C, the polydispersity values are about 1.2 for both 40 and 50% NaOH. The increment of the polydispersity, over a period of 48 hours, for all treatments at 30°C also found negligible. For the 80°C treatment, the polydispersity for the 48 and 96 hours treatments were 1.54 and 2.43, respectively, indicating that the chain degradation was occurred vigorously.

Conclusions

Different deacetylation conditions may have similar deacetylation strength (DaS), but lead to chitosan with different physico-chemical properties. Results showed that different deacetylation combination affects the distribution of the N-acetamide groups greatly. High temperature is the most destructive factor for the degradation of molecular weight of chitosan.

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Deacetylation of chitin by fungal enzymes

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Summary

The aim of this research is to design a method on technical scale for the enzymatic deacetylation of chitin into chitosan. Five fungal strains have been compared for their ability to produce extracellular chitin deacetylase. Three strains, *Colletotrichum lindemuthianum, Aspergillus alliacus* and *Aspergillus nidulans* produce an extracellular deacetylase that can act on natural (10% deacetylated) chitin whereas the extracellular enzyme from *Absidia coerulea, Mucor rouxii* and *Absidia glauca* cannot use natural chitin but act only on partially deacetylated chitin (maximum activity with around 60% degree of deacetylation (DD)). The enzymes have been further characterized by their optimal temperature and pH for enzymatic deacetylation, their thermostability, their sensitivity to the reaction product acetate and for the presence of chitinase. The best enzyme producing strains are *Absidia coerulea* and *Colletotrichum lindemuthianum*. *Absidia* has the advantage that it can be produced easily (maximum activity was produced within 3 days) but cannot act on natural chitin. *Colletotrichum* has the advantage it can act on natural chitin but is less attractive due its plant pathogenicity and its slow growth (maximum activity was produced after 5 days).

The conditions for larger scale application of *Absidia* have been investigated more in detail. In a synthetic medium containing standard amounts of inorganic salts, glucose and lactose appeared to be efficient carbon sources. Yeast extract (2%) gave much better production as compared with peptone or corn steep liquor. Growth was optimal at 30°C, at pH in the neutral range above 4.5 and in the presence of a low amount of detergent. Enzyme levels were significantly enhanced if chitin was included in the medium. This inducer of enzyme activity is most effective at a degree of deacetylation of about 60%. In bioreactor studies, higher CDA levels are produced at higher airflow rate. The role of partially deacetylated chitin as inducer has been confirmed.

Introduction

Chitin, a homopolymer of β(1→4)-linked N-acetylglucosamine, is one of the most abundant, easily obtainable, and renewable natural polymers, second only to cellulose. It is commonly found in the exoskeletons or cuticles of many invertebrates and in the cell walls of most fungi and some algae. Chitin exists in several Zygomycetes species in its deacetylated form referred to as chitosan. Chitin is an extremely insoluble material and has yet to find important industrial applications, whereas chitosan is water soluble and a much more tractable material with a large number and a broad variety of reported applications. At present, industrial manufacture of chitosan is performed by deacetylation of purified chitin with concentrated alkali and high-temperature treatment. Using this preparative method, it is difficult to avoid

degradation of the polymer chain and to circumvent environmental pollution problems. Thus, either enzymatic deacetylation of chitin or utilization of natural chitosan has to be considered especially when a controlled, nondegradative, and well-defined process is required.

As is well known, the chitosan existing as a major component of the cell wall of Zygomycetes is synthesized through the tandem action of chitin synthetases and chitin deacetylase (CDA). In this pathway, chitin synthetase polymerizes N-acetyl glucosamine precursor molecules to chitin; chitin deacetylase catalyzes the deacetylation of the nascent chain of N-acetyl glucosamine residues. CDA plays a very important role in fungal growth of *Zygomycetes*. This action of chitin deacetylase could be very useful industrially in the enzymatic production of chitosan. Chitin deacetylase (CDA) has been purified to homogeneity from mycelia extracts of the fungus *Mucor rouxii* by Kafetzopoulos et al. [1]. The intracellular CDA was also purified from a fungus, *Absidia coerulea*, and characterized by Xiao-Dong et al. [2]. An extracellular chitin deacetylase activity has been purified to homogeneity from autolyzed cultures of *Aspergillus nidulans* and *Colletotrichum lindemuthianum* and further characterized by Carlos et al. [3] and Tsigos et al. [4], respectively.

In this study, chitin deacetylase was produced from different strains and compared using enzymological criteria. Enzyme preparation has been improved by modifying fermentation conditions and partial purification. Finally conditions for scale up of biocatalytic deacetylation of chitin have been investigated.

Materials and Methods

Since there are advantages and disadvantages associated with enzyme located within or outside the cells, both extra- and intracellular chitin deacetylase (CDA) have been tested for all CDA containing strains that were available for this study, namely, *Mucor indicus, Mucor hiemalis, Mucor sp, Aspergillus sajae, Aspergillus awamori, Aspergillus alliacus, Aspergillus usamii van shirousamii, Aspergillus clavatus, Aspergillus sp, Aspergillus foetidus, Aspergillus kawachii, Absidia coerulea, Aspergillus nidulans, Absidia glauca, Cunnighamella echinulata, Gongronella butleri, Rhizopus oryzae, Mucor rouxii, Colletotrichum lindemuthianum*. The cultures were allowed to grow for 5 days for spores formation in the agar slants at 30° C. Spores were harvested by rinsing the culture slants with sterile 0.01% Tween 80 solution and collecting the spore suspensions in a sterile test tube. The spore suspension of each species (10^6 spores/ml) was grown in 250 ml flasks containing 50 ml of medium (2% glucose, 1% peptone, 0.1% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% NaCl, 0.5% $MgSO_47H_2O$ and 0.01% $CaCl_2$ in distilled water at pH 4.5) and incubated at 30° C and 150 rpm. The extracellular enzyme activity of chitin deacetylase grown for 1, 3, 5 days was determined.

Mycelia and extracellular enzyme were separated by filtration through a glass funnel. The filtrate was used as extracellular enzyme. The mycelia were washed with distilled water until the filtrate was clear. The intracellular enzyme was extracted from 0.1g freeze dried mycelium by grinding with mechanical homogenizer (Poly Science UAC 24R, 25,000/min) in 10 ml of 0.02 M phosphate buffer at pH 5.8 for 3 min. The extract was suspended in buffer by gently stirring during 1 hr at 4° C and centrifuged to obtain crude CDA enzyme as a clear supernatant. This supernatant has been used without further purification. Enzyme activity was measured in a standard incubation mixture containing 50 mg of chitin/ chitosan substrate (between 60 and 100 mesh size), 1ml enzyme preparation and 5ml phosphate buffer (pH 5.8). The acetic acid released from chitin by CDA was analyzed by gas chromatography (glass column containing 80/120 carbopack BDA, FID detector, 175, 200, 200°C for column, injector, and detector temperature, respectively) Enzyme activity was expressed in units, one unit being

the amount of enzyme that liberates 1μmole acetic acid per min under standard conditions.

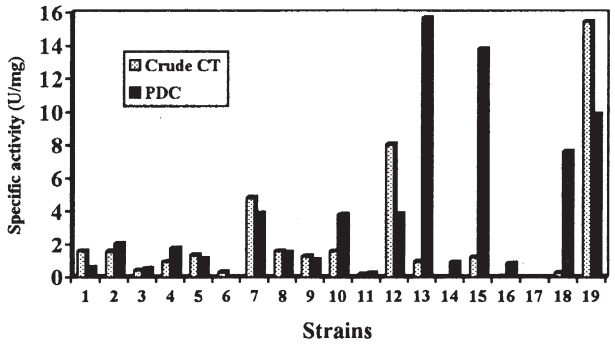
Six different strains which produce highest activity have been characterized for optimal temperature, thermostability, optimal pH, sensitivity to acetic acid inhibition, and chitinase activity.

The organism chosen from the above experiments is *Absidia coerulea*. This fungus is capable to grow on cheap, simple media without the need to add expensive growth promoters. The principal factors have been investigated that influence the course of enzyme fermentation: culture temperature, pH, and the dissolved oxygen tension, which itself is a function of the aeration rate and agitation rate applied to the fermentor system. Many enzymes are synthesized in larger quantities only in the presence of a specific inducer. The following materials have been used as inducer: Crude chitin powder, 30-70% degree of deacetylation (DD) partially deacetylated chitin. Surfactants such as Tween 80 can effect enzyme yield and enhance the release of extracellular enzyme from microbial cells. Therefore, the various concentration of Tween 80 (100 - 500 PPM) have been tested as a surfactant to release more extracellular enzyme from the cell.

The nutrient deficiencies as well as physiological constraints can be the major limiting factors in bioprocessing. Absence of reaction to the addition of a particular nutrient means that nutrient is not limiting whereas an increase of growth or of CDA activity after addition shows that the nutrient considered is present in growth limiting amounts. Therefore, the optimization of carbon source has been investigated by using starch, maltose, sucrose and lactose instead of glucose. Similarly, peptone, yeast extract, ammonium sulfate, corn steep liquor, malt extract have been compared as nitrogen source for optimization. The compositions of the optimized carbon and nitrogen sources have been varied in the range of 0.5 to 10% to increase the production of CDA.

Results and Discussion

Comparison of CDA activity produced by different strains: As shown in Fig 1, chitin deacetylase (CDA) activity varied among the fungi evaluated. *Absidia* species produce CDA which have highest extracellular specific activity. Among *Absidia* species, *A. coerulea* CDA (15.61U/mg) has higher specific activity than *A. glauca* one (13.78U/mg). The specific activity of *C. lindemuthianum* with chitin as substrate (15.4 U/mg) is also comparable to that of *A. coerulea*. The following highest specific activity was produced by *M. rouxii* (7.59U/mg). *A. coerulea* also produces highest extracellular total CDA activity (23.41 μmole/min) among all strains. The following highest total activity was produced by *M. rouxii, C. lindemuthianum, and A. glauca* (22.77, 20.03, and 13.78 μmole/min, respectively). Extracellular specific and total activities are much higher than the data for the intracellular enzyme (Table 1). Moreover extracellular enzyme has the advantage that the fungal mycelium does not have to be disrupted and that the CDA does not get mixed with intracellular protein. So extracellular enzyme (broth) was selected and characterized further. It is remarkable that some fungi have a chitin deacetylase that is more actives on chitin as compared with chitosan. This has been found for *Colletotrichum, Aspergillus kawachii* and *Aspergillus alliacus*.



1: A. *nidulans* 6: *A. awamori* 10: *A. sp* 15: *A. glauca*
2: *M. indicus* 7: *A. alliacus* 11: *A. foetidus* 16: *G. butleri*
3: *M. hiemalis* 8: *A. usamii van* 12: *A. kawachii* 17: *C. echinulata*
4: *M. sp.* *shirousamii* 13: *A. coerulea* 18: *M. rouxii*
5: *A. sajae* 9: *A. clavatus* 14: *R. oryzae* 19:*C. lindemuthianum*

Figure 1. Comparison of extracellular specific CDA activity produced by different strains (left bar = crude chitin substrate, right bar = 58%DD chitin substrate)

Table 1. Comparison of specific and total activity for four strains, which produce highest CDA

Strains	Extracellular CDA		Intracellular CDA	
	Specific activity	Total activity	Specific activity	Total activity
A. Coerulea	15.61	23.41	1.67	15.78
A. Glauca	13.78	13.78	2.7	4.30
C. Lindemuthianum	15.41	20.03	.22	0.56
M. rouxii	7.59	22.77	1.25	7.52

Specific activity = Enzyme activity (U)/mg protein
Total activity = U enzyme/ml* volume (100mL)

Optimization of carbon and nitrogen sources: It is highly important to formulate a medium for enzyme production that is both cheap and where possible, gives constant production over a long production period. To investigate various carbon sources in the fermentation medium, glucose has been replaced by different carbon sources as shown in Fig 2(a). The medium with mannose and lactose as carbon source produced the highest CDA after about 80 hours. The lag phase of the mannose CDA production is longer than that with lactose as carbon source. In a next series of experiments, lactose and glucose have been compared. The medium with 4% of glucose produced more CDA than that with 2% of lactose (33.7 and 24.3 μmole/min, respectively) by using optimum conditions and medium composition (30°C, pH 4.5, 2% of yeast extract and inorganic salts). So, glucose was selected as a carbon source for the production of CDA.

Similarly, using different nitrogen sources the fermentation medium has been optimized for the production of CDA. Only one source of nitrogen has been added to the medium when nitrogen source was varied. It could be seen clearly that the highest CDA is produced by yeast extract and then followed by peptone and corn steep liquor (Fig 2b). Regarding morphology, the three nitrogen sources with higher CDA values produce thick mycelium whereas the other sources are giving only thin hyphae and pellet form. Ammonium sulphate is giving very low activity .

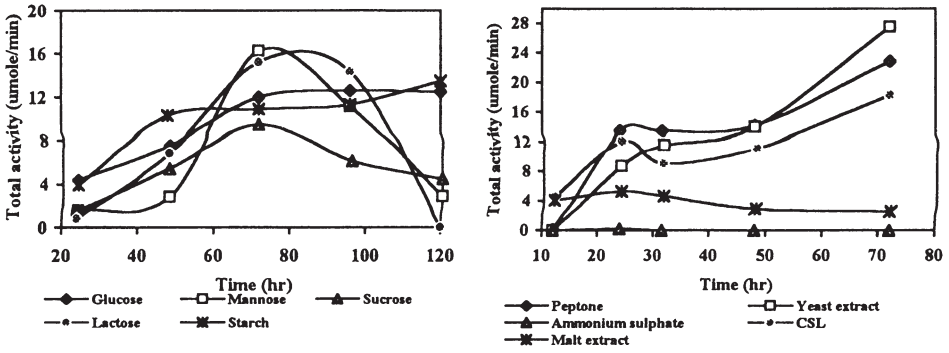


Figure 2. Optimization of carbon source (2% w/v) (a; left) and nitrogen source (2%w/v) (b; right) for the production of CDA.

Effect of temperature and pH on the Absidia *CDA production*: The optimum temperature for the synthesis of a particular enzyme may differ from the optimum temperature for growth. The optimum temperature has been determined by varying the fermentation temperature in the range 20 - 35° C in a rotary shaker. As shown in Fig 3(a), highest total CDA activity is produced at 30°C during 24 hours cultivation. The enzyme activity is lower when the fungus is grown at lower temperature. The fungus, *Absidia coerulea*, could not be grown at 35°C.

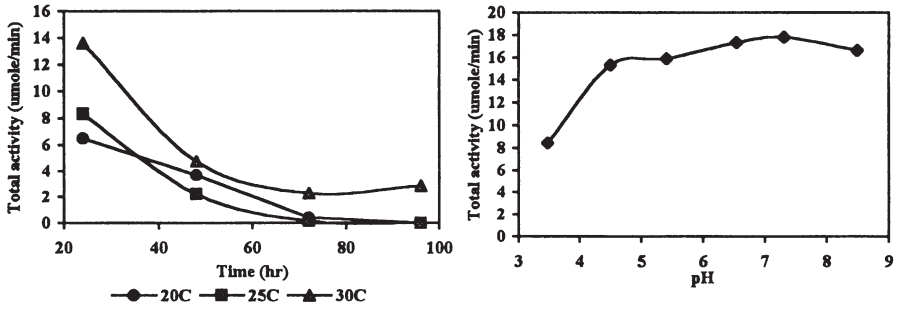


Figure 3. Effect of fermentation temperature (a, left) and medium pH (b, right) on CDA production

For experiments on the effect of pH on CDA production, series of flasks in the range of pH 3.48 to 8.5 have been prepared. At low pH, there is effect of pH on CDA production but above pH 4.5 CDA production is unaffected (Fig 3b). This is the opposite trend with the optimum pH of *Absidia* CDA for substrate. The growth of microbial cells is also influenced by pH and, in many cases, the optimum pH for the achievement of the maximum specific growth rate of the organism differs from the optimum pH for stability of an extracellular enzyme [5].

Effect of induction and surfactant: Fig 4(a) explains that chitin and chitosan are inducer for CDA production. Partially deacetylated chitin with 58% degree of deacetylation, which is the most specific substrate among the partially deacetylated chitins (Stevens et al. 1997), is also the best inducer. The second comparable inducer tested is 70% degree of deacetylated chitosan. Another observation is that the fermentation medium with 58% degree of deacetylated chitin contains the highest concentration of acetic acid (data not shown), probably produced by CDA during fermentation using the inducer added to the medium as substrate.

Although the medium without surfactant and with 100 ppm of Tween 80 produced nearly the same amount enzyme, CDA activity increased with higher Tween 80 concentration as shown in Fig 4(b). These effects may be due to modification of the cell membrane by surfactant causing an increase its permeability and leakiness to the enzyme. Larger amounts of surfactant may affect the enzyme production.

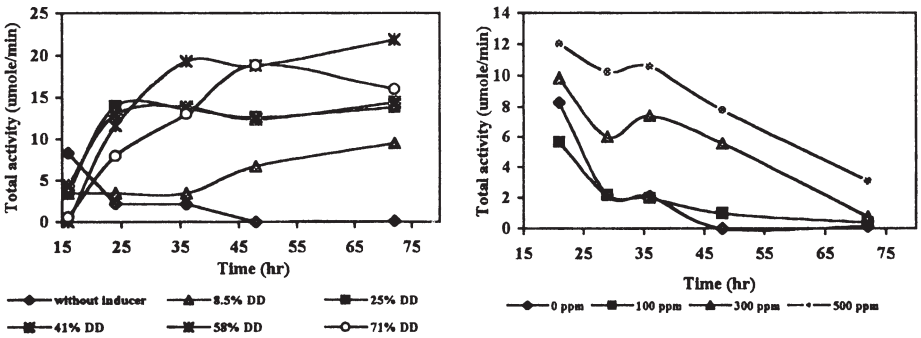


Figure 4. Effect of induction (a; left) and surfactant (b; right) on CDA production

Effect of aeration in bioreactor with controlling pH: Control of aeration and pH throughout the fermentation are two advantages in using a bioreactor. In one liter bioreactors, the airflow rate was varied in the range of 0 - 0.4 L/min. Partially deacetylated chitin (1% of PDC, 58% DD) was added to the reactors with 0.05 and 0.1L/min airflow rate. Fig 5 shows that *Absidia coerulea* requires air to grow because there is no mycelium at 0 L/min airflow. The reactor with 0.2 L/min air flow rate produced more CDA than 0.1 L/min reactor while the media containing 58% degree of deacetylated chitin were giving the remarkable higher activity than the media without inducer. Therefore, it could be clearly concluded that higher CDA levels are produced at higher airflow rate and PDC is an inducer for *Absidia coerulea* to produce CDA.

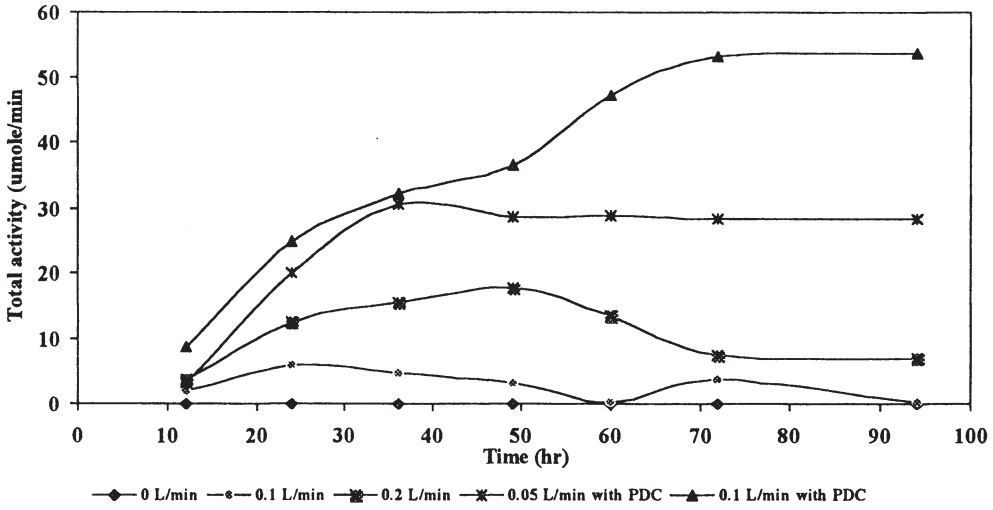


Figure 5. Effect of Aeration in Bioreactor with controlling pH

Conclusions

Among the strains available, *A. coerulea, A. glauca, C. lindemuthianum, M. rouxii* have been selected, based on their specific and total CDA activities. Extracellular specific and total activities are much higher than the intracellular activities. Extracellular enzyme (broth) has been selected for further characterization also because they do not require that the cells be disrupted and separated subsequently although they require a relatively high concentration factor. Fermentation conditions and medium compositions of *A. coerulea*, which produces the highest CDA activity, have been optimized. The highest CDA activity was produced at 30°C and pH 4.5. *Absidia* CDA is inducible by 58% DD chitin, which is the best partially deacetylated chitin substrate. Surfactant (Tween 80) also enhanced the release of CDA into the broth. The optimum carbon and nitrogen sources are glucose and yeast extract, respectively. The standard fermentation conditions and medium composition have been selected as follows: 30°C, 100 rpm, 2% yeast extract, 4% (or 2%) glucose, 70 or 58% DD chitosan (0.1% K_2HPO_4, 0.1% NaCl, 0.5% $MgSO_47H_2O$, 0.01% $CaCl_22H_2O$). In the bioreactor, it can be clearly demonstrated that chitosan is an inducer *for Absidia coerulea* to produce CDA. Higher airflow rates give the higher CDA activity if pH is controlled carefully. By modifying the fermentation conditions and medium composition, CDA activity could be improved from 80 to 330 and then increased to 530 in total activity/L broth by using bioreactor and controlling pH.

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Production of partially degraded chitosan with desired molecular weight

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Summary
Chitosan, a polymer of 2-deoxy-2-amino-D-glucose can be chemically prepared by *N*-deacetylation of naturally occurring chitin. Partially degraded chitosans have been obtained by oxidative degradation at ambient temperature. This product can be prepared by oxidative degradation of 1% chitosan in 1% acetic acid with aqueous 0.1% $NaNO_2$ solution. A desired molecular weight of the product can be obtained by controlling the ratio of nitrite to chitosan. The molecular weight of the product depends mainly on the ratio of nitrite to chitosan.

The optimal amount/percentage of $NaNO_2$ to a particular quantity of chitosan is discussed. This paper reports the synthesis of partially degraded chitosans at ambient temperature by oxidative degradation with $NaNO_2$.

Introduction

Chitosan or polyglucosamine is prepared by N-deacetylation of chitin. Chitosan solutions are viscous aqueous systems. A 1% solution of chitosan is enough to give a viscous solution (system). Various chemical procedures for preparing chitosan with low molecular weight are acidic hydrolysis and oxidative degradation with $NaNO_2$ or with H_2O_2 [1,2]. Much attention has been paid to prepare partially hydrolyzed chitosans for increasing the concentration of chitosan solutions. Concentrated form of chitosan is especially for applications in the field of food industry, pharmacy and medicine [3]. The partially degraded chitosan can be obtained either by chemical or enzymatic hydrolysis. It has been reported that nitrous acid reacts with chitosan causing deamination and depolymerization [4]. The present paper reports the synthesis of partially degraded chitosans at ambient temperature by oxidative degradation with $NaNO_2$.

Materials and Methods

Chitin was prepared from shrimp shell in our Laboratory. Chitosan was prepared from chitin by deacetylation in the Laboratory (see chart, p. 64). The molecular weight was from 2×10^6 to 3×10^6. All other chemicals used were of reagent grade. Chitosan was dissolved in 1% aqueous acetic acid solution to prepare a 1% solution of chitosan (w/v). The oxidative degradation of chitosan in solution by aqueous 0.1% $NaNO_2$ solution with a ratio of nitrite to chitosan 1:10 to 1:100 was performed for a period of 1 to 7 hrs. The reaction was carried out at room temperature ($29 \pm 1^\circ C$).

The viscosity of chitosan samples in acetic acid solution were measured using a digital viscometer (Brookfield Model DV - II) at 25°C. The viscosity of chitosan sample solutions

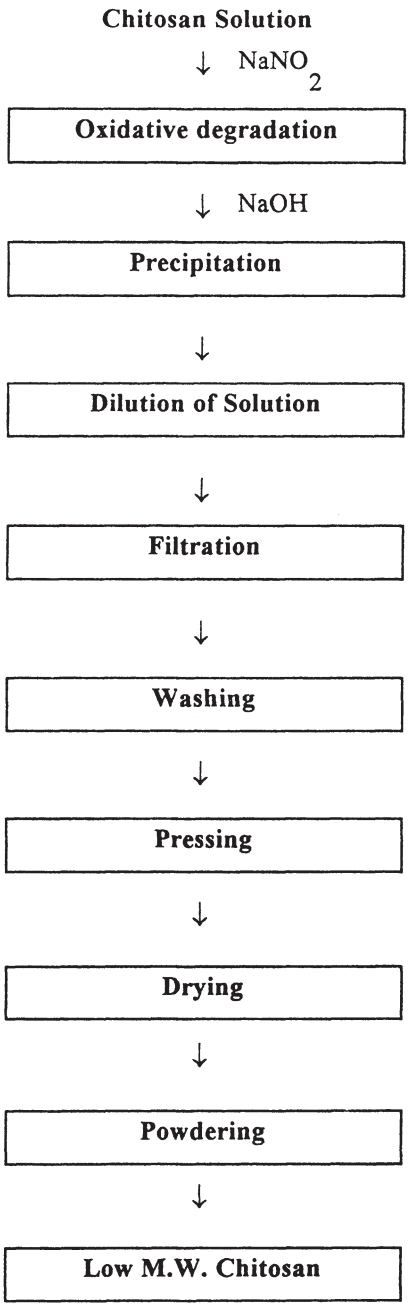

Fig 1. A schematic representation of the preparation of low molecular weight chitosan by oxidative degradation.

were measured with an Ubbelohde Viscometer also. The molecular weight of chitosan was calculated from the Mark-Houwink equation.

The method for preparing low molecular weight chitosan by oxidative degradation involves oxidative degradation of chitosan solution with $NaNO_2$, then precipitation with NaOH. This precipitate has to be filtered, washed and dried. The product will be low molecular weight chitosan.

The partially degraded chitosans with desired molecular weight were prepared according to the scheme outlined in Figure 1.

Results and Discussion

The results of the analysis are presented in Table 1 and Figure 2. There was considerable reduction in the viscosity of chitosan after oxidative degradation which gives low molecular weight chitosan. The viscosity decreased as the ratio of $NaNO_2$ to chitosan increased. Of the different treatment periods, 5 hrs. treatment was found ideal for oxidative degradation process. The degree of deacetylation was above 85% for the degraded chitosans after 5 hrs. of treatment. The oxidative degradation using a nitrite to chitosan ratio of 1:50 decreased the molecular weight of chitosan from 2×10^6 to 2×10^5.

Homogenous depolymerization of chitosan can be achieved by oxidative degradation of chitosan with $NaNO_2$. The amine groups are attacked by the nitrosating species originating from the nitrous acid and the nitrite is being converted to nitrogen gas, thus the reagent is conveniently eliminated from the reaction mixture. Oxidative degradation caused by the reaction of amine groups with $NaNO_2$ favours breaking of long chain chitosan molecules to a shorter length. For each molecule of $NaNO_2$ consumed, a chitosan chain was cleaved at one point by a random stoichiometric cleavage [4,5].

The partially hydrolysed chitosan can be prepared quickly by homogenous oxidative degradation of chitosan solution with aqueous sodium nitrite solution. The molecular weight of chitosan can be controlled by varying the amount of $NaNO_2$ in oxidative degradation. This type of chitosan permits the preparation of relatively compact forms of product and thus the transportation costs can be reduced. By oxidative degradation a series of chitosan products of low molecular weight are obtained which can be used for the preparation of microcapsules containing living cells, such as red blood cells [6] and animal liver cells [7]. In order to use chitosan safely in medical fields, the sodium nitrite must be carefully removed.

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Table 1 : Method for preparing low M.W. Chitosan by oxidative degradation with sodium nitrite

Sl No.	Wt. of parent Chitosan (g)	Viscosity of parent Chitosan (cps)	Ratio of 0.1% $NaNO_2$ soln. to 1% Chitosan soln.	Period of reaction (hrs)	Wt. of oxidatively degraded chitosan (g)	Viscosity of Oxidatively degraded Chitosan(cps)
1	10	420	1:100	5	9.9	69 ± 0.53
2	10	420	1:70	5	9.8	38 ± 0.52
3	10	420	1:50	5	9.8	32 ± 0.52
4	10	420	1:30	5	9.6	30 ± 0.54
5	10	420	1:10	5	9.6	27 ± 0.53

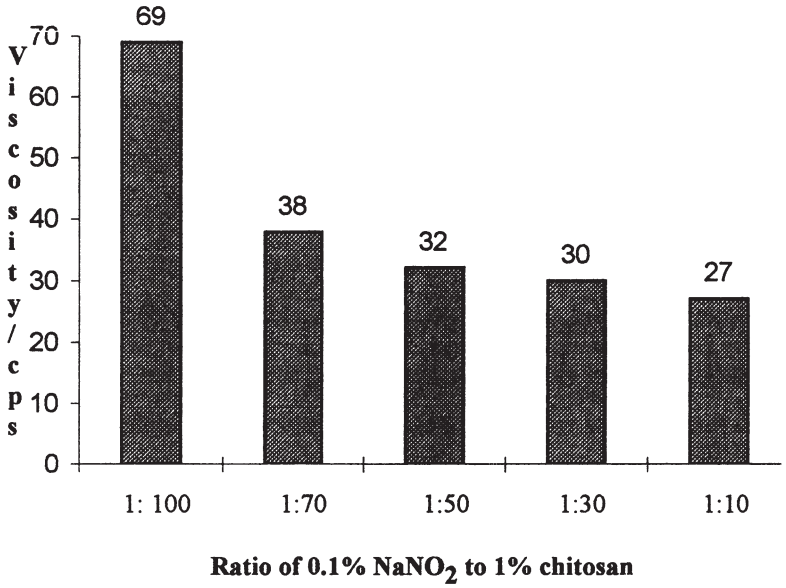

Fig 2 : Method for preparing low M.W. Chitosan by oxidative degradation with sodium nitrite

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Chitin-containing materials *Mycoton* for wound treatment

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Summary
"Mycoton" is the name of new chitin-containing fibrous materials made of cell walls of the fungi (*Higher Basidiomycetes)*. Mycoton can be used for wound treatment in the form of a powder, as a cotton-like material and as a non-woven napkins. The clinical approbation of Mycoton was carried out in the Center of Liver Surgery and in the Clinic of Department of Obstetrics and Gynecology of National Medical University in Kiev. Mycoton inhibits the development of pathogenic bacteria such as *Staphylococcus aureus, St. epidermidis, St. hemoliticus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris.* Chitin adsorbs wound toxins and bacteria. They have hemostatic and anesthetic properties. The important properties of a new dressing materials are atraumaticity and biodegradability in alive tissues. As a result Mycoton reduces patient's stay at a hospital for 5-7 days.

Introduction

Cotton-wool, bandages and gauze, being products of natural cellulose fibers from the cotton plant, are widely employed as a bandaging material. Among other natural materials able to compete with the cotton plant, chitin has many indices favorable for the use in medicine. There are a great number of patents on the production of bandaging materials from crustacean chitin. But the known technologies for the production of fibers from such raw material are rather complex and expensive.

Fungi are other source of chitin. Known are methods for producing chitin dressing materials of fungal source (Pat. DE N 2923802, 1979; Pat. GB N 2165865, 1986; Pat. EU N 460774, 1991; Pat. JP NN 04.343762, 1992; 04.300362, 1991). But fungi-producers used for this purpose and methods of the fungal biomass from lower fungi treatment do not permit obtaining qualitative materials.

The found approach of this task makes it possible to produce from fungal biomass of *Higher Basidiomycetes* the chitin hollow fibres 3-5 mkm in diameter which have good elasticity and strength both in the humid and dry states (Pat. Russia 2073015, 1997). These fibers permit obtaining paper-like and nonwoven sheet dressing materials which were named "Mycoton" [1, 2]. They also may be used as a substitute for cotton-wool and stitching materials for covering wounds, burns and ulcers. The investigations on laboratory animals and clinical tests on patients have shown that Mycoton is intoxic and has styptic and analgetic

properties, inhibit the inflammatory processes, prevent suppuration and accelerate healing of wounds. These materials are biodegradable in human tissues remaining after wounds no scars and also have effects similar to tattoo.

Materials and Methods

Chitin-containing materials Mycoton can be used for wound treatment in the form of a powder, as a cotton-like material and as non-woven napkins. Before applying Mycoton the primary surgical treatment, necrotomy and toilet of a wound with antiseptic solutions such as 3% peroxide , furacillin (1:5000) and others with the subsequent drying by gauze tampons were performed for all patients. Mycoton in the form of a powder and cotton-wool was used as applications to the wound surface by a thin layer (1-3 mm). Mycoton napkins have thickniss of 0.1 and 0.2 mm. The remedy being fixed with the aseptic gauze bandage. The preparation was used from the moment of the rise of the purulent process in a wound up to the origin of granulations. The further treatment of the wound was carried out according to the principles of purulent surgery.

The clinical approbation of Mycoton was carried out at the Kiev Center of Liver Surgery in 87 patients aged from 24 to 75 (main group 1) and at the Clinic Department of Obstetrics and Gynecology of National Medical University in 24 women with traumas of perineum after child birth (main group 2). The action of Mycoton in treatment of patients of main groups is compared by all clinical and laboratory indices with the wound process in patients of similar control groups 1 and 2 which were treated by traditional methods. In control groups for comparison were used the multicomponent preparations: ointment Levomykol on the hydrophilic base, 10% ointment Methyluracil on the petrolatum-lanolin base, Vishnevsky liniment and Dioxidine preparation.

Depending on the etiotropic factor of the origin of wound and its localization all patients of main and control groups 1 were divided into 6 subgroups:

1 - 19 patients with the traumatic infected wounds of soft tissues;

2 - 14 patients with abscesses and phlegmons of soft tissues;

3 - 13 patients with suppuration of postoperative wound;

4 - 18 patients with chronic venous insufficiency complicated by trophic ulcers and trombophlebitis of subcutaneous veins of lower extremeties;

5 - 12 patients with obliterating atherosclerosis of lower extremeties complicated by trophic ulcers of the cruses;

6 - 11 patients with bullous-necrotic forms of erysipelatous inflammation.

The common clinical and laboratory methods were used to study the local and general therapeutic action of the remedy under hospital conditions. The local application of the remedy was estimated by studies of the following parameters:

1. Adsorption-detoxication properties of Mycoton in comparison with medical carbon adsorbents SKN P-1 and SKN P-2.

2. Inoculation of the wound content to determine a degree of wound dissemination with the pathogenic microflora (*Staphylococcus aureus, St. epidermidis, St. hemoliticus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris*).

3. Cytology of wound prints which included:

3.1. Dynamics of the inflammatory process in a wound.

3.2. Periods of epithelization and granulation rise in a wound.

3.3. Periods of complete healing of the wound and preparation to its closure.

The general clinical and therapeutic effect of Mycoton was estimated in patients by studies of the clinical and laboratory indices of the functional state of the liver, kidneys, pancreas, peripheral blood and certain indices of the coagulating blood system.

Results and Discussion

Mycoton has passed tests under the requirements which are shown to dressing materials both on medicobiological properties, and on physics and chemical parameters. By the index "Nontoxicity for an organism" Mycoton has received the positive conclusion of Pharmacological Committee of Ukraine.

According to requirements for surgical cotton-wool, index "Hygroscopicity" should be no lower than 20 g/g. Under analogous conditions Mycoton has hygroscopicity 20 g of water per 1 g of dry matter. Index "Capillarity" for cotton-wool should be no less than 75 mm. Mycoton has capillarity 100 mm.

Index "Traumaticity" of a bandaging material is associated with adhesive properties. Bandaging material fibres overgrow with renewing tissues. During the removal of cotton-wool and gauze bandages a part of wound surface tissues is also pulled off together with fibres having high thickness. That results in secondary traumatization and renewal of bleeding. This may be the reason of the repeated infection, a sharp rise of treatment periods and of the additional pain during bandaging. Due to the fact that chitin fibres of Mycoton are considerably thinner than the cotton plant ones (10 times in a diameter and 100 times in the transversal section) they readily destroyed on removal from a wound and cause no destruction to the tissues which have already begun to heal. Thus, Mycoton is atraumatic. This is one of its most important advantages promoting more rapid healing of wounds and the absence of pains on removal of bandages. Cotton plant fibres are not able to biodestruction in human tissues. This demands to remove them obligatory from wounds after their healing, that causes painful sensation in the patient. As a result scars form at the place of the wound. Mycoton possesses this valuable property – "Biodegradability". It completely and without a trace decomposes in wound tissues, without leaving scars or effects resembling tattoo on the skin.

Cotton-wool fibres have no therapeutic properties. Chitin-containing material Mycoton has a whole complex of own therapeutic properties. It stops bleeding, anesthetizes wounds, inhibits the development of main microorganisms which cause suppuration. Besides, chitin and glucans exert immunomodulating and antitumor activity. Thus, by this index chitin material Mycoton has important advantages over cotton plant and other bandaging materials.

The important problem at treatment of wounds is the control of the infectious microflora. Therefore the dressing material should have ability to adsorb bacterial cells and to suppress their development. Mycoton has these abilities due to a fine fibrous structure and chemical composition.

The tests of sorption properties of adsorbents were carried out on pure cultures of microorganisms in a physiological solution. 1 g of adsorbent brought in 10 ml of a solution and added 0.08 ml of bacteria culture with titer of 10^9. Test-tube with a solution was shaked during 20 minutes. Then adsorbent was separated from a liquid and residual concentration of microorganisms in a solution was defined. On a difference of concentration percent of bacteria extraction was expected (Table 1). After five-fold washing of Mycoton only 11.3% of bacteria were washed off from it.

Table 1. Ability of different adsorbents to take bacteria from a water suspension

Adsorbent	Microorganisms extraction on adsorbents, %			
	Escherichia coli	*Staphylococcus aureus*	*Enterobacter*	*Klebsiella*
SKN P1	78.8 ± 0.4	25.4 ± 0.3	63.2 ± 0.6	19.3 ± 0.8
SKN P2	91.3 ± 0.7	31.5 ± 0.7	88.2 ± 0.2	33.4 ± 0.4
Mycoton	95.3 ± 0.6	81.7 ± 0.4	88.6 ± 0.3	82.4 ± 0.7

In vitro dynamics of the bacteriostatic titre lowering in such pathogenic microorganisms as *St. aureus, E. coli, Ps. aeruginosa* demonstrates that Mycoton by its antibacterial activity is on the average 7 times as high as ointment Levomykol and 49 times as high as ointment Methyluracil (Table 2a). The similar results are received at a comparison of Mycoton with Vishnevsky liniment and Dioxidine preparation (Table 2b). It means that Mycoton surpasses by its activity the main groups of preparations which these ointments represent.

Table 2a. Antibacterial properties of Mycoton in comparison with other remedies (group 1)

Remedy	Bacteriostatic titer, *mkg / l*		
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Escherichia coli*
Methyluracil	180.0	40.0	40.0
Levomycol	6.5	6.5	25.0
Mycoton	2.2	2.2	2.2

Table 2b. Antibacterial properties of Mycoton in comparison with other remedies (group 2)

Remedy	Bacteriostatic titer, *mkg / l*			
	St. aureus	*St. epidermidis*	*Ps. aeruginosa*	*Escherichia coli*
Vishnevsky liniment	4.2	18.1	4.7	15.4
Dioxidine	6.3	20.5	6.1	13.2
Mycoton	2.1	2.1	2.2	2.3

Clinical data show that in main group 1 there is a tendency to a decrease in the microbic dissemination of a wound and to disappearance of a such pathogenic flora as *St. aureus, Ps. aeruginosa, P. vulgaris* as compared to control group 1. When using the conventional methods of treatment with ointment Levomykol in patients of the control group the pathogenic dissemination of wounds remained higher than the "critical" level even on the 7^{th}-10^{th} day after the beginning of the medicinal action (Table 3a).

At the patients of main and control groups 2 degrees of the microbic dissemination of wounds studied on the 1st and 3rd day of treatment. Mycoton also has shown higher efficiency (Table 3b).

A comparative cytological study of wound prints in patients of main and control groups 1 has shown a rapid (on the average 3-6 days) transition from the degenerative-inflammatory process into the reparative one. That was manifested clinically by disappearance of local inflammatory reaction in the wound, clearance of the inflammatory region from the excudation products and tissue inviability.

Table 3a. A comparative estimation of the microbic dissemination of a wound in patients after the action by Mycoton (main group 1) and Levomykol (control group 1)

Preparation	Degree of wound dissemination, *m-o/g*					
	St. aureus		*Ps. aeruginosa*		*Proteus vulgaris*	
	2^{nd} day	7^{th} day	2^{nd} day	7^{th} day	2^{nd} day	7^{th} day
Mycoton	10^9-10^6	10^2-10^1	10^8-10^7	10^3-10^1	10^8-10^6	10^2-10^1
Levomykol	10^8-10^6	10^6-10^4	10^9-10^6	10^6-10^3	10^8-10^6	10^6-10^3

Table 3b. A comparative estimation of the microbic dissemination of a wound in patients after the action with Mycoton (main group 2) and Vishnevsky liniment (control group 2)

Prepara-tion	Degree of woumd dissemination, *m-o/g*							
	St. aureus		*St. epidermidis*		*Ps. aeruginosa*		*E. coli*	
	1^{st} day	3^{rd} day	1^{st} day	3^{rd} day	1^{st} day	3^{rd} day	1^{st} day	3^{rd} day
Mycoton	10^8-10^6	10^2-10^1	10^8-10^6	10^2-10^1	10^7-10^6	10^3-10^1	10^9-10^6	10^3-10^1
Vishnev-sky lini-ment	10^7-10^6	10^6-10^5	10^8-10^6	10^6-10^5	10^7-10^6	10^6-10^5	10^9-10^6	10^8-10^5

When using Mycoton the periods of wound clearance reduced by 3-5 days as compared to the control. In patients of all subgroups of control group the processes of wound clearance, origin of granulations and the beginning of epithelization took place in later periods (Tables 4a, 4b). The general positive clinical effect was observed in most of patients beginning from the 3^{rd} day and preserved up to the end of treatment, that evidences for the bactericidal and sorption-deintoxication properties of Mycoton. The application of Mycoton permitted decreasing (5-7 days) the average number of patient days under hospital conditions.

When studying the general clinical and therapeutic effect of Mycoton it was possible not only to analyze the general clinical symptomatics arising during local wound infection and intoxication but also to estimate the functional state of basic organs and systems by the clinical (Table 5) and laboratory indices.

Table 4a. A comparative effectiveness of Mycoton (main group 1), Levomykol and Methyluracil (control group 1) on treatment of purulent wounds

Assessment criterion	Average term of manifestation, *days*			P
	Mycoton	Levomycol	Methyluracil	
Hyperemia and edema reduction	2.7 ± 0.4	3.1 ± 0.2	4.1 ± 0.2	0.1
Necrolysis	3.5 ± 0.7	4.4 ± 0.5	6.4 ± 0.3	0.1
Granulation of a wound	4.1 ± 0.2	6.3 ± 1.4	7.7 ± 1.3	0.1
Beginning of a wound Epithelization	5.6 ± 0.3	8.9 ± 1.3	9.2 ± 1.2	0.1
Average days of recovery	6.9 ± 0.9	12.3 ± 0.3	14.4 ± 0.7	0.1

Table 4b. A comparative effectiveness of Mycoton (main group 2), Vishnevsky liniment and Dioxidine (control group 2) on treatment of purulent wounds

Assessment criterion	Average term of manifestation, *days*			P
	Mycoton	Vishnevsky liniment	Dioxidine preparation	
Hyperemia and edema reduction	1.1 ± 0.6	2.9 ± 0.6	2.7 ± 0.6	0.1
Necrolysis	1.9 ± 0.4	3.5 ± 0.7	3.9 ± 0.3	0.1
Granulation of a wound	2.8 ± 0.4	4.7 ± 0.6	5.3 ± 0.2	0.1
Beginning of a wound epithelization	3.5 ± 0.3	5.3 ± 0.1	6.0 ± 0.4	0.1

Table 5. Dynamics of changes in certain clinical symptoms 7 days after application of preparation Mycoton (main group 1)

Clinical symptom	Degree of changes, %			
	Reduction	Disappearance	Without changes	Improvement
Body higher temperature	23	77	-	-
General weakness	73	16	11	-
Allergy	-	-	-	-
Improvement of a general condition	-	-	24	76

To estimate more reliably the therapeutic effect of Mycoton we have studied the clinical and laboratory indices of the functional state of the liver, kidneys, pancreas, peripheral blood, certain indices of the coagulating blood system in patients of subgroup 3 of main and control groups 1. The analysis of experimental data has shown that Mycoton exerts no essential influence on biochemical indices of life-important organs and system of an organism when using it as a wound-healing remedy [3]. It has no toxic effects on the life-important organs and systems.

The results of clinical approval of Mycoton on patients with different wounds (suppuration of a wound, trophic ulcers, phlegmons, abscesses of different localization) have shown its high clinical effectiveness. It manifested in a significant decrease of intoxication, characters of local inflammation and in positive dynamics of the clinical and laboratory indices. The application therapy with Mycoton lowers significantly dissemination in a wound to the minimal level (10^2-10^3 m-o/g), accelerates the transition of the hydration phase into the dehydration one. The latter reduces patient's stay at a hospital for 5-7 days and also prevents the development of possible pyoseptic complications.

Mycoton represents new generation of effective dressing chitin-containing materials. It surpasses known materials in many unique properties valuable to medicine. This makes Mycoton promising for a wide use.

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Biological activity of selected forms of chitosan

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Summary
Plants infected with a necrotic pathogen often develop long lasting resistance against broad range pathogen infections, called systemic acquired resistance (SAR). Nevertheless, naturally induced SAR occurs rarely in nature, is not predictable in timing and level of the expression therefore, it can not be used for crop protection in practice. Numerous studies have shown that an external application of various compounds can mimic biological SAR. To this group belong, among others, salicylates, fatty acids, jasmonates, benzothiadiazole (the first commercial resistance inducer) etc. There are only few reports on resistance responses induced by natural elicitors such as β-1,3-glucan and chitosan. We compared activity of benzothiadiazole (Bion) and chitosans: chitosan acetate, microcrystalline chitosan and chitosan oligomers in induction of resistsance. Chitosan induce broad spectrum of resistance in plants. Chitosan contrary to Bion can act dually: i) by blocking of the first steps of the infection and ii) by SAR induction. Bion does not affect pathogen directly but induces SAR only. SAR induced by chitosan is host dependent. Both, chitosan forms and Bion induce SAR with different kinetics and probably via different signal transduction pathways. Chitosan, harmless to human and the environment offers a great potential for ecological farming practice.

Introduction
Plants possess various defense mechanisms against pathogens including both, stable and inducible ones. Pathogens induce natural resistance responses, particularly the phenomena of localized acquired resistance and systemic acquired resistance (SAR). Induction of SAR can be achieved also by treatment (immunization) of plants with natural and synthetic compounds. Conceptually, induced resistance is reminiscent of the immunization system found in animals. Synthetic compounds such as benzothiadiazole (BTH), analog of salicylic acid, have produced resistance to broad spectrum of pathogens and is the first commercially applied chemical plant defense activator [1]. Natural products such as β-1,3-glucan [2] and chitosan also induce resistance in plants to pathogen infections. Treatment of plants with chitosans protects them against fungi, bacteria, viruses and viroids [3,4,5,6]. We present a comparison of the ability of selected forms of chitosan and benzothiadiazole (BTH) in the induction of resistance in plants.

Materials and Methods

In our experiments following products were used: standard chitosan (flakes, MW = 150000, DA = 15%), microcrystalline chitosan (hydrogel, MW = 160000, DA = 20%), chitosan oligomers and BTH (Bion, Novartis). To obtain chitosan acetate, flakes of chitosan were dissolved in diluted acetic acid and pH of the solution was adjusted to 5.5 – 5.7 with 1*N* KOH. The hydrogel of microcrystalline chitosan (MCCh) was suspended in water by homogenizaton. Chitosan oligomers were obtained by degradation of standard chitosan by fungus *Aspergillus fumigatus* according to the method described previously [7] and analyzed by HPLC [8]. Bion in granulated form was suspended in water.

Most of experiments were performed using an Alfalfa mosaic virus (AlMV) and a Tobacco mosaic virus (TMV) both causing local necrotic lesions in bean and tomato plants, respectively. Another model was also used i.e. tomato plants and bacteria *Pseudomonas syringe* pv. tomato. Young plants were treated protectively with chitosan forms or Bion at various intervals between treatment and pathogen inoculation. Induction of SAR was checked by challenge of treated plants with pathogens and was calculated as the percentage of the reduction of the number of lesions produced by pathogen on treated leaves in comparison to non-treated ones (control). Experiments were carried out under greenhouse conditions at 20-25^{0}C. An effect of products on growth of bacteria *in vitro* was determined by minimum inhibitory concentration (MIC) tests [5].

Results and Discussion

Optimization of the activator concentration causing immunization of plants to induce the systemic acquired resistance (SAR) needed definition of the highest concentration of the preparation which does not cause phytotoxicity. In the case of chitosan acetate in solution this effect was observed for concentrations ranged between 0.3 - 0.5%, and depended on plant species and conditions of experiment. Bean and cucumber plants were more sensitive than tobacco so, high temperature and strong sun exposure made plants more sensitive for chitosan. Microcrystalline chitosan and chitosan oligomers in concentration 0.5% showed week phytotoxicity. On the other hand 0.01% Bion demonstrated high phytotoxicity for all plants tested. Taking above observations and efectivity of preparations into consideration, we used 0.1% chitosan forms and 0.01% Bion solutions for induction of resistance in plant.

Classic inducers of SAR do not act directly on pathogen but activate metabolism of plant so, infection is more difficult and even when happened, progress and mobility of pathogen are limited. Direct influence of chitosans and Bion on viruses and their infectivity were tested via inoculation of plants with the mixture of both, inducer and pathogen.

Chitosan acetate in 0.01% solution added to AlMV inhibited the virus infection completely (Table 1). Microcrystalline chitosan and chitosan oligomers inhibited the infection on the lower level. However, Bion did not act at above conditions. Differences in properties of chitosan forms and Bion may be explained in two ways: chitosan in the presence of the virus inhibits its infectivity and/or the first steps of viral infection. The first presumption is excluded by the fact that when bean and tobacco were inoculated with the mixture of TMV and chitosan, in the first case total inhibition was observed contrary to second one. This is an evidence that chitosan does not affect virus directly and its infectivity.

According to above, an inhibition of the local infection of bean bases on the effect of chitosan on the first steps of infection, e.g. absorption of the virus to the cell wall what is necessary for infection. When virus particles were removed from *milieu* of chitosan by e.g.

high - speed centrifugation through the layer of saccharose did not show infectivity. We think that virus particles were firmly surrounded by the polycation what made its contact with cell wall impossible. Other properties of chitosan also came out. They can be defined as specificity to the species of plant. This host specificity can derive, in some aspects, from a compatibility or noncompatibility of cell wall and chitosan chemistry.

Table 1. Effect of chitosan forms and Bion on Alfalfa mosaic virus (AlMV) and Tobacco mosaic virus (TMV)

Treatment [1)]	**Inhibition of lesions production (%)**	
	Bean	Tobacco
AlMV		
in 0.01% chitosan acetate	100	
in 0.01% MCCh	98.5	
in 0.01% ch. oligomers	95.0	
in 0.01% Bion	0	
TMV		
in 0.01% chitosan acetate	100	0
in 0.01% MCCh	100	0
in 0.01% ch.oligomers	100	76
in 0.01% Bion	0	0

[1)] *Plants were inoculated with mixtures of viruses and preparations*

0 - *no inhibition*

MCCh - *microcrystalline chitosan*

ch. oligomers - *chitosan oligomers*

In the case of bean compatibility can be manifested by strong and easy binding to the cell wall what results in mechanical blocking of infection and, on the other hand, by induction of SAR. Weak efficiency of SAR induction in tobacco can be explained by lack of similar affinity between chitosan and plant. It is worthy of note that this trouble can be eliminated or limited by use of chitosan oligomers. Chitosan oligomers contrary to chitosan acetate induced resistance in tobacco plants [7].

When leaves of bean were sprayed with chitosan acetate solution and MCCh suspension followed by daily inoculation with virus, from the first and several next days bean leaves were protected against infection in almost 100% (Table 2). In the case of Bion the level of induction of the virus infection increased day by day and 7 days after treatment reached the maximum. This type of dynamics is characteristic for SAR induction. Results for chitosan forms presented in Table 2 are significantly different from that obtained for Bion. It can be explained by forming of chitosan film on bean leaves. When leaves are mechanically inoculated, chitosan and virus enter together scratched wall and chitosan acts similar to one added to the inoculum i.e., it blocks absorption of virus by cell wall.

When, during mechanical inoculation, conditions did not allow for contact of virus with chitosan what was reached by treatment of bottom side of leaf and infection of upper one viral infection was also inhibited showing defined dynamics. Maximum of such inhibition was observed after 2 – 3 days after treatment and than slowly decreased (Table 3). Dynamics of induction of SAR by chitosan and Bion are therefore different.

Table 2. Effects of chitosan forms and Bion on the production of local lesions by Alfalfa mosaic virus on bean leaves.

Interval between treatment and inoculation (days)	Inhibition of lesions production (%)				
	Chitosan		MCCh		Bion
	0.1%	0.01%	0.1%	0.01%	0.01%
1	100	100	100	63.5	32.0
2	100	99.9	99.5	70.5	39.5
3	100	100	99.5	65.5	59.5
4	100	99.5	100	55.5	74.5
5	100	99.5	99.0	60.0	92.8
7	100	99.0	99.5	58.5	96.5

Table 3. Induction of resistance by chitosan acetate and microcrystalline chitosan against Alfalfa mosaic virus in non-treated parts of the treated bean leaves

Interval between treatment and inoculation (days)	Inhibition of lesions production (%)	
	Chitosan	MCCh
1	90.5	52.5
2	93.0	85.0
3	75.5	70.0
4	60.5	58.0
5	55.5	50.5

Therefore we conclude that antiviral activity of chitosan in leaves treated followed by mechanical inoculation can be realized in two ways: i) via direct blocking of first steps of infection; ii) via induction of systemic resistance. It can be important in the case of viruses and viroids, transmitted in the nature only mechanically.

Both, chitosan acetate and Bion induce resistance not only in leaves treated but also away from them, systemically. After treatment with these preparations the new leaves which were not developed at the time of treatment and did not receive or receive low dose of product became resistant to viruses in 60% - 90%. Similar results however, but at the lower level, were obtained using microcrystaline chitosan and chitosan oligomers.

Natural SAR induced by pathogens is directed against wide broad spectrum of various pathogens and appears in wide range of plant species. SAR induced by chitosan and Bion has similar properties but in some aspects is different as was shown in the case of virus – bean and virus – tobacco. Also in the case of phytopathogenic bacteria which infects plants in different way than viruses similar problems were observed. As it arises from Table 4 chitosan in solution after long lasting treatment (24 h) of bacteria *Pseudomonas syringe* pv. tomato (PSt) inhibited their growth *in vitro*. For short time (2 h) their inhibition in environment is limited to reduction of activity only. Microcrystalline chitosan and chitosan oligomers like Bion did not inhibit the growth of bacteria. It is most essential that chitosans induce resistance in tomato at similar level as Bion (Table 4).

Table 4. Effects of chitosan forms and Bion on bacteria *Pseudomonas syringe* pv. tomato (PSt)

Product	Growth of bacteria *in vitro* (MIC) [1)]	Inhibition of infection (%) [2)]
Chitosan acetate	0.1	75.0
MCCh	(0.5)	60.0
Chitosan oligomers	(1.0)	65.0
Bion	(0.5)	80.0

[1)] *The lowest concentration of product that inhibited the growth of bacteria. Ineffectivness at the concentration used is presented in parentheses*

[2)] *Three days before infection tomato plants were sprayed with 0.1% chitosans or 0.01% Bion*

Induction of SAR occurs via mechanisms where, through various signal pathways, systems of resistance genes are run. Chitosan induces among others synthesis and accumulation of endogenous salicylic acid (SA) and pathogenesis related proteins (PR) [9]. We studied an influence of chitosans and Bion on the level of peroxidase activity in bean leaves. All chitosan forms tested contrary to Bion caused significant increase of the level of peroxidase activity. Chitosan in solution was the most effective (data not shown). Peroxidase is involved in lignification processes what was manifested by secondary thickening of cell walls in cells of bean treated with chitosan [10].

Chitosan represents a novel disease control compound that activates the plant inherent disease control mechanisms. SAR induced by chitosan acts against broad spectrum of pathogens. Antiviral activity of chitosan forms can be manifested in two ways: i) by inhibition of the first steps of infection and/or ii) by induction of the systemic resistance to virus infection. Chitosan oligomers applied to the tobacco plants gave significantly higher level of SAR that original polysaccharide. These results demonstrate: i) that SAR induced by chitosan is host dependent, and ii) the importance of elicitor formulation when considering the potential to the control of plant diseases. It is worthy of note that microcrystalline chitosan in water suspension has different properties than dissolved in acid one but showed similar biological activity. Bion, the first commercial resistance activator and chitosan induce SAR with different kinetics, may be due to different signal transduction pathway involved in SAR induction. Chitosan is harmless to human and offers a great potential in crop protection strategies, oriented towards efficacy and protection of the environment.

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Application of chitosan on the keeping quality of cut flowers

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Abstract

Chitosan has antimicrobial function, and it also can inhibit the synthesis of ethylene. For these reasons, acid and water soluble β-chitosan with different degrees of deacetylation (DD) were used in preserving cut flowers, and the effects of these treatments on the cut flowers were studied.

The senescence of gladiolus cut flowers was not very sensitive to ethylene, nor did acid soluble or water soluble chitosan affect the vase life of cut flowers. However water soluble chitosan improved the pedicle bending and raise the total number of bloomed florets; and at the same time, it increased the diameter of the third florets. The optimal dosage was 20 ppm of water soluble chitosan with >90% DD.

For carnation, the osmotic pressure caused by high concentration of chitosan decreased the rate of water uptake and the fresh weight of cut flowers, but the vase life of flowers were prolonged 8 to 9 days and the improvement of percentage of sleepiness were prominent by using acid soluble chitosan with 80-90% DD. It also softened the pedicle though, and this was an undesirable side effect.

Introduction

Chitosans has antifungal function and can also prevent plants from insect attacks. When chitin and chitosan come in contact with plants, they stimulate the secretion of chitinase or chitosanase in the host cells, which decompose chitin and chitosan in the insects and microbes and kill them. The enzymes decompose them into bioactive chitooligosaccharide, and this stimulates plants to produce phenol compounds. These materials are also effective in depressing the growth of microbes, besides being harmful to insects [1].

Chitosan is widely applied in agriculture, especially for the post-harvest handling of kiwi and strawberries [2,3].

Many researches concerning the keeping qualities of cut flowers by chemical preservatives were reported [4-7], but it seems difficult to keep the qlality of cut flowers by a single chemical preservative. In general, the preservatives that keep the quality of cut flowers are mainly composed of bactericides, anti-ethylene agents, and metabolizing substances.

In this research, the objective was to observe if chitosan could be used to inhibit ethylene formation and to delay senescence and thereby to prolong the vase life of cut flowers.

Materials and Methods

β-Chitin which was locally produced was used. *Gladiolus*: the bud stage "Pink Giant" was purchased from the local wholesale market. Miniature carnation: the bud stage *Dianthus caryophyllus L.* carnation was obtained at a wholesale market.

Chitosan: the sheath of cuttlefish was dried and ground to pass a 120 mesh screen before use. β-chitin was treated with aqueous 45% (w/v) NaOH solution under heterogeneous condition at 55°C and β-chitosans with different degrees of deacetylation (DD: 60-70, 70-80, 80-90, and >90%) were obtained after different periods of reaction time.

Water soluble chitosans: chitosans with DD 80-90 and >90% were resolved in 0.2 M acetic acid and made into 1.4% chitosan solution. Then they were immersed in an ice bath and ultrasonic degradation was conducted for 12 hours at 10 W by sonicator (Sonicator Vc-20T, Sonics, Inc.) then filtered through a 0.45 um filter. Finally, dialysis was conducted by a MW 12-14,000 tube to remove any salts or ions left, and then freeze dried to improve the solubility of chitosan in distilled water.

Cut flowers: *Gladiolus*: they were originally 100-110 cm in length, later cut to 85 cm, leaving 3-4 leaves and 12-16 florets and placed in glass jars containing 500 ml of acid or water soluble chitosan solution. Each treatment was tested on 3 samples.Carnation: they were originally about 60cm in length, later cut to 45 cm, leaving 2-3 leaves and 5 florets. The measurements were the same as the gladiolus flowers.

Measurement of water balance:

(1) Rate of water uptake (mg/g FW/day) = (wt. of the day before – wt. of that day) × 1000/days/FW of that day (FW: fresh weight)

(2) Changes in fresh weights (%) = (wt. of that day – wt. of the day before) × 100/days/wt. of the first day

Measurement of vase quality:

(1) Vase life: Gladiolus: the day when 4 flowers wilted was recorded. Carnation: the 2nd day when the flowers started to shrink was recorded.

(2) Diameter of flowers: Gladiolus: the 3rd largest floret was measured. Carnation: the largest flower was measured.

(3) Number of blooming flowers: the numbers of flowers at the 3rd day, and at the last day of the test were recorded.

(4) Bending percentage: the day when the angle of bent neck exceeded 90^0 was counted.

(5) Sleepiness: the day when the petals of the carnation flowers were curled by ethylene was recorded.

Results and Discussion

A. Preparation of chitosans: After treating β-chitin with 45% NaOH at 55°C for 2 to 6 hours, and the antifungal effect of chitosan would decrease with the decrease of DD, so chitosans with 60-70, 70-80, 80-90 and >90% DD were obtained.

B. Application to gladiolus cut flowers

a. Effects of pH values: As shown in Fig.1 and 2, the rate of water uptake and fresh weight were most significant at pH 6. Water balance was the most common reason for termination of vase life for cut flowers, and the water potential of cut flowers declined with time as well as water uptake, water loss and water conductivity in the flower stem. The solution under pH 5 could not maintain the water balance of cut flowers, and all the articles tested were more significant at pH 6 (Table 1).

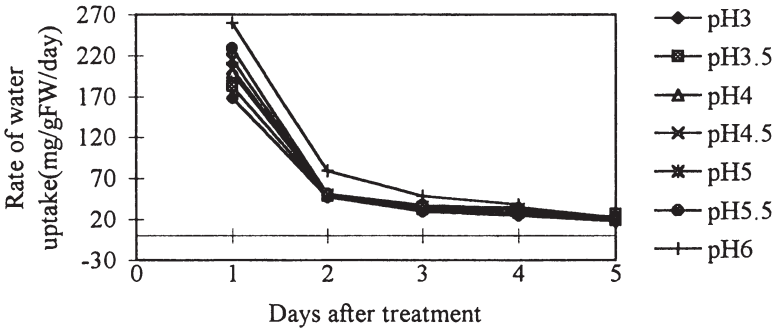


Figure 1. Effect of different pH value treatments on the rate of water uptake of cut gladiolus flowers.

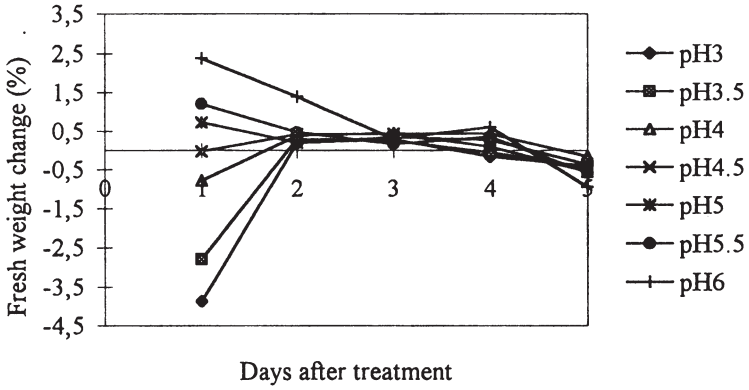


Figure 2. Effect of different pH value on the fresh weight changes of cut gladiolus flowers.

Table 1. Effect of different pH value treatments on the vase life, total florets opened and bending percentage of cut gladiolus flowers.

Treatment	Vase life (days)	Total no. florets opened per spike	Total no. florets opened % of max.	Bending percentage (%)
pH6.0	8.00^{a*}	7.67^{a}	42^{a}	33^{c}
pH5.5	7.33^{ab}	6.67^{ab}	41^{a}	33^{c}
pH5.0	7.00^{b}	5.33^{cb}	30^{b}	67^{b}
pH4.5	6.00^{c}	3.33^{d}	17^{c}	100^{a}
pH4.0	5.67^{cd}	3.67^{cd}	20^{bc}	100^{a}
pH3.5	5.00^{de}	2.00^{d}	11^{c}	100^{a}
pH3.0	4.33^{e}	2.67^{d}	16^{c}	100^{a}

*Means with the same letter on the same column were not significant difference ($p<0.05$) by Duncan's Multiple Range Test (DMRT)

b. Effect of DD: The effects of DD on the water uptake for gladiolus cut flowers are shown in Fig. 3 and all chitosans with different DD could stimulate the uptake on the first day. Chitosan with DD > 90% could maintain water balance better than other DD and the control, because the higher the DD, the better the antifungal effect. The growth of microorganism in the vessel contributed to a progressive impairment of the water conducting system. But this is not the sole reason, since the phenomenon was observed also in sterilized water. Fig. 4 shows the effects on the change of fresh weights; all the test flowers lost weight after the second day. As chitosan is a polyelectrolyte, it is positively charged and might react with the negatively charged sites of the vessel of the cut flowers, which might have caused the vascular blockage.

Chitosans were not effective in prolonging the vase life of flowers. Even though chitosans had an antifungal function, its polypositive charges decreased the fresh weight of cut flowers. However, the higher the DD, the higher the diameter of the florets (Fig. 4). Periods of rapid expansion of cut roses were accompanied by decreased in starch and increase in soluble sugars in the petals, but the osmotic pressure gradients were decreased. Chitosans could also stimulate florets to bloom earlier, and increase the number of blooming flowers, for example, the treated cut flowers had 4-5 florets on the third day, while the control had only 1-2 florets. Woltering and Van Doorn reported that treatment with ethylene for 24 hours revealed little to no sensitivity as indicated by slight acceleration of petal wilting [8]. Several polyamines like AOA (α-aminooxyacetic acid) had anti-senescence properties by inhibiting ACC (1-amino-cyclopropane carboxylate) synthase, a key enzyme in ethylene biosynthesis from methionine [9]. In this study, chitosan might have inhibited the synthesis of ethylene with its amino groups, and thus improved the keeping quality of cut flowers.

c. Effects of concentration: The acid soluble chitosan with DD > 90% was used here, and the higher the concentration of chitosan, the lower the rate of water uptake in cut flowers; but only the sample with 20 ppm showed that water uptake exceeded the control one (Fig. 5).

As for fresh weights, as the concentration increased, the fresh weights decreased. This was more apparent for those concentrations higher than 60 ppm (Fig. 6). Because the higher the concentration, the more the positive charges the vascular blockage would be more serious.

The effects of acid soluble chitosan with DD >90% are shown in Table 3. The senescence of gladiolus cut flowers had low sensitivity to ethylene, even though chitosan could inhibit the synthesis of ethylene. So the concentration of chitosan had no significant effects on the vase life and diameter of the 3 rd floret, whereas it could stimulate the development of florets; so that the total numbers bloomed were quite different.

d. Water soluble chitosan: Water soluble chitosan with DD >90% was used in comparison with that of the acid soluble one. The trend of water uptake was almost the same as the acid soluble one (Fig. 7). As for the change in fresh weights, both of them had the same results, but water soluble chitosan showed better keeping of fresh weight than that of the acid soluble one (Fig. 8).

As water soluble chitosan has lower positive charges than the acid soluble one, it had less effect on the transportation of water in tissues and also a lower ability to depress ethylene biosynthesis. Due to these reasons, the vase life of the flowers was extended 2 more days. As for the bloomed florets, the water soluble chitosan made as many as 11-12 florets bloom, but the acid soluble one had only 6-8 florets (Table 4).

C. Application to cut carnation

Effects of DD: As shown in Fig.9 and 10, the acid soluble chitosan with different DD had less effect on the rate of water uptake and fresh weights. On the other hand, qualities such as vase life, the diameter of florets, and percentage of sleepiness were improved, but there were few

differences among those with different DD (Table 5). As the results show, the rate of water uptake and the fresh weight decreased because the growth of microorganism contribute to the vascular blockage, not because its positive charge reacted with the negatively charged sites of the vessel in cut flowers.

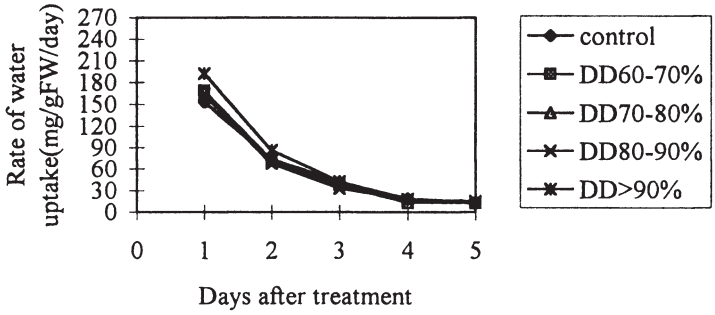


Figure 3. Effect of the acid soluble chitosan with various degree of deacetylation on the rate of water uptake of cut gladiolus flowers. *DD: degree of deacetylation

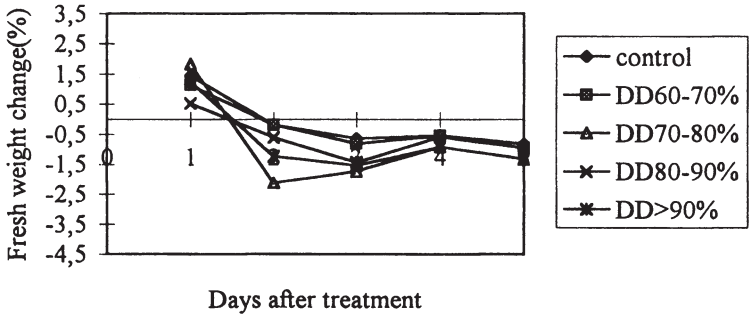


Figure 4. Effect of the acid soluble chitosan with various degree of deacetylation on the fresh weight changes of cut gladiolus flowers. *DD: degree of deacetylation

Table 2. Effect of the acid soluble chitosan with various degree of deacetylation on the opening of florets, vase life, floret diameter and total florets opened of cut gladiolus flowers.

Treatment	Vase life (days)	3rd floret diameter(mm)	No. florets opened on 3rd day	Total no. florets opened per spike	%of max.
control	6.00^{a*}	71.67^{ab}	1.33^{b}	3.67^{b}	25^{a}
DD**60-70%	5.00^{ab}	72.33^{ab}	1.00^{b}	6.00^{ab}	40^{a}
DD70-80%	4.67^{b}	69.00^{b}	4.00^{a}	6.00^{ab}	39^{a}
DD80-90%	5.00^{ab}	77.00^{ab}	5.00^{a}	5.67^{ab}	36^{a}
DD>90%	6.00^{a}	84.33^{a}	4.67^{a}	6.33^{ab}	40^{a}

*Means with the same letter on the same column were not significant difference ($p<0.05$) by Duncan's Multiple Range Test (DMRT) **DD: degree of deacetylation

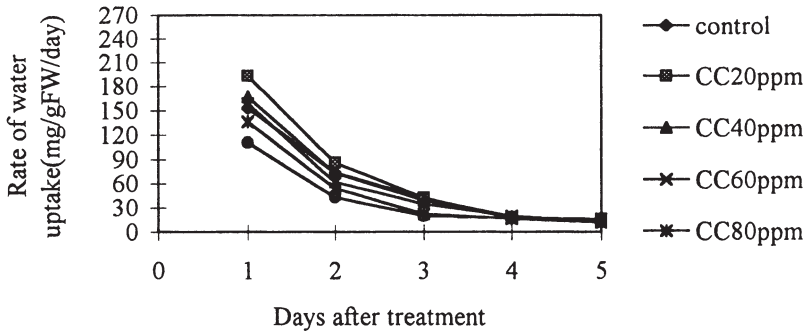


Figure 5. Effect of >90% degree of deacetylation acid soluble chitosan with various concentration on the rate of water uptake of cut gladiolus flowers. *CC: concentration of chitosan

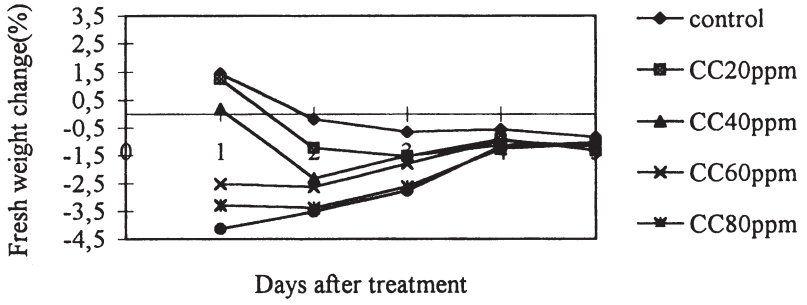


Figure 6. Effect of >90% degree of deacetylation acid soluble chitosan with various concentration on the fresh weight changes of cut gladiolus flowers. *CC: concentration of chitosan

Table 3. Effect of >90% degree of deacetylation acid soluble chitosan with various concentration on the opening of florets, vase life, floret diameter and total florets opened of cut gladiolus flowers.

Treatment	Vase life (days)	3rd floret diameter(mm)	No. florets opened on 3rd day	Total no. florets opened per spike	%of max.
control	6.00^{a}*	71.67^{a}	1.33^{b}	3.67^{b}	25^{b}
20ppm	6.00^{a}	84.33^{a}	4.67^{a}	6.33^{a}	40^{ab}
40ppm	5.00^{b}	70.67^{a}	3.67^{a}	6.00^{ab}	37^{ab}
60ppm	5.00^{b}	72.00^{a}	4.67^{a}	6.00^{ab}	38^{ab}
80ppm	5.00^{b}	77.00^{a}	4.00^{a}	6.33^{a}	37^{ab}
100ppm	5.33^{ab}	81.00^{a}	4.33^{a}	8.00^{a}	43^{a}

*Means with the same letter on the same column were not significant difference ($p<0.05$) by Duncan's Multiple Range Test (DMRT)

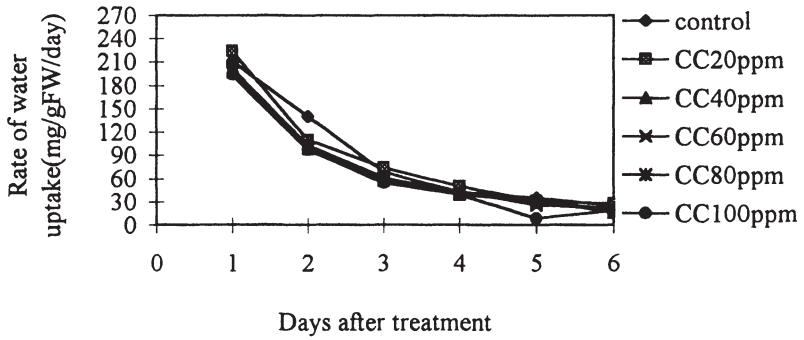


Figure 7. Effect of >90% degree of deacetylation water soluble chitosan with various concentration on the rate of water uptake of cut gladiolus flowers. *CC: concentration of chitosan

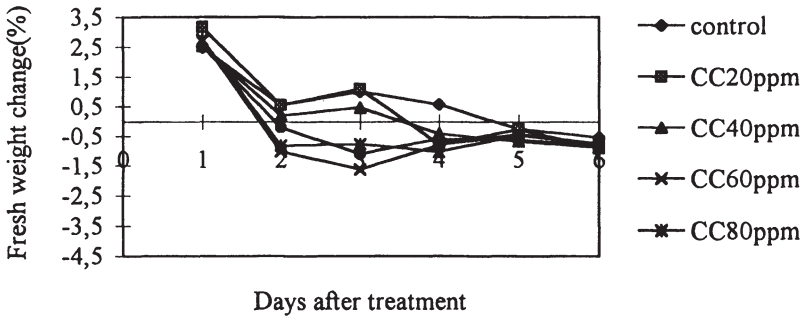
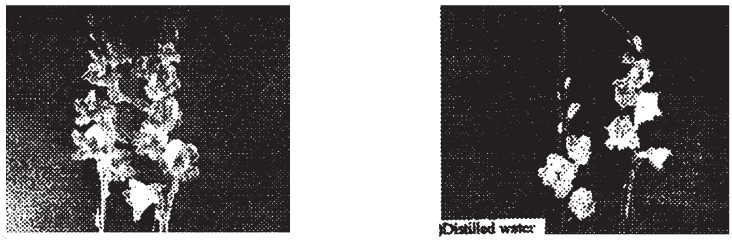


Figure 8. Effect of >90% degree of deacetylation water soluble chitosan with various concentration on the fresh weight changes of cut gladiolus flowers. *CC: concentration of chitosan.

Table 4. Effect of degree of deacetylation >90% water soluble chitosan with various concentration on the opening florets, vase life, floret diameter and total florets opened of cut gladiolus flowers

Treatment	Vase life (days)	3rd floret diameter(mm)	No. florets opened on 3rd day	Total no. florets opened per spike	%of max.
control	7.33^{a*}	77.00^{c}	0.67^{a}	6.33^{b}	35^{b}
20ppm	8.00^{a}	94.67^{a}	1.67^{a}	11.67^{a}	82^{a}
40ppm	8.00^{a}	94.00^{a}	1.67^{a}	10.33^{a}	78^{a}
60ppm	7.67^{a}	91.67^{ab}	1.33^{a}	11.00^{a}	68^{a}
80ppm	7.33^{a}	86.00^{abc}	1.00^{a}	10.67^{a}	70^{a}
100ppm	7.00^{a}	81.00^{bc}	0.67^{a}	10.67^{a}	67^{a}

*Means with the same letter on the same column were not significant difference ($p<0.05$) by Duncan's Multiple Range Test (DMRT)



(a) (b)

Figure 9. Effect of >90% degree of deacetylation water soluble chitosan on the keeping quality of gladiolus cut flowers. (a) >90% degree of deacetylation water soluble chitosan, 20 ppm; (b) distilled water.

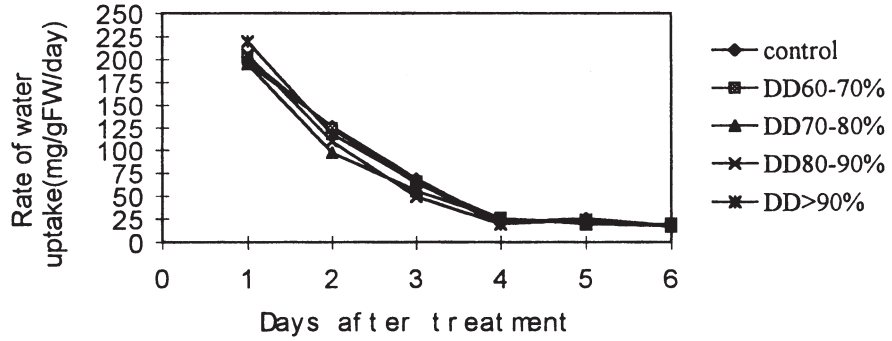


Figure 10. Effect of the acid soluble chitosan with various degree of deacetylation on the rate of water uptake of cut carnation flowers. *DD: degree of deacetylation.

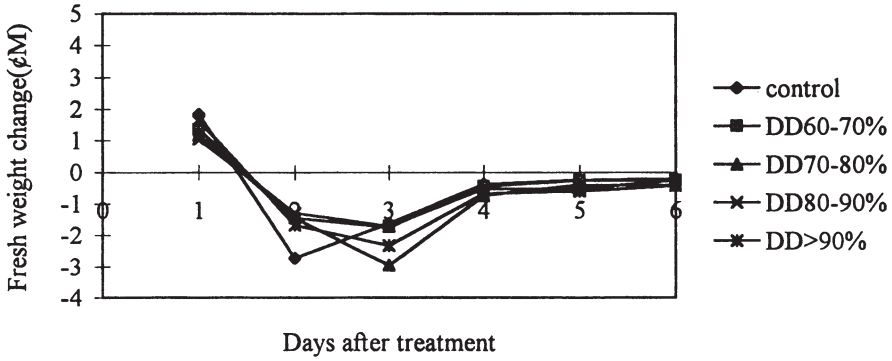


Figure 11. Effect of the acid soluble chitosan with various degree of deacetylation on the fresh weight changes of cut carnation flowers. *DD: degree of deacetylation.

Table 5. Effect of the acid soluble chitosan with various degree of deacetylation on the opening of florets, vase life, floret diameter and total florets opened of cut carnation flowers

Treatment	Vase life (days)	Largest flower diameter(mm)	%of Sleepiness
control	7.00^{b*}	51.67^{b}	100.00^{a}
DD**60-70%	9.67^{a}	57.33^{a}	66.67^{b}
DD70-80%	10.00^{a}	57.00^{a}	66.67^{b}
DD80-90%	10.33^{a}	61.00^{a}	26.67^{c}
DD>90%	9.67^{a}	59.67^{a}	33.33^{c}

*Means with the same letter on the same column were not significant difference ($p<0.05$) by Duncan's Multiple Range Test (DMRT) **DD: degree of deacetylation

a. Effects of concentration: There were no significant differences among such indicators as vase life, the diameter of flowers, and the percentage of sleepiness with chitosan with >90% DD. Therefore acid soluble chitosan with 80-90% DD was used in this experiment, since it was easier to prepare. As Fig. 11 shows, while the concentration of chitosan increased, the water uptake increased as well; but after it reached 100 ppm, the uptake decreased, because of its osmotic pressure.

The same phenomenon was observed for the fresh weights (Fig. 12).

Table 6 shows the effects on the quality. Chitosan might delay the senescence process of cut flowers. When the concentration was 80 ppm, the vase life of the flowers were 15 days, an extension of 8 more days. On the other hand, it also improved the sleepiness and diameter of the flowers. The senescence of carnation cut flowers had high sensitivity to ethylene that induced sleepiness, and terminated the vase life of these bloomed flowers and could shorten the life of the flowers.

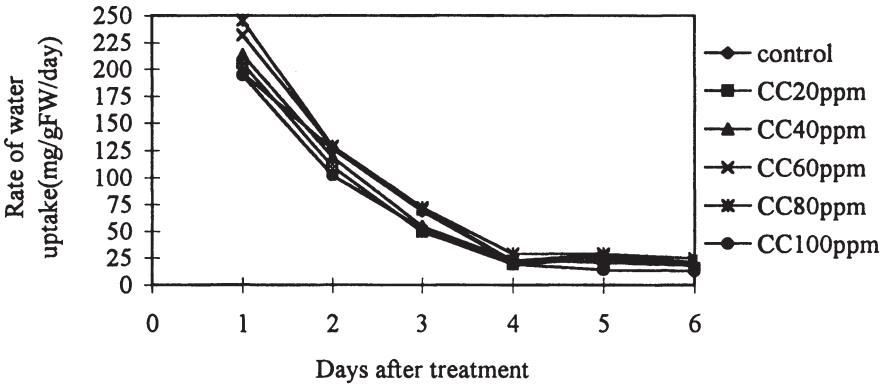


Figure 12. Effect of 80-90% degree of deacetylation acid soluble chitosan with various concentration on the rate of water uptake of cut carnation flowers. *CC: concentration of chitosan.

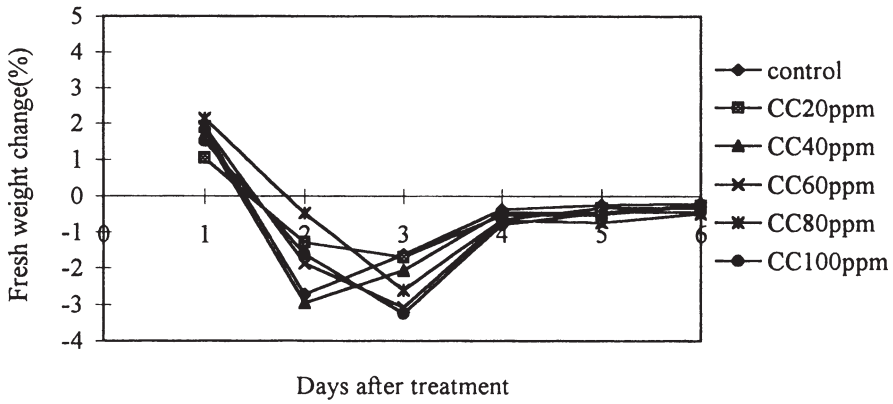


Figure 13. Effect of 80-90% degree of deacetylation acid soluble chitosan with various concentration on the fresh weight changes of cut carnation flowers. *CC: concentration of chitosan

Table 6. Effect of 80-90% degree of deacetylation acid soluble chitosan with various concentration on the opening of florets, vase life, floret diameter and total florets opened of cut carnation flowers

Treatment	Vase life (days)	Largest flower diameter(mm)	%of Sleepiness
control	7.00^{e}	51.67^{b}	100.00^{a}
20ppm	10.33^{d}	61.00^{a}	26.67^{b}
40ppm	12.00^{c}	61.33^{a}	6.67^{c}
60ppm	13.33^{b}	61.67^{a}	0.00^{c}
80ppm	15.33^{a}	63.00^{a}	0.00^{c}
100ppm	14.67^{a}	62.67^{a}	0.00^{c}

*Means with the same letter on the same column were not significant difference ($p<0.05$) by Duncan's Multiple Range Test (DMRT)

(a) (b)

Figure 14. Effect of 80-90% degree of deacetylation acid soluble chitosan on the keeping quality of cut carnation flowers. (a) 80-90% degree of deacetylation acid soluble chitosan, 80 ppm; (b) distilled water.

c. Water soluble chitosan with 80-90% DD was used in this experiment. Although several concentrations of chitosan were tried, none of them could improve the water uptake and fresh

weights. While when 60 ppm chitosan was applied, the decline of both water uptake and fresh weights were improved (Fig. 14 and 15), and there were more significant differences among such indicators as vase life, the diameter of the flowers and the percentage of sleepiness compared to the control (Table 7). The results of sleepiness shows that water soluble chitosan's ability to inhibit the synthesis of ethylene of was decreased.

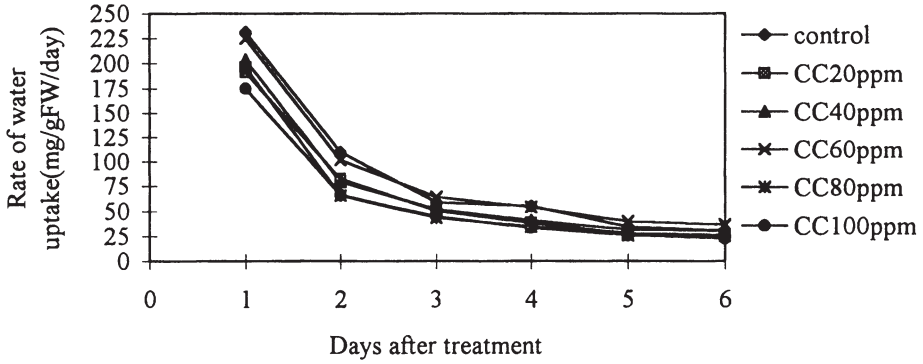


Figure 15. Effect of 80-90% degree of deacetylation water soluble chitosan with various concentration on the rate of water uptake of cut carnation flowers. *CC: concentration of chitosan

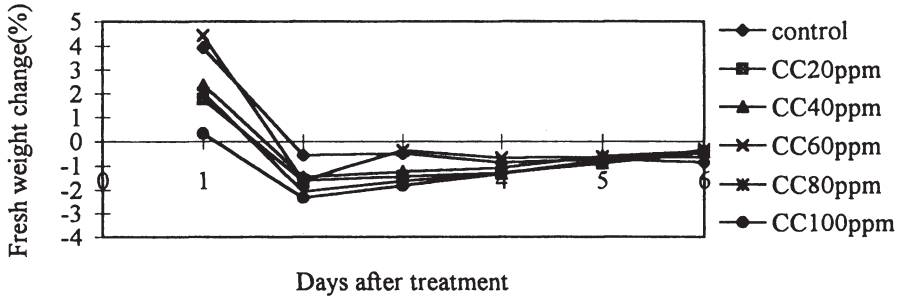


Figure 16. Effect of 80-90% degree of deacetylation water soluble chitosan with various concentration on the fresh weight changes of cut carnation flowers. *CC: concentration of chitosan.

Conclusions

Chitosans with different degrees of deacetylation were prepared, and that with 60-70% DD was made with 45% NaOH solution at 55°C for 2 hours, while that with 80-90% DD was made under the same condition but for 4 hours.

Two representative flowers, namely gladiolus and carnation, were used to observe the effects of chitosans on keeping qualities of cut flowers.

Table 7. Effect of degree of deacetylation 80-90% water soluble chitosan with various concentration on the opening of florets, vase life, floret diameter and total florets opened of cut carnation flowers

Treatment	Vase life (days)	Largest flower diameter(mm)	% of Sleepiness
control	5.33^{c*}	47.33^{c}	100.00^{a}
20ppm	8.67^{ab}	53.67^{b}	86.67^{ab}
40ppm	8.67^{ab}	54.67^{b}	73.33^{cb}
60ppm	10.67^{a}	63.33^{a}	60.00^{c}
80ppm	7.33^{cb}	51.33^{bc}	66.67^{cb}
100ppm	6.67^{cb}	48.33^{c}	80.00^{abc}

*Means with the same letter on the same column were not significant difference ($p<0.05$) by Duncan's Multiple Range Test (DMRT)

Gladiolus: They were not sensitive to ethylene; therefore, even though chitosan might depress the formation of ethylene, it could not delay their senescence process. When chitosan with >90% DD was applied, the concentration of 20 ppm could increase the blooming of floret buds from 6 to 12, and the ratio of blooming florets increased from 35 to 82%, and the largest diameter of florets increased from 77 mm to 95 mm.

Carnation: At the concentration of 80 ppm, chitosan with 80-90%DD could extend vase life to 8-9 days. Water soluble chitosan also showed effects on the extension of vase life and decreased the percentage of sleepiness, but it was not so significant in comparison to the acid soluble one.

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Preparation and characterization of chitosan films: application in cell cultures.

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SUMMARY
Chitosans from different sources and with different preparation methods were obtained. The molecular weight and deacetylation degree for each one were determined. Chitosan films were obtained and their thickness were tested. These films were used as a support of cell cultures. The results show a cell growth depending on the characteristics and the source of chitosan.

INTRODUCTION
Chitin is, as it is well known, a structural polymer found in insects, crustaceans and cell walls of fungi. It is considered to be the second or third most abundant natural polymer after cellulose (and possibly lignin). The biopolymer used in our work is chitosan, which is obtained by N-deacetylation of chitin. Chitosan is a linear polymer of β-1,4-linked glucosamine units, and the primary amine functionality at position 2 provides interesting properties to this polymer.
The natural sources and the procedure of preparation supply chitosan with different characteristics such as degree of deacetylation, molecular weight, microscopical structure, etc. These properties must be considered depending on its subsequent applications.
The development of films compatible with cell attachment and growth is a major goal of biomaterial research. Films to which cells can easily be attached will have a wide application as cell culture substrates and tissue substitutes. In general, as cells have a predominantly negative charge on their surfaces, they are known to adhere much more strongly to substrates with acidic and/or neutral groups [1]. Chitosan is a natural product and biologically compatible, so it can be used as substrate with basic groups.
Several authors reported that epithelial cells grow on films of chitosan [2] and fibroblast cells on collagen-chitosan blended films [3].
The purpose of the present report is to evaluate the behaviour of different chitosan films as a support of cell cultures and to test the influence of source and method of chitosan preparation on cell growth.

Materials and Methods

Chitin was obtained from crustacean shells of different sources including Cuban lobster (*Polinurus vulgaris*), and prawns (*Penaeus caramote* and *Pleoticus mülleri*).

Commercially available chitin was purchased from Sigma, while hydrochloric acid, sodium hydroxide and acetone were supplied by Merck.

Isolation of chitin: The procedure used to isolate chitin was a modified version of a previously reported one [2].

***Preparation of chitosan*:** We have obtained chitosan by three different methods:

a)Homogeneous hydrolysis. The chitin obtained was brought into contact with a 50% NaOH solution at 60°C for 48 (sample B) and 136 h (sample E), after which it was washed with distilled water, filtered and dried.

b)Heterogeneous hydrolysis. Chitin was treated with a 50% NaOH solution at 136°C for 1 h, under N_2. Then, it was washed, filtered and dried, samples A and D.

c)Microwave thermochemical process [5]. Chitin was added NaOH 30% in a 100 mL bottle, placed on a microwave-oven digestion system operated at 440 w for 35 min. Cold distilled water was added, then it was filtered, thoroughly washed with water and dried at 25°C. Sample C.

Characterization procedures

a)Degree of deacetylation. First derivative ultraviolet spectrophotometry [6]. Chitosan was dissolved in 0,01M acetic acid to prepare solutions containing 1 mg of dry chitosan per ml. Solutions were also prepared with N-acetyl-D-glucosamine in 0,01 M acetic acid to be measured against acetic acid. The derived spectra are obtained at a slit width of 1 mm, a scanning speed of 30 nm/min and a time constant of 1 s.

b)Degree of deacetylation. ^1H NMR spectroscopy[7]. ^1H NMR was performed on a AMX500 Bruckker NMR spectrometer under a static magnetic field of 125 MHZ and 500.13 MHZ respectively at 70°C. For these measurements, 5 mg sample were introduced into a 5 mm diameter NMR test tube, to which 1 ml of 2 wt% DCl/D_2O solution was added.

c)Viscometry. Viscosities of chitosan solutions (1% in 0,1 M acetic acid-0,2 M NaCl) were determined with a rotational viscometer and an Ubbelohde viscometer at 25 ± 0,1°C.

d)Viscosity-average molecular weight. The equation used to relate LVN (Limiting Viscosity Number) values to molecular weights was the Mark-Houwink equation:

$[\eta] = k M^a_v$

where $[\eta]$ = LVN and the constants for our experimental conditions were [8]:

$k\ (cm^3\ g^{-1}) = 1{,}81 \times 10^{-3}$ and $a = 0{,}93$

e)Thickness of films. It was determined with an Olympus microscope, using a suitable micrometer.

f)Scaning electron microscopy (SEM). The SEM technique was used to characterize the surface of chitosan films and chitin and chitosan particles. Thus, dried particles were coated with Au-Pd on a SEM Coating Unit under a nitrogen atmosphere for 70 s and then examined under a JSM 64 CO scaning electron microscope.

g)Preparation of chitosan films. 1% chitosan was dissolved in 50 mM acetic acid. The homogeneous suspension was poured into Petri dishes that were kept at room temperature until they were dried.

h)Cell cultures. C3H10T1/2 cell lines were obtained from the American Type Culture Collection (Manassas, Virginia). All the cell lines were maintained on 25 cm^2 flasks with DMEM containing 10 % Foetal Bovine Serum plus antibiotics (100 U/mL penicillin and 100μg/mL streptomicin sulfate). Cell experimental cultures were achieved on 48-well plates

with chitosan films previously formed. Trypsinized cells were seeded in the growing medium previously described at 4000 cells/cm^2.

Results and Discussion

In this work, we have obtained chitosan from prawn chitin (*Penaeus caramote* and *Pleoticus müller*i)) and lobster chitin (*Polinurus vulgaris*), by three different methods: homogeneous hydrolysis, heterogeneous hydrolysis and microwave thermochemical process.

Preparation method and characterization of chitosan: Heterogeneous and homogeneus hydrolysis are two conventional methods used to prepare chitosan, while microwave thermochemical process has been developed recently [5].

We have obtained chitosan from prawn by the three methods and tried to find a correlation between their characteristics.

We have determined in all the samples the degree of deacetylation calculated by ^{1}H NMR and first derivative UV spectroscopy, and the viscosity-average molecular weight.

Table 1. Characteristics of prawn chitosan

Sample	A	B	C
Preparation method	Heterogeneous hydrolysis	Homogeneous hydrolysis	Microwave process
Deacetylation degree ^{1}HNMR	74	82	98
Deacetylation degree UV	75	79	98
MWv	467.735	251.188	131.825

Table 1 shows the results of chitosan prawn characteristics: deacetylation degree and viscosity-average molecular weight. It can be seen that heterogeneous hydrolysis produces chitosan with a low deacetylation degree, while microwave thermochemical process produces higher values. As regards viscosity-average molecular weight the relationship is the opposite: the heterogeneous hydrolysis produces chitosan with higher viscosity-average molecular weight. The results demonstrate that the microwave method produces a reduction in the polymer length.

The results of lobster chitosan are shown in Table 2. There are not appreciable diferences between the acetylation degree viscosity-average molecular weight in samples obtained by homogeneous and heterogeneous hydrolyisis.

Table 2. Characteristics of lobster chitosan

Sample	D	E
Preparation method	Heterogeneous hydrolysis	Homogeneous hydrolysis
Deacetylation degree ^{1}HNMR	82	79
Deacetylation degree UV	83	73
MWv	157.290	181.134

Chitosan films: Chitosan films were obtained from each sample, using our own procedure. The thickness (table 3) and surface were tested by SEM.

Table 3. Thickness of films

Samples	A	B	C	D	E
Thickness (μm)	20.2	22.5	20.2	10.0	20.2

The films obtained with chitosans (B and E, homogeneous hydrolysis) show an irregular surface. The films of chitosan obtained by the microwave thermochemical process have a discontinous and irregular surface. Its microphotograph is shown in figure 1. This special aspect and its characteristics are not appropiate for cell culture. The films of chitosan obtained by heterogeneous hydrolysis show a regular surface (fig.2).
The macroscopic aspect is also different. Films obtained with chitosan processed by microwave were easily broken. The others were flexible.

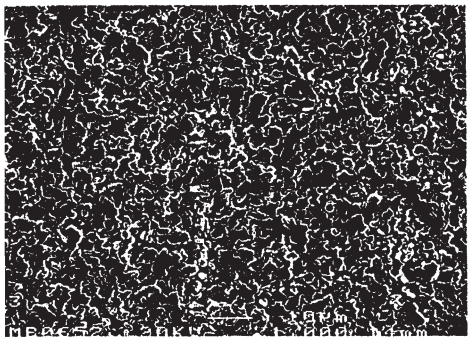

Figure 1. SEM of film obtained with prawn processed by microwave (sample C)

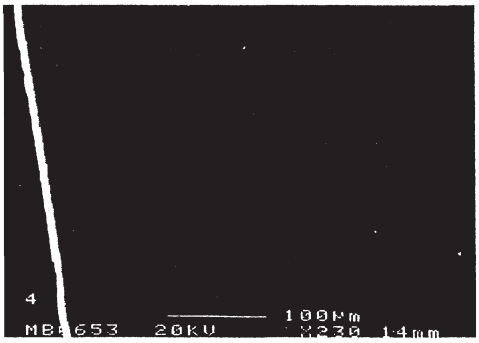

Figure 2. SEM of film obtained with prawn chitosan by heterogeneous hydrolysis (sample A)

Behaviour of chitosan films on cell cultures: All the samples that gave good films were assayed as a support for cells growth. A comparative study was developed and the best results are shown in figures 3 and 4 corresponding to chitosans E (lobster) and A (prawn).
The cell growth is possible on films obtained from homogeneous and heterogeneous chitosan. However the best results were reached in the case of film from chitosan prepared by heterogeneous hydrolysis.
In the case of prawn the microphotograph show clusters of rounded cells that seem to grow out of the film (fig. 4).
On the other hand cell growth on chitosan films from lobsters is possible with both kinds of sample, but the best results are shown when chitosan are obtained by homogeneous hydrolys. Cells are spread on the film adopting a fibroblast-like form (fig.3).

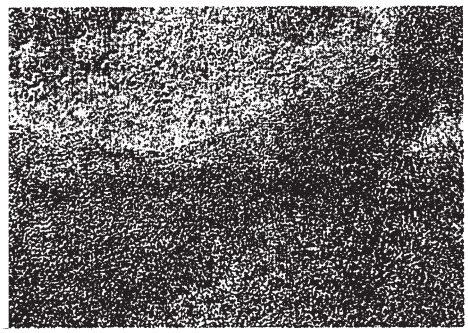

Figure 3. Cells growth on lobster chitosan film

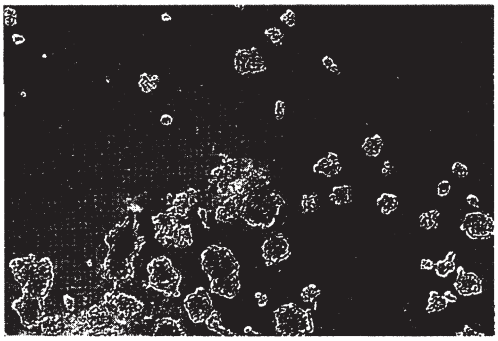

Figure 4. Cells growth on prawn chitosan film

In conclusion, the results show the capacity of chitosan films to be used as support of cell cultures. The growth of cells depends on the source and characteristics of chitosan films. The chitosans prepared by microwave thermochemical process are not suitable to form film. The more appropiate deacetylation degree for cell culture is ranged 73-80. The MWv in the studied interval does not affect the results.

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Transport phenomena in chitin gels

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Summary
The aim of this paper is to study different transport phenomena in chitin gels obtained by N-acetylation of chitosan in a water-alcohol mixture. Three kinds of transport were studied on these gels : the desorption of solutes when they do not interact with chitin, the sorption of solutes when they interact with chitin and the solvent osmosis. In order to investigate interactive sorption, dyes having different chemical structures such as Acid Blue 74 or Congo Red were used. Concerning the non interactive desorption, the role of different parameters on the diffusion was tested, peculiarly the role of the size of the diffusing substrates. For that, solutes such as PP vitamin, B1 vitamin and caffeine were used. Finally, the solvent osmosis of the gel when it is immersed in a concentrated solution of gelatin was examined.

Introduction

The two classical kinds of gels can be obtained either with chitin or chitosan. They correspond to chemical and physical gels. Chemical gels are formed by means of covalent crosslinkings. They are generally obtained thanks to the use of glutaraldehyde[1] or epichlorohydrin[2]. The formation of physical gels must be related to the presence of low energy and reversible interactions which can be created with a diacid (oxalic acid)[3], with polyoxyanions of molybdate[4] or by chemical modification of chitosan. We have chosen to work on a physical gel obtained by acetylation of chitosan in an acetic acid-water-alcohol solution[5,6,7]. Physical gels have two advantages compared to chemical gels. The first advantage corresponds to their reversibility. The second concerns the chemical structure of polymer chains which, in this case, is not modified. As a consequence, the whole of the physicochemical and biological properties of chitosan or chitin chains is preserved.

This paper deals with the study of different transport phenomena in these gels : the sorption of interactive solutes (Acid blue 74 and Congo Red), desorption of non interactive molecules (PP vitamin, B1 vitamin and caffeine) and the solvent osmosis phenomena.

Materials and method

Gel formation: 10 ml. of an aqueous acetic acid (0,5%) solution of chitosan (1%) was mixed with 8 ml of 1,2-propanediol. An acetylating solution (acetic anhydride and 1,2-propanediol) was slowly added to the solution of chitosan under stirring and the mixture was transferred into a cylindrical mould (∅=26mm, H=30mm).

Vitamins and caffeine diffusion: After syneresis and washing in deionised water, gels (∅=18 mm, H=21mm) were immersed in an excess of a concentrated aqueous solution of the studied solute over 24 hours. The study of the solute release was then carried out in 1 liter of deionised water at room temperature. The amount of released solute was measured by UV spectrophotometry (KONTRON instrument) at a wavelength : λ =262 nm for PP vitamin, λ = 272nm for caffeine and λ = 240 nm for B1 vitamin.

Dye sorption: The gels were immersed in 1liter of the studied dye. The concentration of the solution was determined at room temperature by a UV spectrophotometer. The calibration wavelength used were : λ = 346 nm for Congo red, λ = 560nm for Remazol 5 and λ = 610 nm for Acid blue 74.

The coefficient partition Kd is calculated from the following equation :

$$Kd\ (ml/g) = \frac{(C_0 - C_\infty)}{C_\infty}\frac{V}{m}$$

C_0 and C_∞ are the dye concentrations in the bath at the beginning and at the end of the sorption respectively, m is the weight of the polymer and V is the volume of the coloured bath (ml).

Osmosis solvent: The gel was immersed in 250ml of a concentrated solution of gelatin (33.3% (w/w)) at a temperature equal to 50°C. The molecular weight of the gelatin used was $\overline{Mw}$ = 50 000 g.mol^{-1}.

Results and Discussion

Desorption of non interactive solutes: Molecules of various steric hindrance such as PP vitamin, B1 vitamin and caffeine (see Table 1) were chosen in order to determine the role of the size of purely diffusing agents on the transport phenomena. These solutes were also chosen in relation with their good biological properties. As a consequence, the gel containing them can be considered as a possible system for sustained release of drugs.

PP vitamin is the anti-pellagra vitamin. B1 vitamin has an antineuritic effect and caffeine is well known for its tonic power and is enclosed in the composition of various mixtures particularly slimming creams.

Diffusion in chitin gels is a Fickian process. As a consequence, the transport rate was evaluated by a diffusion coefficient D calculated from the second Fick's law.
The following equation is proposed for a plane gel[8].

$$\frac{M_t}{M_\infty} = 4\left[\frac{D}{\Pi}\frac{t}{l^2}\right]^{\frac{1}{2}},$$

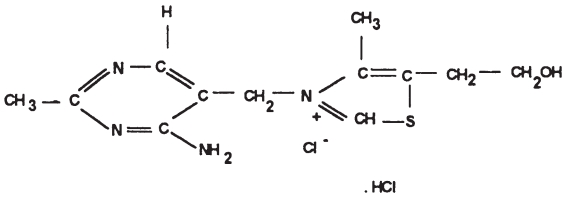
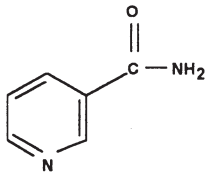
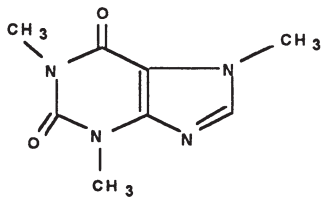
l is the thickness of the gel, t, the time, M_t and M_∞, the amount of released solute for a given time t and infinite time respectively.

In the case of a cylindrical gel, the diffusion coefficient is calculated by fitting experimental data with the relation proposed by *Ritger* and *Peppas*[9] :

$$\frac{M_t}{M_\infty} = 4\left[\frac{D}{\Pi}\frac{t}{a^2}\right]^{\frac{1}{2}} - \Pi\left[\frac{D}{\Pi}\frac{t}{a^2}\right] - \frac{\Pi}{3}\left[\frac{D}{\Pi}\frac{t}{a^2}\right]^{\frac{3}{2}} + 4\left[\frac{D}{\Pi}\frac{t}{l^2}\right]^{\frac{1}{2}},$$

with a : radius of the cylinder.

Table 1. Chemical structure of the different diffusing agents used for desorption studies.

Diffusing agents	Chemical Structure
B1 Vitamin	
PP Vitamin	
Caffeine	

Results are presented in Table 2. Diffusion coefficients appear to be located in a range between 3.7 and 5.6 10^{-6} $cm^2.s^{-1}$. These values are close to those published for poly(ethylene glycol)[10] and poly(methacrylic acid-g-ethylene glycol) hydrogels[11]. Nevertheless, they are a decade lower than those observed for organic solutes with the same molecular weight in pure water.

Moreover, neither the steric hindrance nor the chemical structure of the diffusing solutes strongly affects the diffusion coefficient. This result must be related to the fact that the pore size of the gel is much greater than the size of diffusing solutes[12] but also that interactions between diffusing solutes and the network are very weak.

Sorption of interactive molecules: Sorption of Acid blue 74 was tested on two gels : acetylated at 92% and at 98%. An important decrease of the dye concentration in the bath was observed. The sorption tends toward a plateau near 80 hours and depends on the degree of acetylation. Indeed, the amount of sorbed dye is higher for low DA's and the values of partition coefficient Kd are inversely proportional to DA's revealing thus the importance of the electrostatic interactions between chitin gel (NH_3^+) and Acid Blue 74 (SO_3^-).

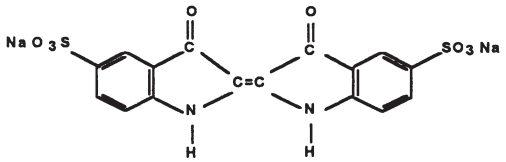
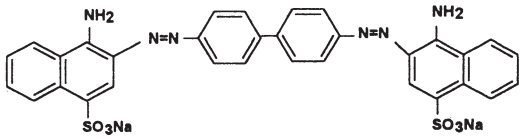
Contrary to acid blue 74 which is sorbed in the bulk of the gel, Congo Red is only sorbed at the surface. The first layers of the sorbed dye give rise to a barrier effect (negatively charged surface) and inhibit the further sorption of dye by electrostatic repulsions. When the amino functions of chitin are highly charged (pH4) or free (pH 10), no desorption is observed in relation with the fact that electrostatic interactions are

not essential for the sorption of this dye. On the other hand, H bonding between amino groups of the dye and hydroxyl or amide group of the network should be predominant. We observe the same situation for hydrophobic interactions between the cyclic aromatic systems of the dye and the methyl groups of the network.

Table 2. Diffusion coefficients of various water soluble solutes in chitin gels

	Diffusion Coefficients D ($cm^2.s^{-1}$)
PP Vitamin(122.1g.mol^{-1})	
Cylindrical gel DA = 98%	$4.5\ 10^{-6}$
Cylindrical gel DA = 92%	$5.6\ 10^{-6}$
Plane gel DA = 98%	$4.5\ 10^{-6}$
B1 Vitamin(337.27 g.mol^{-1})	
Plane gel DA = 98%	$4.2\ 10^{-6}$
Caffeine(94.2 g.mol^{-1})	
Cylindrical gel DA = 98%	$3.7\ 10^{-6}$

Table 3. Chemical structures of the used dyes for sorption studies.

Dyes	**chemical structure**
Acid Blue 74	
Congo Red	

Solvent osmosis of the gel: In order to study the rejection of the solvent of the gel by osmosis, a sample is placed in a concentrated aqueous solution of gelatin (33%(w/w)). In this condition, the chemical potential of water is lower inside the network compared to outside. Then, there are two possibilities to equilibrate the chemical potentials of water between the inner and the outer parts of the gel. The first corresponds to the migration of water from the bulk of the gel to the gelatin solution. The second corresponds to the gelatin diffusion from gelatin solution to the bulk of the gel.

Gelatin diffusion seems to be limited because, at this concentration (33,3%(w/w)), the triple helix of the gelatin forms aggregates which size is greater than the pore size of the network. As a consequence, an important migration of water from the network to the gelatin solution and an important dehydration of the gel are observed.

The weight of the gel after osmosis represents only 4.2% of the weight of the gel at the gel point and the syneresis observed in this case is the most important syneresis never observed[13]. This result points out that it is possible to go beyond the syneresis limit by playing on the difference of chemical potential of water between the inner and the outer parts of the gel. This behaviour is very interesting if we consider that it allows us to simply multiply by 25 the concentration of the gel which then become close to 15 %.

Conclusions

The network corresponding to a chitin gel can interact with dyes either with electrostatic interactions or with lower energy interactions such as hydrophobic interactions. In the case of Congo red the transport is limited by electrostatic exclusion and then the gel can be considered as a way to colour specifically a surface. This property can be used in cosmetic and nutrition fields.

On the other hand, the porosity of the gel is so high that non interactive and isolated molecules of low molecular weight can diffuse easily throughout the gel. As a consequence, chitin gels can be used as drug vectors for these systems.

Finally, transport of solutes can be limited by size exclusion and as a consequence the thermodynamic imbalance created in this case leads to the osmosis of the solvent out of the network and thus allows us to regulate the size of the gel.

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Symplex membranes of chitosan and sulphoethylcellulose

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Summary
Polyelectrolytecomplex membranes were used to dehydrate alcoholic solutions of. They were prepared by simultaneous interfacial reaction of aqueous solutions of two oppositely charged poly-ions. The cellulose derivative sulphoethylcellulose (SEC) was used as the polyanionic component and chitosan as the polycationic component. Membrane properties such as degree of swelling, morphology and separation parameters were investigated.

Introduction
In recent years membrane technology has been the subject of intensive studies due to its wide variety of applications. One area of membrane technology is the dehydration of alcoholic solutions, since alcohols form azeotropes with water that cannot be separated by conventional methods. A large number of materials have been used in order to improve the dehydration effect, but the separation parameters can still be improved. The use of hydrophilic polyelectrolytes as a component of the membranes yields better separation parameters. The formation of polyelectrolyte complexes (symplexes) provides an elegant method of rendering these water-soluble systems insoluble, and is therefore useful in solving dehydration problems [1-4].

In this investigation sulphoethylcellulose (SEC, figure 1) and chitosan were used as components of membranes. Sulphoethylcellulose is a cellulose derivative that is water soluble even at very low degrees of substitution (DS). It is easily accessible by polymer-analogous substitution reactions of cellulose, adjusting the molecular parameters as molar mass and degree of substitution as desired. Due to its polyanionic character it reacts with a solution of polycationic chitosan. Membranes were produced by flocculation of the symplexes. Chitosan is already familiar as a membrane material but it is generally applied as a covalent, cross-linked network. In this research the electrostatic properties were used to cross-link the polymers. The strong Coulomb interaction between the oppositely charged polyelectrolytes causes the formation of the polyelectrolyte complex.

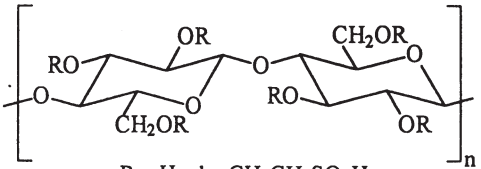

Figure 1. Repeating unit of sulphoethylcellulose

The aim of this investigation was to introduce SEC and chitosan as suitable raw materials for symplex membranes. Discussion centres on characterisation of the molecular parameters of the components that play a role in membrane synthesis and the properties that are important in the separation process.

Materials and Methods

Sulphoethylcellulose was produced industrially by sulphoethylation of cellulose with vinylsulphonic acid. Chitosan with different molar masses and degrees of deacetylation were obtained in commercially available form and used without any further purification. Solutions with a content of 2-5 % polyanionic polyelectrolyte were obtained by dissolving the samples in water or 2 % polycationic polyelectrolyte in 1 % acetic acid.

The properties of the membranes are dependent upon the molecular parameters of the base materials, which also influence the solution structure and hence the properties of chitosan and SEC. Therefore the samples were characterised by determining the degree of substitution, the degree of deacetylation, the molar mass and the molar mass distribution. Therefore polyelectrolyte titration, IR-spectroscopy, viscosimetry and size exclusion chromatography were used. Further details about the methods used can be found in [5-15].

To prepare symplex membranes, a simultaneous interfacial reaction of aqueous solutions of the two oppositely charged poly-ions was used. The solution of sulphoethylcellulose was spread as a 0.5 mm thick film on a glass plate and carefully put into a solution of chitosan. A spontaneous surface reaction between the two components led to formation of the polyelectrolyte complex membrane. After a reaction time of 1 h the membrane on the glass plate was washed with distilled water to remove the adjacent unreacted polycationic compound. The membrane was loosened from the support and dried. The thickness of the membranes obtained was in the range of 10-30 µm, depending on the SEC concentration in the casting solution.

The membranes were characterised in terms of swelling behaviour, network structure, morphology and separation parameters. The degree of membrane equilibrium swelling was determined according to

$$SD\ (\%) = 100\ \% \cdot (m_s - m_d) / m_d$$

where m_s and m_d are the weights of membrane strips in the swollen and dried states respectively. Rheological oscillation measurements of the swollen membranes were carried out on a Bohlin CS 50 stress-controlled rheometer [16-17]. Pervaporation experiments were performed with a laboratory-scale apparatus using a P28 stainless steel measuring cell supplied by Celfa AG, Switzerland. Water-isopropanol mixtures at 50°C were used for membrane testing. The permeate flux was determined gravimetrically and the compositions of feed and permeate by density measurements. The separation efficiency is generally described by two main values [18]:

- separation capacity: described by the permeation rate J: J = mass / (time · area)
- separation selectivity: described by the separation factor α

$$\alpha = \frac{c(H_2O)_{feed} / c(H_2O)_{permeate}}{c(alcohol)_{feed} / c(alcohol)_{permeate}}$$

Results and Discussion

Characterisation of the components: The molecular parameters of the components used are summarised in table 1. The degree of substitution of the SEC samples covers a small range. The lower the DS, the more the chains are aggregated in solution and cannot be handled as single chains. The molar masses of the sulphoethylcellulose are quite high in comparison with the chitosan samples.

Table 1. Molecular parameters of the samples used.

sample	DS	DD [%]	[η] [ml/g]	M_w [g/mol]	M_n / M_w
SEC 1	0.39	-	374	370 000	4.8
SEC 2	0.35	-	432	550 000	3.5
SEC 3	0.28	-	524	1200 000	5.2
CHT 1	-	99	25	9 000	1.4
CHT 2	-	75	62	40 000	3.3
CHT 3	-	88	327	90 000	1.5

Characterisation of the membranes: The swelling behaviour can be correlated with the pervaporation properties because the ability of the medium to dissolve in the membrane is important for the separation process. The swelling ratio decreased with increasing ethanol concentration in the medium. Ethanol is dissolved less readily than water in the membrane, consequently ethanol should not permeate the membrane as well as water.

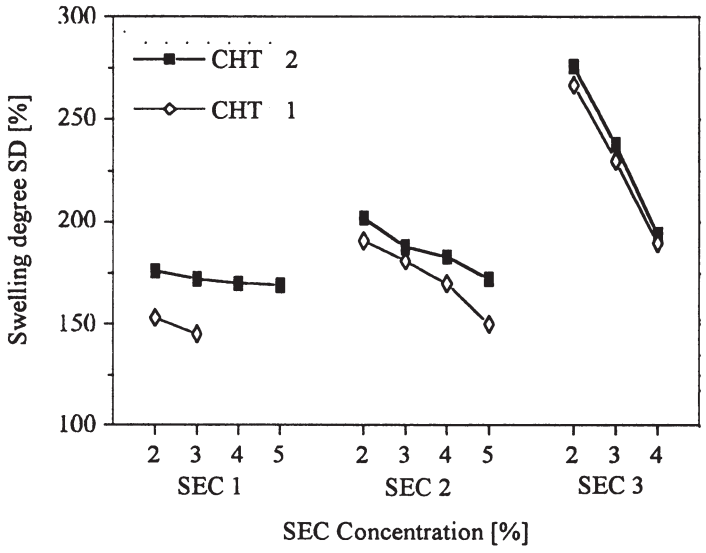

Figure 2. Swelling degree of different membrane samples.

The degree of swelling is dependent upon the components used and their concentration in the casting solution. Figure 2 shows the results in pure water. The SD increases with increasing molar mass of sulphoethylcellulose or chitosan and with decreasing concentration. This can be explained by the change in network meshes. The largest degree of swelling is exhibited by the

membranes of sample CHT 2 or SEC 3, which have longer chain lengths than sample CHT 1 or SEC 1. The longer the polymer chains of the components, the greater the meshes of the membrane network are, and the more the membrane may swell. If the concentrations are low, there are large distances between the chains, and big meshes can be formed in which water can intercalate into the membrane. However, with increasing concentration the volume between the chains is filled and the resulting meshes become smaller.

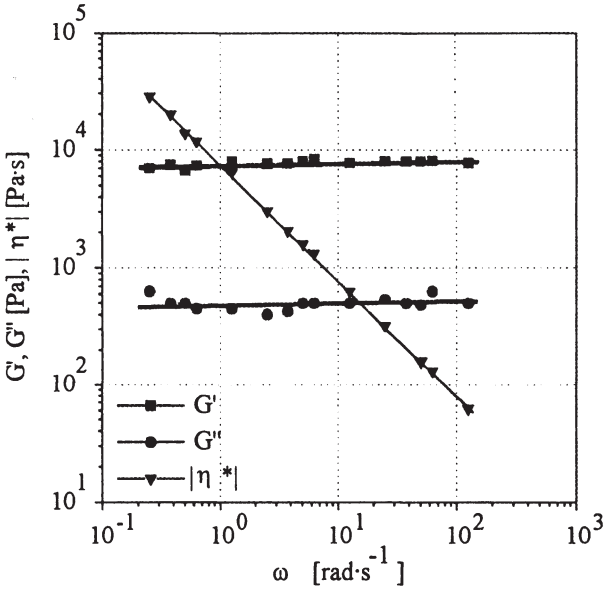

Figure 3. Dependence of the oscillation frequency upon the storage and loss moduli.

The swollen membranes are a network system and can be characterised by means of rheological oscillation measurements. Figure 3 shows schematically how the oscillation frequency depends upon the storage and loss moduli. In the case of networks a plateau modulus, G'_P, exists from which the molar mass between two cross-links M_e can be calculated. The higher the plateau modulus, the smaller the meshes are, and the more compact the network will be [17].

$$M_e = \frac{\rho \cdot R \cdot T}{G'_P}$$

The plateau modulus increases with increasing concentration, increasing charge density and decreasing molar mass. The membranes become more compact, which influences the separation properties. Table 2 shows the dependence of mesh size and network density upon SEC concentration, molar masses of SEC and chitosan, degree of substitution and degree of deacetylation.

The swelling behaviour of the membranes can be combined with the mesh size as seen in figure 4. The larger the meshes of the network, the more the system swells.

Table 2: Dependence of network properties of molecular parameters of SEC and chitosan.

increasing	c (SEC)	M (SEC)	M (CHT)	DS	DD
mesh size = M_e	decreasing	increasing	increasing	decreasing	decreasing
network density	increasing	decreasing	decreasing	increasing	increasing

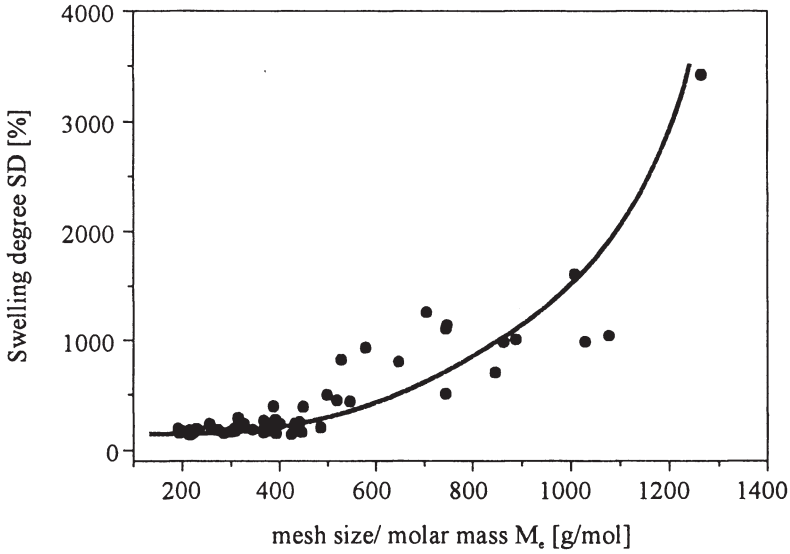

Figure 4. Dependence of mesh size upon swelling degree.

The morphological structure of the membranes was examined by scanning electron microscopy. A cross-sectional view of a freeze-dried symplex membrane shows that the membranes are dense, homogeneous and symmetric (figure 5). There is no difference between the top and the bottom of the membrane surface.

Figure 6 illustrates the permeation rate and separation factors of some membranes. They show excellent separation performances with high separation factors and high permeate fluxes. With increasing water concentration in the feed mixture, the separation factor decreases and the permeation rate increases. This is caused by the correlation between permeation and swelling. The more swollen a membrane is, the better a solution can permeate, because of the increasing mesh size. But this also implies that the separation efficiency decreases, because the alcohol molecules can also permeate better. So the membranes with sample CHT 3 show very good separation factors but quite low permeation fluxes and the membranes of sample CHT 1 have high fluxes and low separation factors.

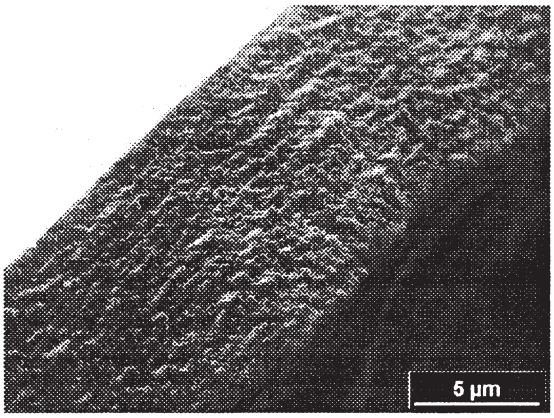

Figure 5. Cross-sectional view of a symplex membrane.

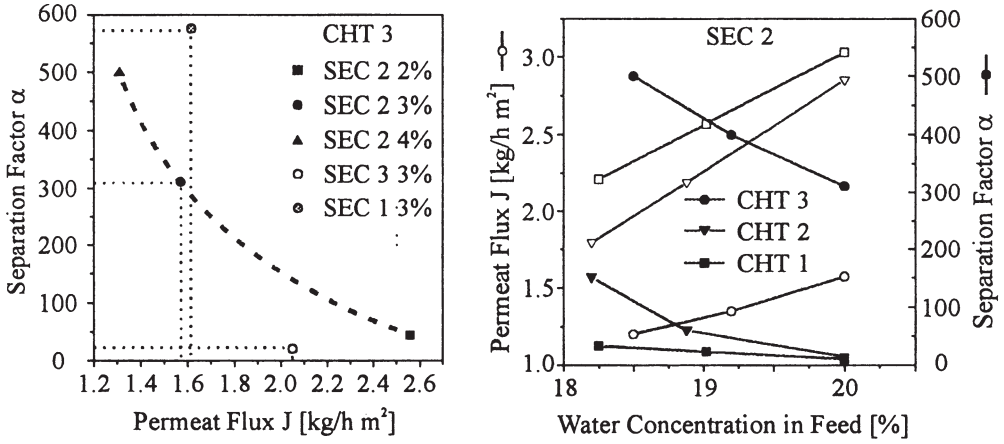

Figure 6. Separation properties of membranes with isopropanol-water mixtures at 50°C. a: different SEC samples, b: different chitosan samples.

The membranes of sample SEC 2 show different separation properties as a function of SEC concentration in the casting solution: the permeation rate J decreases and the separation factor α increases. With increasing concentration the membrane structure becomes more compact. The larger the molecules are, the more time they need to permeate, which improves the separation efficiency.

Conclusions

Sulphoethylcellulose and Chitosan are suitable raw materials for synthesising symplex membranes. Characterisation of the components gives useful information about their molecular parameters and their solution structure. The samples investigated mainly differ in molar mass and charge density.

The membranes produced are dense, homogeneous and symmetrical. They swell in water but not in alcoholic solutions. With increasing SEC concentration in the casting solution the membranes become more compact. This influences the plateau modulus in oscillation measurements and the separation parameters. The membranes show good separation factors at high permeation rates and are suitable for the dehydration of alcoholic solutions.

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Preparation and use of chitosan – Ca pectinate pellets

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Summary
Chitosan is a biopolymer with the capability of adsorbing heavy and transition metal ions in aqueous systems.

With the aim of improve some metal ions adsorption, chitosan - Ca pectinate pellets were prepared by adding powder chitosan to a Na-pectinate solution (2 % w/v). The solution was dropped into a $CaCl_2$ solution (4%) and the pellets were washed with distilled water and kept refrigerated. It was concluded the pellets obtained by this way actually improved the performance of controlled adsorption compared for the same conditions and for the same ions Pb(II), Hg (II) and Cd (II), according to the respective adsorption isotherms, at 20 ± 1°C.

Introduction

The removal of heavy metals in aqueous systems as contaminants is very difficult and shows problems, not only because of methodology but also because its industrial cost. Some techniques, such as chemical precipitation and reverse osmosis are not effective in this situation, and adsorbents as activated carbon and ion – exchange resins have their limitations.

The chitosan has a high affinity for virtually all non alkali, group transition – metal cations [1]. A very attractive route appears in these days: the bioadsorbents. Chitosan – Ca alginate pellets were introduced during this decade [2] for the remotion heavy and transition metal ions. The chitosan is usually obtained in powered form that is both nonporous and soluble in acidic media [3]. The low internal surface area of the nonporous material limits access to interior adsorption sites and hence lowers metal ion adsorption capacities and adsorption rates.

For metal ion adsorption applications, the high internal surface area of the porous beads could boost the metal binding capacity and also increase the transport rate of metal ion into particle [4].

The solubility of chitosan in acidic media prevents its use in recovery of metal ions.

In the present work we describe the immobilization of two biopolymers, chitosan (cationic) and Na pectinate (anionic), process which involves the metal ions union to the hydroxil and amino groups on the chitosan [5], and to the carboxyl group on the pectinate.

The purpose of this work was the presentation of a methodology to obtain chitosan – Ca pectinate pellets and the feasibility of their use for the remotion of this kind of contaminants in waste water.

Materials and Methods

The chitin was isolated from crawfish exoskeleton. The chitosan used was prepared by deacetylating chitin with NaOH 70% (w/v) at 136 °C. Following that step, it was washed with distilled water until the pH value of chitosan suspention reached 7. Then it was treated with ethanol and acetone, dried at 105 °C during an hour, and the obtained powder was sieved through mesh 140-200 (deacetylation 83% and viscosity 160 mPa s.).

Pectin used was from SIGMA (cytric source, deacetylation grade 84%).

First it was considered essential to establish the best Pectin-Chitosan (P/CH) ratio for obtaining uniform and with right firmness pellets, and also pellets which could stay stable through time and during stirring.

Studies were conducted with different ratios, obtaining the following results:

- P/CH ratio 0.45 : beads formation was not observed.
- P/CH ratio 1.80: beads with the demanded features were formed.
- P/CH ratio 5.00: beads features obtained were better than with ratio 1.80
- P/CH ratio 7.20: beads properties were similar to those obtained with ratio 5.00
- P/CH ratio 10.0: irregular rings were obtained.

Therefore, 1.80, 5.00 and 7.20 ratios were used.

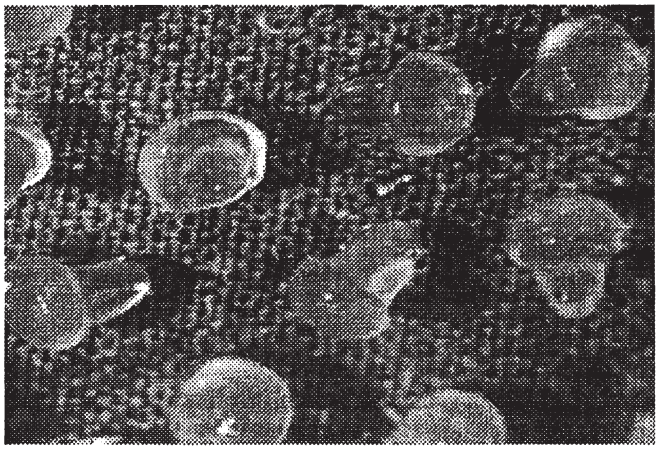
With the aim of choosing one of these three ratios, we studied the adsorption of Pb (II), Hg (II) and Cd (II),that is, which was the ratio that gives pellets with the best retention of these species.

The stirring times were 1,5,10,20,40,45,60,75,90,115,120 and 150 days.

According to these preliminary tests the P/CH ratio 5.00 showed the best adsorption in the lower time and the beads were not deformed during stirring.

The pellets were prepared mixing powder chitosan with 100 mL suspention Na-Pectinate 2% (w/v).

The suspention was homogenized with a magnetic stirrer and immobilized by dropping it into a solution of $CaCl_2$ (4% w/v). The resulting beads were kept in this solution for 12 to 24 hours at ambient temperature in order to hardened them.



Second, the beads were distilled water washed. The resulting beads were kept refrigerated between 4 –7° C until they were used. The species involved in the adsorption

study were determinated by plasma induced emision Spectrophotometer SHIMADZU ICPS 1000 III equipped with ultrasonic aerosol generator UAG 1.

Results and Discussion

To show the pellets useful life span we studied the Hg (II) adsorption. The pellets can be used after 5 months since they were prepared, as shown in figure 1.

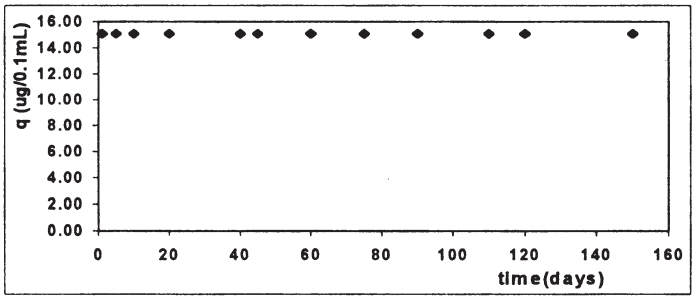


Figure 1. Hg(II) 7.56 10^{-4} M. $NaClO_4$ (ionic strength 0.01M) pH 5. Batch stirring, temp. 20 ± 1°C

To show the pellets efficiency during adsorption, individual experiences were performed with Pb(II), Hg (II) and Cd (II), and they were compared with chitosan and pectin as adsorbents themselves.

The optimal operating conditions as stirring time, pellets quantity, temperature and ionic strength were also studied.

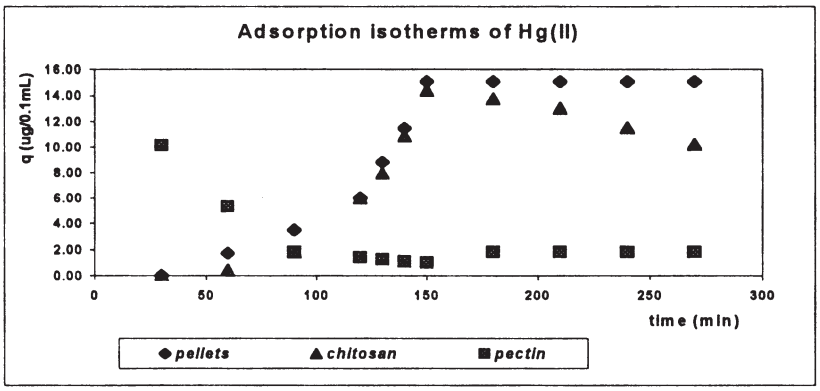


Figure 2. Hg(II) 7.5 x 10^{-4} M. $NaClO_4$ (ionic strength 0.01M) pH 5. Batch stirring, temp 20± 1°C

As shown in figure 2, Hg(II) shows a different behaviour with chitosan ,pectin and with the pellets.

The chitosan adsorbs following the parabolic law of diffusion, but once it has reached a maximum at 150 min, a slow desorption takes place. The pectin shows an absolutely different adsorption isotherm compared to the chitosan pellets isotherm. In the experimental results showed by the pectin, first adsorption takes place and second a slow desorption,

reaching equilibrium at a very low concentration. Pellets behave quite similar to chitosan, but doesn't show desorption during our experiences.

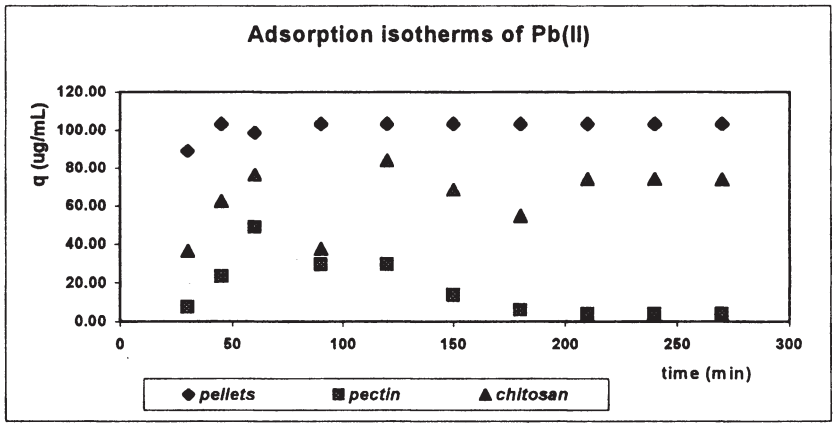


Figure 3. Pb(II) 5 x 10^{-4} M. $NaClO_4$ (ionic strength 0.01M) pH 5. Batch stirring, temp 20± 1°C

Doing a similar analysis to Hg(II) in Pb (II) (figure 3), chitosan shows consecutive adsorption-desorption cycles, and then reaching equilibrium. The pectin shows a valuable initial adsorption, and second a slow desorption. The pellets show a greater adsorption, reaching equilibrium after 80 minutes.

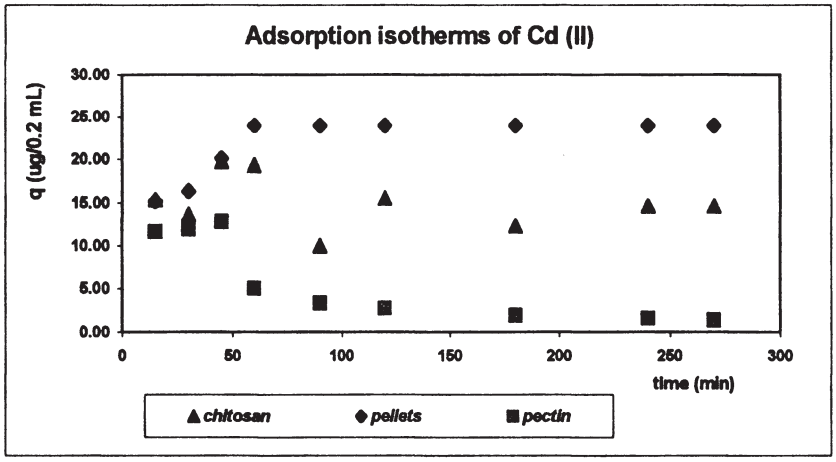


Figure 4. Cd(II) 1.06 x 10^{-3} M. $NaClO_4$ (ionic strength 0.01M) pH 5 Batch stirring, temp 20± 1°C

Cadmium shows (figure 4) a similar behaviour than Pb (II). The adsorption process for the pellets shows two steps until it reaches a maximum adsorption. First, a quick

superficial adsorption takes place, and after that diffusion trough superficial porous appears and this is the rate-limiting step [1].

In every experimental step for the adsorption control for Pb(II), Hg (II) and Cd (II)it was concluded that the pellets showed the best efficiency for the three species adsorption.

This reveals that the functional groups involved that appear in each chitosan and pectin are in a better position in the pellets, regarding to adsorption.

Adsorption sites will involve positively charged amino groups (NH_3^+) and to secondary ionized alcohols (alcohol-O^-) due to the fact that ph is 5.

Supporting on theoretical calculation done in our laboratory using EHMO[6] and modify by Calzaferri et al. (ICONC program)[7] we have enough reason to explain that the different behaviour are due to a different binding energy between metal and corresponding functional group, and this would support the different chitosan adsorption-desorption processes, being this binding weaker than the binding in the pellets[8].

It was concluded that chitosan - Ca pectinate pellets constitute an excellent system for eliminating contaminants from water systems.

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Bioseparation of protein from cheese whey by using chitosan coagulation and ultrafiltration membranes

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Summary
The protein removal from cheese whey was highest by using 50 mg/L of chitosan solution in comparing with other coagulants. Consequently, the supernatant was separated by ultrafiltration using chitosan membranes. Performance of chitosan membranes were measured by the flux and percent rejection of the protein. It was found that the flux value was higher than that of the flux without chitosan coagulation. Chitosan coagulation was used mainly to increase the flux as the reduction of dissolved solids which were achieved by coagulation. The protein rejection was decreased for the same reason. The result showed that chitosan can be utilized including coagulation and ultrafiltration for bioseparation of the protein.

Introduction
Chitin is polymer of [β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose]. It is one of the most abundant organic materials on earth and second only to cellulose and murein, which is the main structural polymer in the cell wall of bacteria. The principal derivative of chitin is chitosan, produced by alkaline deacetylation of chitin. Chitosan is polymer of [β-(1→4)-2-amino-2-deoxy-D-glucopyranose] [1]. Chitin and chitosan have wide application in food, pharmaceutical, and other industrial uses such as wastewater treatment, bioseparation, coating and protective materials. Regarding to the applications of chitosan, the purpose of this study is to evaluate the activity of chitosan as coagulant in comparison to other commercial products and the performance of chitosan membrane in ultrafiltration for concentration of cheese whey protein [2-4].

The main purpose of using ultrafiltration to treat whey is to concentrate the native whey proteins to obtain a protein powder. A number of whey-pretreatment have been developed to improve UF membrane flux rate for fractionating the whey protein concentrate (WPC). The flocculation of cheese whey protein by chitosan can also improve UF membrane flux rate for fractionation [5-8].

Materials and Methods

The cheese whey was collected from local dairy factory having 1008 mg/l total kjeldahl nitrogen (TKN), 6300 mg/l protein nitrogen, 62177 mg/l total solid and pH of 4.7. Protein present in the supernatant of cheese whey were primarily in dissolved form. However, they may precipitate out after pH adjustment. Accordingly, effect of pH adjustment on protein precipitate and settlement from cheese whey supernatant was first investigated. 1 liter of supernatant cheese whey was taken into a number of 1 liter beakers and pH ion each beaker was adjusted to a specific value ranging from pH 6.0 to 10.0. Turbidity and protein content of the supernatant before and after pH adjustment were recorded for estimation of protein removal efficiency.

Investigation were conducted by jar test to estimate effect of operating parameters like pH and coagulation dosage on protein removal. Four different coagulants were used viz. chitosan, polyaluminium chloride (PAC), alum sulfate ($Al_2(SO_4)_3 \cdot 14H_2O$), and carrageenan. Separate stock solution of the four coagulants were first prepared with concentrations of 5g/l of chitosan in 1% acetic acid [1], 50g/l of PAC, 50 g/l alum, and 5 g/l of carrageenan in water. The operating conditions are summarized in Table 1.

Table 1. Various operating conditions in coagulation

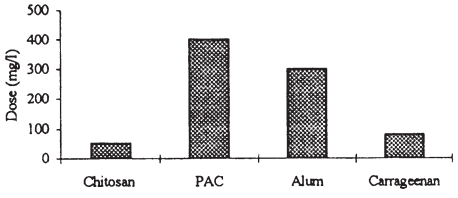
Operating conditions		Unit	Values
Coagulant:	Chitosan	mg/l	20-90
	PAC	mg/l	100-500
	Alum	mg/l	100-500
	Carrageenan	mg/l	20-100
pH		-	6.0-10.0
Rapid mixing:	Speed	rpm.	150
	Contact time	min.	2
Slow mixing:	Speed	rpm.	20
	Contact time	min.	15
Sedimentation in Imhoff cone		min.	60
Filtration		min.	10

Cheese whey separation was done by using chitosan as coagulant and then let to separate protein from supernatant cheese whey by chitosan membrane with the selected operation of ultrafiltration. Chitosan membrane is prepared from local commercial chitosan products form shrimp shells with a moisture content of 10.00%, ash content of 0.50% and degree of deacetylation of 74.85%.

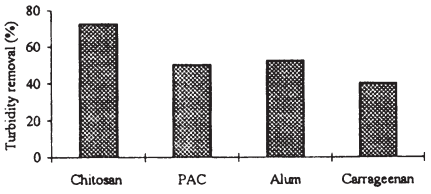
The ultrafiltration experiments were conducted in DDS mini lab 10 module by chitosan membrane of 20000 Dalton MWCO. The experiments were investigated by varying applied pressure at 0.9, 1.2 and 1.5 bar while keeping retentate flowrate constant at 0.6 GPM. The retentate flowrate was then varied at 0.2, 0.6 and 1.0 GPM keeping applied pressure constant at 1.2 bar. Temperature was maintained at 25°C for both experiments. The pH of feed concentration was fixed at 7.0 for chitosan membrane. Cheese whey without and with chitosan coagulation was tested for each set of ultrafiltration experiment. The permeate solution was collected every hour for 10 hours to analyze flux [9] and protein rejection [7]. Protein concentration of cheese whey in feed tank and permeate stream was measured spectophotometrically using calibration curve developed by Bradford Assay [10].

Results and Discussions

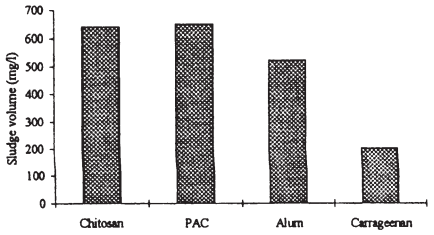
Figure 1 illustrates the performance of different coagulants at their selected operating conditions. As can be seen, performance by chitosan is superior than other coagulants in all respects.



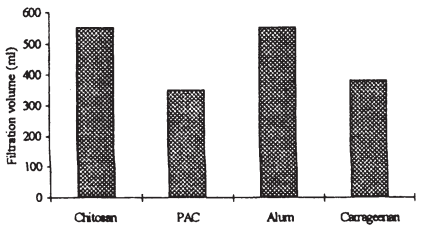
(a) Dosage for coagulants at optimum pH



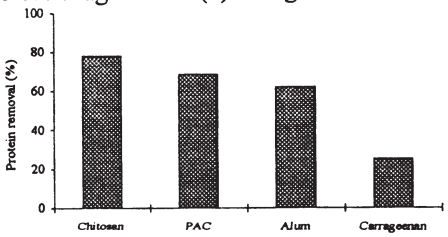
(b) Turbidity removal for different coagulan



(c) Protein removal for different coagulant



(d) Sludge volume at different coagulant



(e) Filtration volume at different coagulant

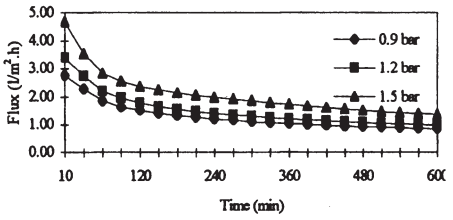
Figure 1. Effect of coagulation on coagulants

Chitosan yielded better protein as well as turbidity removal at lower dosage value. Coagulant dosage at selected operating conditions for chitosan was only 50 mg/l as compare to 400 mg/l for PAC, 300 mg/l for alum, and 80 mg/l for carrageenan while superior protein removal of 77.8% was obtained by chitosan in comparison with 68.5%, 62.0%, and 25.0% by PAC, alum and carrageenan, respectively. Turbidity removal of 72.5% was obtained by chitosan under selected conditions while only 50.0%, 52.5% and 40.0% removal were recorded by PAC, alum and carrageenan, respectively. This data also suggests turbidity removal is not proportional to protein removal from cheese whey. Raw Cheese whey was necessary to maintain at a pH of 7.0 otherwise chitosan membrane was damaged and/or dissolved by lactic acid if pH lower than 5.8.

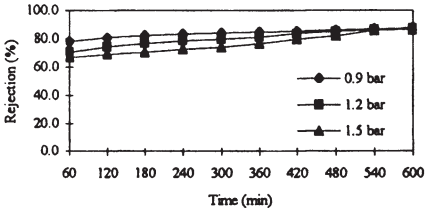
Effect of applied pressure on chitosan membrane without coagulation: The effect of applied pressure on permeate flux and protein rejection was investigated by performing experiments with three different applied pressures viz. 0.9, 1.2, and 1.5 bar. The flowrate from retentate stream was kept constant at 0.6 GPM in all the experiments.

Permeate flux: Permeate flux in all the batches showed marked reduction of 48.4%-54.3% for chitosan membrane during first hour of operation. Steady state flux was obtained after about 7 hours of operation in case of chitosan membrane. Steady state permeate flux for chitosan membrane (CTS) were 0.88, 0.68, and 0.61 l/m^2.h for the applied pressure of 1.5, 1.2, and 0.9 bar, respectively (Figure 2a). The decrease in permeate flux during a typical batch operation can be attributed to the increase in thickness of protein gel layer which increases the resistance to permeate flux. Deposition and adsorption of colloidal proteins on external surface and internal pores of the membrane leads to reduction on membrane permeability, causing reduction of permeate flux [6].

Protein rejection: Average protein rejection during each hour of sampling interval with respect to batch time is plotted in Figure 2b. Protein rejection is lowest of about 66.8% with CTS for 1.5 bar applied pressure as compare to 70.5% and 78.0% with CTS for 1.2 and 0.9 bars during first hour of batch operation. This is primarily because at the beginning of batch operation, the external surface as well as the internal pores of the membrane are clean and hence more protein molecules can escape through the membrane at higher operating pressure [5]. The steady state protein rejection are 81.4%, 82.8% and 85.6% with CTS for 1.5, 1.2, and 0.9 bars respectively (Figure 2b) implying higher steady state protein rejection for higher operating pressures. It is expected that this values will eventually reach time averaged protein rejection as batch operation time further increases.



(2a) Permeate flux



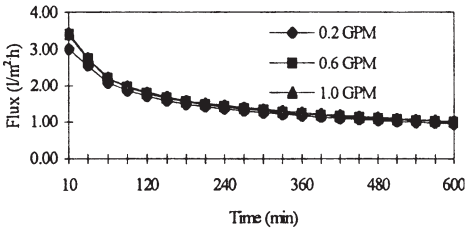
(2b) Protein rejection

Figure 2. Effect of applied pressure on chitosan membrane without coagulation

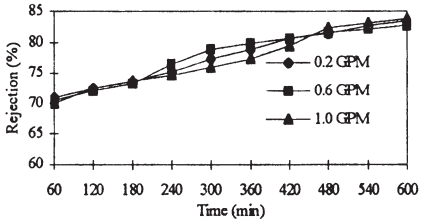
Effect of flowrate on chitosan membrane without coagulation: *Permeate flux*: Figure 3a shows that permeate flux was higher for higher retentate flowrate after first hour of batch operation. Steady state permeate flux of 0.71, 0.68, and 0.65 l/m^2.h with CTS were recorded for retentate flowrate of 1.0, 0.6, and 0.2 GPM, respectively. The increase in permeate flux with increased retentate flowrate can be attributed to the effect of high flowrate to remove gel layer on membrane surface [6].

Protein rejection: Since gel layer on membrane surface during batch operation could be partially removed by high flowrate [5], it was expected that protein rejection would decrease with increased flowrate. Accordingly, the variation of protein rejection with batch duration based on cumulative values was presented in Figure 3b. As can be seen from these

figures, protein rejection during batch operation does not show improvement while the variation with flowrate from 0.2 to 1.0 GPM.



(3a) Permeate flux



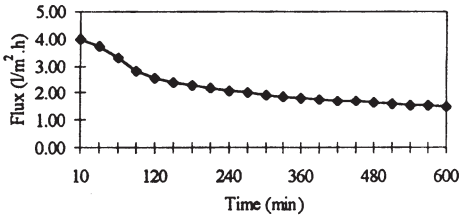
(3b) Protein rejection

Figure 3. Effect of flowrate on chitosan membrane without coagulation

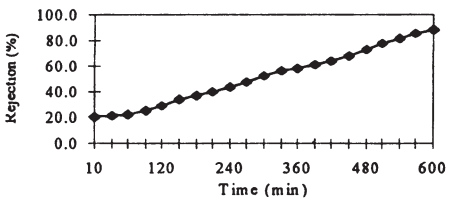
Chitosan membrane of MWCO 20000 Da ultrafiltration for separation of proteins from cheese whey showed the steady state permeate flux of 0.68 l/m^2.h and protein rejection of 86.5% at optimum pressure of 1.2 bar and retentate flowrate of 0.6 GPM.

Ultrafiltration of chitosan membrane with coagulation: *Permeate flux*: Figure 4a shows the cumulative permeate flux decreased around 17.6% during first hour of operation for chitosan (CTS) membrane . Steady state flux was obtained after about 8 hours of operation. The cumulative flux reduction for CTS membrane 62.6% at the end of operation. Permeate flux for both membranes in case of cheese whey with chitosan coagulation was higher than that of without coagulation. .

Protein rejection: Figure 4b shows protein rejection in case of cheese whey with chitosan coagulation were lower than in case of cheese whey without chitosan coagulation. The cumulative protein rejection started at 20% and increased to 88.8% for CTS membrane. Steady state protein rejection was found when it reached at 8 hours. The nature of the curve was similar although this reduces the permeate flux due to the increased resistance caused by protein deposition on membrane and partial blocking of inter membrane pores also reduces any further leakage of protein molecules through the membrane which results in improved protein rejection and this operation occurs lower when applied coagulation.



(4a) Permeate Flux



(4b) Protein rejection

Figure 4. Permeate flux and protein rejection with time for chitosan membrane with coagulation

Conclusions

Coagulation of cheese whey using chitosan and other coagulant varied with pH of the cheese whey. For optimum pH at 7.0 chitosan showed better and more effective coagulant compared to alum, PAC, carrageenan. Chitosan flat sheet membranes showed their high performance for ultrafiltration of cheese whey proteins. Permeate flux and protein rejection depends on effect of applied pressure and effect of retentate flowrate. Permeate flux decreasing and protein rejection increasing caused by deposition and adsorption of colloidal proteins on external surface and internal pores of the membrane leads to reduction on membrane permeability. Cheese whey protein ultrafiltration followed by chitosan coagulation improved the flux as chiotsan coagulant removed portion of protein during coagulation. A decrease in protein rejection occurs during ultrafiltration with chitosan coagulation as solid to a large extend has already been removed during coagulation.

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Preparation of silk fibroin/chitosan fiber

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Summary

85% phosphoric acid was selected as a cosolvent for dissolving chitosan and silk fibroin mixture, and then the mixed solution was wet-spun into coagulation bath (3M ammonium sulfate) through the nozzle of 0.02 cm diameter. Spinnable concentration was about 16% and the viscosity of solution was lower than 1000 poise.

In FT-IR spectra of chitosan and silk fibroin/chitosan fiber, carbonyl peak of silk fibroin was shifted to longer wavelength by hydrogen bonding formation between amino group of chitosan and carbonyl group of silk fibroin. SEM photographs of the surface and cross-sectional view of the silk fibroin/chitosan fiber and that of chitosan-extracted has shown the fibril structure of silk fibrion.

The degree of orientation was increased with the take-up speed and tenacity of silk fibroin/chitosan fiber was about 1.26 g/d. And its average elongation of fiber was about 20% at break point. Antimicrobial activity was evaluated by using *Staphylococcus aureus*, and tested by Shake Flask Method. Bacteria reduction rates of the silk fibroin/chitosan fibers were about 50%.

Introduction

Nowadays, natural polymers have become more and more important for their rich resources and many specific properties such as nontoxicity, biodegradability, and good biological compatibility. Chitosan, poly-(1,4)-2-acetamido-2-deoxy-β-D-glucose, is the second most abundant natural polymer. Chitosan, structurally cationic polysaccharide, is produced by alkaline deacetylation of chitin which occurs mainly in the exoskeleton of crustacea. It is widely used in the fields of medicine, food, materials, cosmestics, environment, agriculture and also in textile industry because it has antimicrobial activity, low toxicity, biocompatibility, and cationic character. Silk fibroin, which is derived from silkworms. Silk fibroin has been used as a high valued textile fiber because of its superior elasticity, strength, softness, lustre, absorbency and a good affinity for dyestuffs. Recently silk fibroin is catching the attention as a biosensor system and also a biological compatibility. The objectives of this study are to select the suitable wet-spun-cosolvent system and spinnable condition for silk fibroin/chitosan solution and to characterize the theological, mechanical, microstructure and antimicrobial properties of the fiber formed.

Materials and Methods

Raw silk fibroin was provided by Korea Silk Research Institute. It was degummed by boiling in 0.3% sodium oleate. Four commercial chitosan samples of similar degree of deacetylation (DDA) (ca. 85~90%) having different molecular weight were used. Other chemicals of first grade were used without further purification.

DDA was determined by the titration method; Chitosan was dissolved in 0.5 mole/L of 0.3 N HCl. Titration was performed with a standard solution of 0.1 N NaOH. There were two inflection points on the titration curve when pH was plotted against the amount of titrant (0.1 N NaOH). DDA was calculated from the amount of NaOH consumed between two inflection points.

16%(wt) mixed solutions dissolved in 85% phosphoric acid were extruded directly into a coagulant bath containing 75% ammonium sulfate. Suitable take up speed at spinning was from 2~12 m/min. The threadlines were coagulated immediately upon emerging from the tip of the nozzle (diameter of 0.02 cm and pressure of nitrogen gas was 1 kg/cm^2). The fibers produced were washed several times to remove the residual solvent and coagulant.

The viscosity of the mixed solution was measured by programmable viscometer (Brookfield Model LV, 25 °C).

The structure of fiber was evaluated by using IR-spectrometer and SEM analysis. The tensile property and orientation at different take up speed were determined by UTM and X-ray diffractometer. The antimicrobial property of silk fibroin/chitosan fiber was evaluated by using *Staphylococcus aureus*, and tested by Shake Flask Method.

Result and Discussion

The solubility of silk fibroin and chitosan was determined in a number of potential dope solvents at room temperature. Some cosolvent systems of silk fibroin and chitosan were chosen among acidic solvents because chitosan is soluble in an acidic condition. Phosphoric acid was chosen for the suitable cosolvent because the solution was stable up to 2 hrs (Fig. 1).

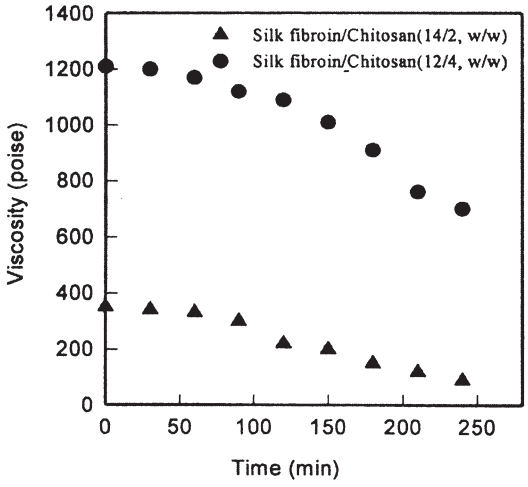


Fig. 1. Viscosities of silk fibroin/chitosan mixture with the change of time (spinndle number 6, r.p.m. 1.0, 23°C)

Suitable spinnable viscosity range was 300~1000 poise and the solution was stable within 2 hrs. When the viscosity was above 1000 poise, it required a lot of time to remove air bubbles formed by stirring. And the viscosity was decreased very quickly because of the degradation of silk fibroin.

16%(wt) mixed solutions dissolved in 85% phosphoric acid were extruded directly into a coagulant bath containing 75% ammonium sulfate. Suitable take up speed at spinning was from 2~12 m/min. The threadlines were coagulated immediately upon emerging from the tip of the nozzle (diameter of 0.02 cm). The fibers produced were washed several times to remove the residual solvent and coagulant.

Interactions between chitosan and silk fibroin was identified by using the technique of FT-IR. In FT-IR spectra of chitosan and silk fibroin/chitosan fiber, carbonyl peak of silk fibroin was shifted to longer wavelength at hydrogen bonding formation between amino group of chitosan and carbonyl group of silk fibroin for blend fiber (Fig.2). The interaction was confirmed by observing the SEM photographs of the surface and cross-sectional view of the silk fibroin/chitosan fiber(Fig. 3).

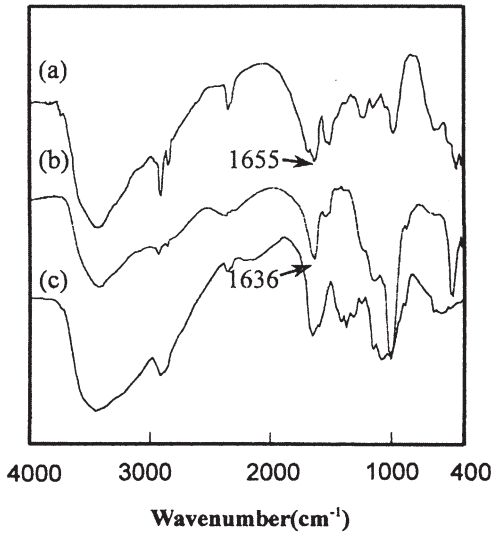
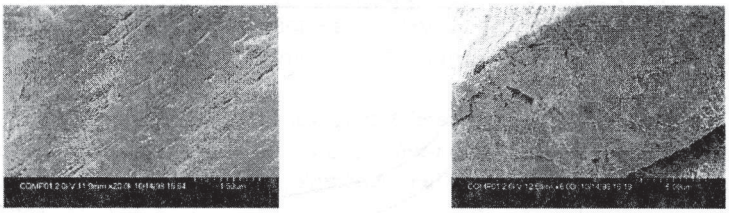
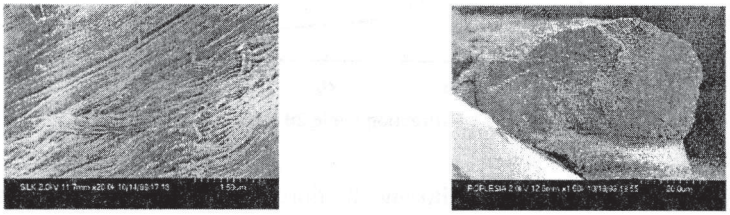


Fig. 2. FT-IR spectra of silk fibroin, chitosan and silk fibroin/chitosan fiber ((a): silk fibroin, (b): silk fibroin/chitosan, (c): chitosan)

Tensile strengths of silk fibroin/chitosan fiber was determined by UTM (STM-5, USA). Tenacity of silk fibroin/chitosan fiber was 1.26 g/d in dry condition and the average elongation was about 20% at break point (Fig. 4). Elongation at break point was somewhat irregular because of air bubble, especially when the viscosity of mixed solution was above 1000 poise. The strengths of silk fibroin/chitosan were slightly increased with chitosan component to silk fibroin. And the degree of orientation and strength of fiber was also increased with take up speed (Fig. 5).



(a) Silk fibroin/Chitosanfiber



(b) Chitosan-extracted from (a)

Fig. 3 Surface and cross-sectional view of silk fibroin/chitosan fiber and chitosan-extracted fiber from (a)

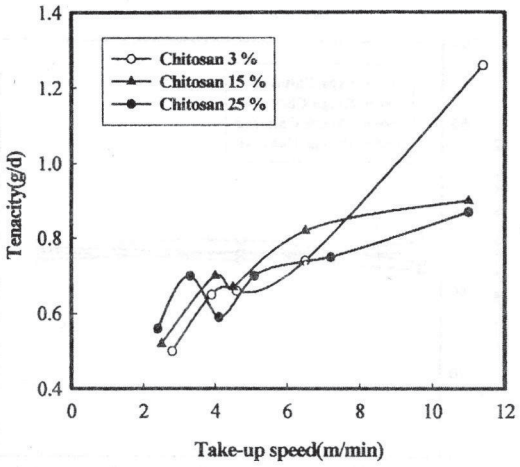


Fig. 4 Tensile properties of the silk fibroin/chitosan fiber with the take-up speed and component ratio of chitosan and silk fibroin (Chitosan (50 cps)).

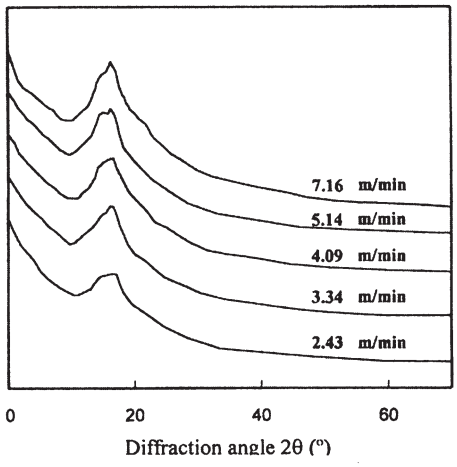


Fig. 5 WAXD equatoria scans of chitosan/silk fibroin fiber (2/14 w/w) with the take-up speeds

Antimicrobial activity was evaluated by using *Staphylococcus aureus*, the designated microorganism for the test, by Shake Flask Method. Bacteria reduction rates of the chitosan fibers were about 70%. But Bacteria reduction rates at silk fibroin chitosan fiber were about 50% (Fig. 6).

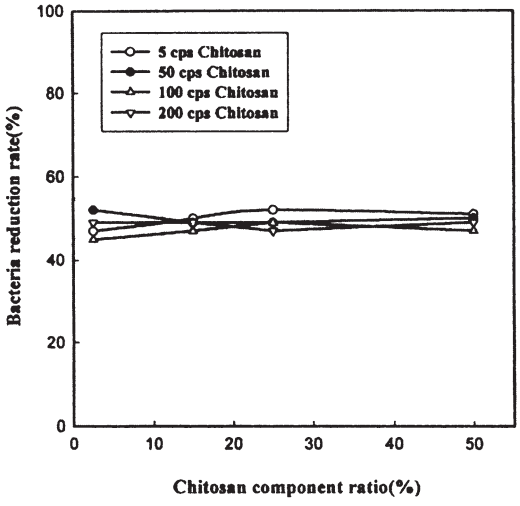


Fig. 6 Bacteria reduction rates of the silk fibroin/chitosan fibers at different silk fibroin/chitosan ratio.

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Preparation of paper sheets containing microcrystalline chitosan

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Summary
Paper sheets were prepared from cellulose pulp to which microcrystalline chitosan (MCCh) had been added (based on the direct introduction method). Alternatively, MCCh was precipitated on paper sheets at pH 10. In addition, papers containing a gel-like dispersion of MCCh and proteins (casein or keratin) were prepared.

Dry or wet paper sheets containing MCCh showed improved mechanical strength and lower swelling as compared with the unmodified paper. Papers with mixtures of MCCh and protein showed lower water retention values and mechanical strength as compared with papers prepared with MCCh alone. These differences were lower in wet paper sheets as compared with dried specimens. The paper sheets prepared according to the direct introduction of MCCh indicated better susceptibility to the biodegradation. Moreover, the addition of proteins caused the alteration of the biological decomposition of the paper sheets.

Introduction
Non-degradable composites of paper containing synthetic polymers which are resistant to enzymatic and microbial actions are widely produced all over the world. Well-known is the problem with utilization of these products and the necessity for the exploration of new packaging materials. Chitosan as a natural, biodegradable polymer having polycationic behaviour becomes an issue of significant interest and importance for applications in the packaging industry.

The aim of this study was to determine the properties of paper sheets prepared with addition of microcrystalline chitosan gel-like dispersions (MCCh), including formultions with proteins (keratin or casein). The alteration of the mechanical properties and the susceptibility to the biodecomposition of modified paper sheets were determined.

Materials and Method
Chitosan (DD=73.5%, Mv=187 000 Da, WRV=171.1%) was prepared from chitin of Antarctic krill (*Euphausia superba*) by heterogeneous deacetylation as described previously [1].

Preparation of paper modified by microcrystalline chitosan (MCCh) with or without protein: MCCh gel-like dispersion prepared by the method described previously [2] was added to 1 dm^3 cellulose fibre suspension (2.4 $g \bullet dm^{-3}$, equilibrated to 75 ± 2 $g \bullet m^{-3}$ of dry paper -

according to DIN 54358 standard). The amount of MCCh was 0.25, 0.5, 1.0, 2.0, 4.0 or 10 wt% of the dry weight of cellulose. Then the mixture was homogenized using a Rapid Homogenizer PTI-31 (Paper Testing Instruments GmbH, Austria), according to German standard ISO 5263, for 10 min at 3000 rpm. Paper sheets were formed using paper-forming machine V/8/76 (Paper Testing Instruments GmbH, Austria) based on ISO 5269/2 and DIN 54358 standards (Figure 1). The paper sheets were dried at 0.96 bar and at 93.0 ± 0.05 °C for 9 min and then air-dried in accordance with DIN 50014 standard. MCCh gel-like dispersion containing 20 wt% proteins (casein or keratin) for paper modification was also used.

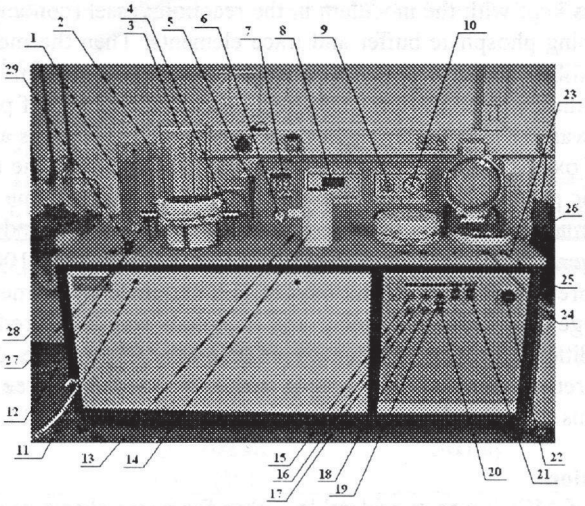


Figure 1. Paper-forming machine V/8/76 (Paper Testing Instruments GmbH. Austria): 1) water container, 2) water heater, 3) forming cistern (10 dm^3), 4) vacuum valve, 5) vacuum indicator, 6) indicator of water valve, 7) SPS console, 8) temperature indicator, 9) indicator of drying range, 10) time indicator for drying, 11) cover lock, 12) lock of forming cistern, 13) Silicon plate for paper forming, 14) forming roller, 15) "START" button for pump 1, 16) "STOP" button for pump 1, 17) "START" button for pump 2, 18) "STOP" button for pump 2, 19-20) Option of run (0 – "Automatic", 1 – "Manual"), 21) Option of water course (0 – "fresh water", 1 – "circular water"), 22) main switcher, 23) drying console, 24) "START' button for drying, 25) "STOP" button for drying, 26) vacuum valve, 27) "STOP" button for automatic run, 28) "START" button for automatic run, 29) 6-position scale for manual run.

Preparation of paper modified by precipitation of MCCh: To the cellulose-fibre dispersion (2.4 g of dry weight) 50 cm^3 distilled water was added and the mixture was adjusted to pH=5.0 by adding of 1 wt% aqueous acetic acid during agitation. Then, a 1.0 wt% aqueous solution of chitosan acetate (1.0, 2.0, 4.0 or 10.0 wt%, resp., of dry fibre weight), with or without 20 wt% protein (casein or keratin), was added. The cellulose-chitosan pulp was stirred overnight under vigorous agitation and then an aqueous solution of NaOH (2 wt%) was added dropwise until pH=10.0. Cellulose-MCCh pulp was stirred for 1 h and the pH was lowered to 8.0 with 1 wt% aqueous acetic acid solution. The suspension was filled up to 1 dm^3.

Homogenization and paper-forming procedure was carried out according to the condition described above.

Biological decomposition of paper sheets containing MCCh, with or without proteins, in communal waste (mineralization test).: The paper sheets containing MCCh with or without proteins (casein or keratin) prepared according to the direct introduction or precipitation of MCCh were used during this stage of study. As a reference, a cellulose sheet prepared by a similar procedure was used [3]. The investigated sample was milled using the Analytical Mill (IKA-WERK GmbH – Germany) at 20 000 rpm for 2 min before introduction to the sapromat. 25 mg of sample was kept with the inoculum in the reaction vessel (containing manometer) in the sapromat containing phosphate buffer and trace elements. Then the medium was filled up to 250 cm^3 using distilled water to obtain a concentration of 100 $mg \bullet dm^{-3}$. As inoculum, 2.5 cm^3 fresh, unclear slime obtained from sewage-treatment plant Stahnsdorf per vessel was used. The decomposition was carried out during 24 days at 25°C. The CO_2 was absorbed with soda. The consumption of oxygen causes the reduction of the gas volume in the reaction vessel that trough the electrolytic production of oxygen is balanced, so that the volume of gas in the vessel is constant. The demand for the current during the electrolysis responds to the biological demand on the oxygen and it is recalculated to ($mg\ O_2 \bullet dm^{-3}$ = $mg\ O_2 \bullet 100\ mg^{-1}$ investigated substance). The theoretical demand on the oxygen is determined by elementary analysis. The consumption of oxygen for inorganic media and inoculum was measured using a reference sample without addition of other substances containing carbon. The difference between biological and theoretical demand for oxygen responds to the degree of the biological decomposition of films or paper sheets.

Results and Discussion

The retention yield of MCCh was considerable higher for paper sheets prepared by means of precipitated chitosan with a lower magnitude of the initial chitosan dose. For initial concentration higher than 4-wt%, the highest productivity was yielded by the direct MCCh introduction method. The modification of MCCh gel-like dispersion by protein affected a decreasing tendency in the MCCh retention rate, especially for casein, resulting in the best linkage of protein with MCCh and the blockade free amine groups of chitosan (Figure 2).

The mechanical strength of paper sheets prepared according to the direct introduction as well as precipitation of MCCh increases at least 80% compared with non-covered paper sheets. When the highest amount of MCCh was added, the increase in Young's module by ca. 43% and burst resistance by ca. 85% were observed, whereas the precipitation method yielded increase by only 24% of Young's module and 48% of burst resistance (Table 1., Figs. 3 and 4).

The direct introduction of MCCh with a concentration from 0.25 wt% to 4 wt% resulted in increase in mechanical strength. However, further increase in chitosan content did not significantly increase this parameter.

Because of the rupture role plays water molecules during the paper formation, it was important to investigate the mechanical properties of paper sheets in high humidity.

Increase in humidity caused a reduction in burst resistance and Young's module of paper sheets and a weak increase in elongation at break. However, the increase of the mechanical properties compared to the reference remained. The lowest reduction in burst resistance and Young's modules was observed for all paper sheet samples containing MCCh with incorporated proteins. The introduction of protein affected a reduction of the distinction between the mechanical properties at low and high rel. humidity (Figure 5.).

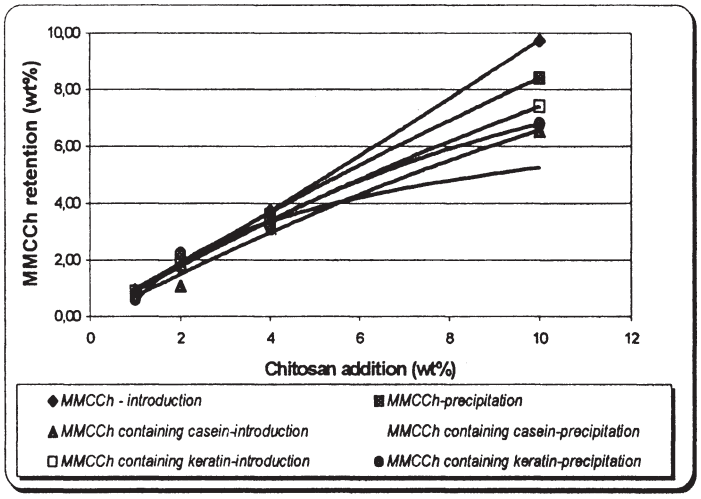


Figure 2. Introduction of MCCh vs. retention of MCCh in the paper sheets

Table 1. Mechanical properties of paper sheets produced with different amount of MCCh with or without protein at rel. humidity of 60%

Concentration of MCCh	Elongation at break (%)	Burst resistance (MPa)	Young's module (MPa)
	Direct introduction method		
0	1.79 ± 0.15	7.20 ± 0.28	1190 ± 100
0.25	1.85 ± 0.19	7.23 ± 0.26	1110 ± 90
1.0	1.85 ± 0.18	7.37 ± 0.20	1080 ± 100
2.0	1.73 ± 0.20	8.20 ± 0.47	1100 ± 150
2.0 containing casein	1.85 ± 0.29	7.70 ± 0.40	1330 ± 115
2.0 containing keratin	1.00 ± 0.20	5.50 ± 0.70	1170 ± 80
4.0	2.36 ± 0.31	10.56 ± 0.39	1310 ± 100
4.0 containing casein	1.74 ± 0.37	7.90 ± 0.70	1330 ± 50
4.0 containing keratin	2.00 ± 0.10	8.80 ± 0.30	1340 ± 60
10.0	2.57 ± 0.19	13.32 ± 0.56	1700 ± 130
	Precipitation method		
0	1.79 ± 0.15	7.20 ± 0.28	1190 ± 100
1.0	2.27 ± 0.24	7.70 ± 0.49	960 ± 120
2.0	2.43 ± 0.14	9.54 ± 0.22	1310 ± 102
2.0 containing casein	2.30 ± 0.20	8.20 ± 0.30	1010 ± 180
2.0 containing keratin	1.90 ± 0.30	7.70 ± 0.30	1130 ± 120
4.0	2.36 ± 0.24	10.88 ± 0.41	1340 ± 180
4.0 containing casein	2.20 ± 0.20	9.80 ± 0.50	1300 ± 200
4.0 containing keratin	2.00 ± 0.20	8.70 ± 0.30	1230 ± 150
10.0	2.20 ± 0.38	10.69 ± 0.42	1480 ± 150

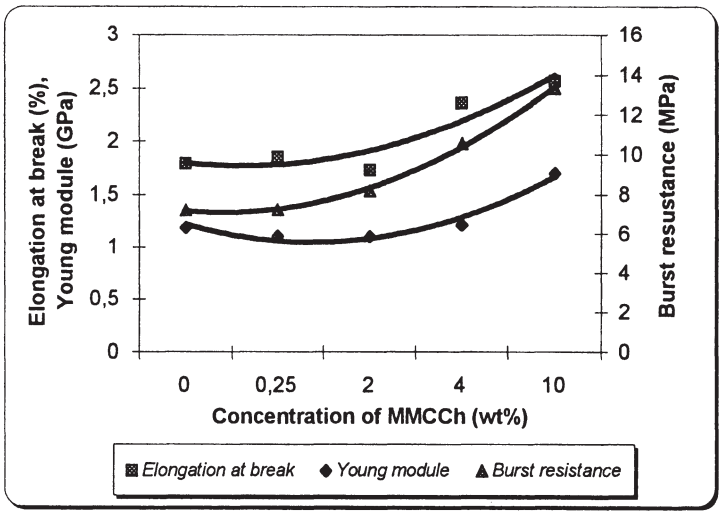


Figure 3. The influence of the concentration of MCCh on mechanical properties of paper sheets prepared by direct introduction method

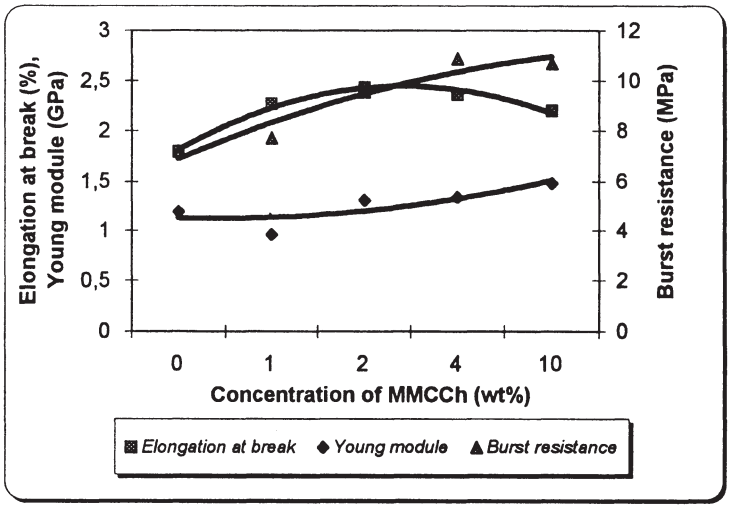


Figure 4. The influence of the concentration of MCCh on mechanical properties of paper sheets prepared from suspension of fibres with precipitated chitosan

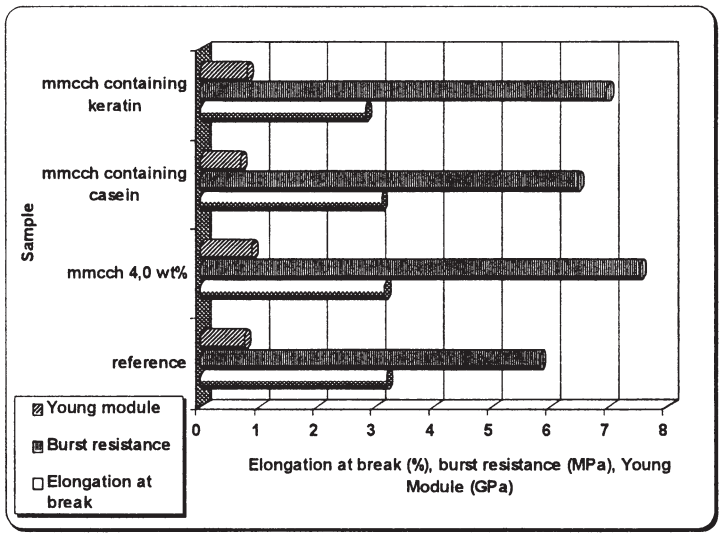


Figure 5. Mechanical properties of paper sheets in wet form prepared by the MCCh direct introduction method

A similar phenomenon was observed for paper sheets prepared according to the precipitation of MCCh. (Figure 6.).

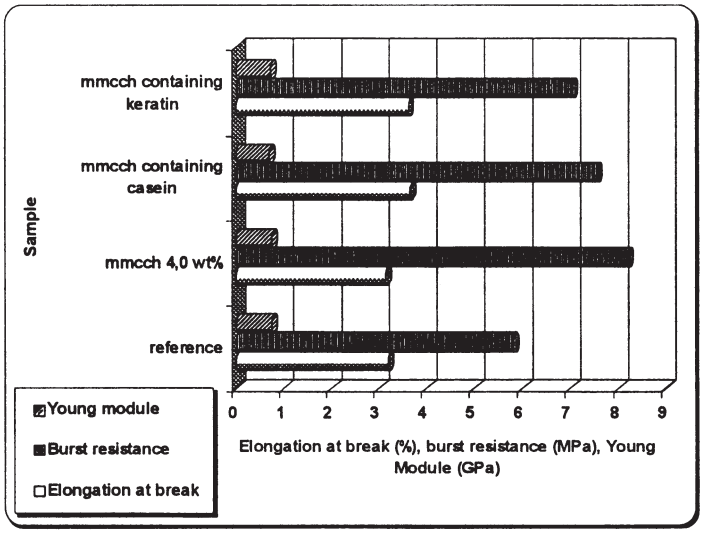
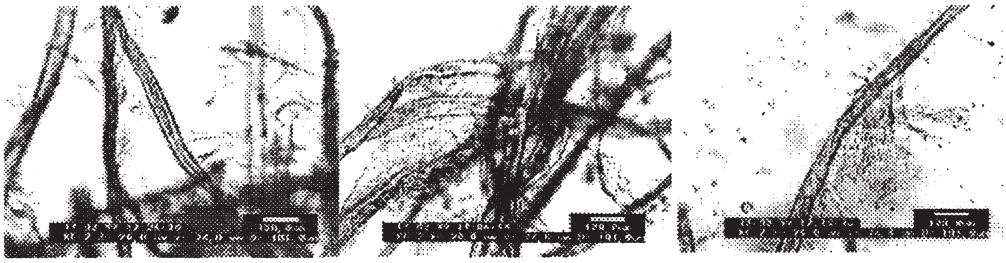


Figure 6. Mechanical properties of paper sheets in wet form prepared by the MCCh precipitation method

A cross-section of the samples allowed their morphological analysis (Figure 7.).



a) b) c)

Figure 7. Microphotographs of: a) cellulose fibres (x100), b) cellulose fibres with direct introduced MCCh gel-like dispersion prepared in a bath containing 20 wt% casein (x100), c) cellulose fibre with precipitated MCCh gel-like dispersion prepared in a bath containing 20 wt% casein (x100)

The coat-like structure of MCCh was observed among the separated fibres of the paper sheets containing its high amount. At lower concentration of MCCh, the surface of fibres was smoother, deprived of thinner fibres, which frequently was sticed together. The direct introduction method resulted in the "web"-like structure but the precipitation of MCCh gel-like dispersion preferred the formation of coating fibres. The coat-like fibres are often visible for the papers sheets prepared by the introduction of protein.

The biological degradation of paper sheets lead to fast decomposition (65%-83%) similar to cellulose paper used as a reference sample (Fig. 8).

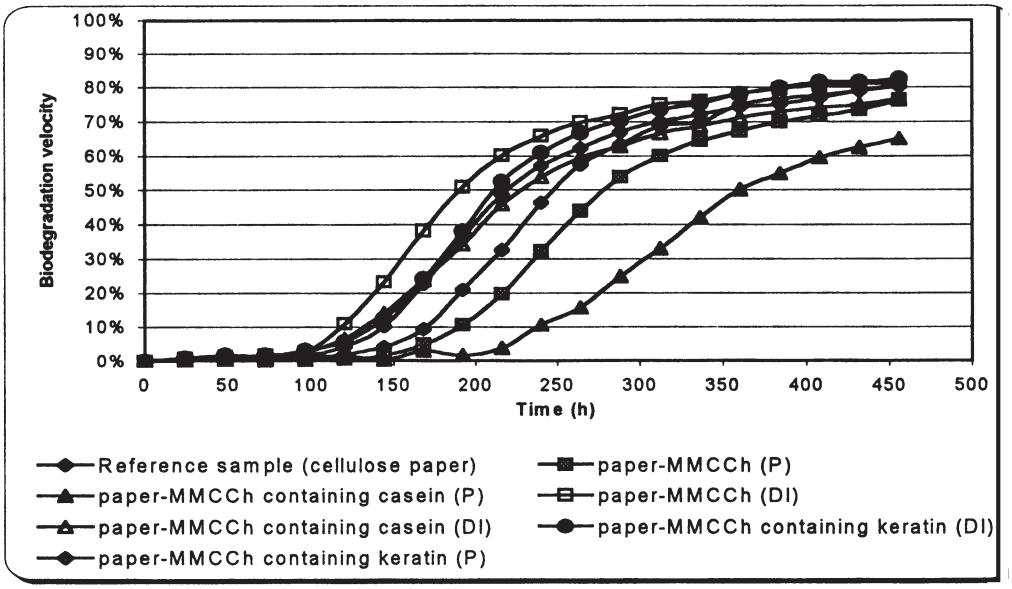


Figure 8. Biological decomposition of paper sheets prepared by direct introduction (DI) or precipitation (P) of MCCh, with or without proteins

The susceptibility to biodecomposition depended mainly on the method of MCCh addition applied. Moreover, the effect of the increase in swelling behaviour was also shown for acceleration of the degradation of the unmodified MCCh. This phenomenon is confirmed by the observation carried out by optical microscope. The coat-like composition of precipitated MCCh onto cellulose fibres affected a decrease in the decomposition, but web-like structure caused more rapid and deeper biodegradation. The modification of MCCh by the introduction of protein prolonged the decomposition of paper sheets especially for the precipitated MCCh containing casein. In a case of keratin, this effect did not differ so strong on the decomposition velocity of paper sheets containing MCCh without proteins.

Direct introduction of MCCh caused an increase in the degradation level of 5-10% compared to cellulose paper (reference) and precipitated MCCh reduced it by a range of 10-20% from that of the reference sample.

Conclusions

1. The mechanical properties measured also at high rel. humidity, increase after addition of MCCh. The precipitation of MCCh with proteins yields better mechanical strength in contrast to the direct introduction method (7%-29%). However, unmodified MCCh gel-like dispersion added by the direct introduction of MCCh results in higher mechanical resistance of paper sheets.
2. At higher rel. humidity, the mechanical strength of paper sheets containing MCCh with proteins is relatively higher than the strength of unmodified sheets (with or without MCCh).
3. "Coat"- or "web"-like fiber structures of MCCh are detected by microscopy. Direct introduction of MCCh resulted more in "web"-like structure of MCCh among the fibres and the precipitation of MCCh resulted in formation of coated fibres. The coat-like fibres are often visible at paper sheets prepared with introduction of protein, but the layers of MCCh are thicker.
4. The biodecomposition rate of paper sheets is correlated with the method of MCCh addition. The direct introduction method, resulting with web-like structure of the fibers, results in faster degradation, whereas the precipitation method, producing a coated fibre structure results in slower biological decomposition. Introduction of proteins decreases the biodegradation velocity.

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Application of chitosan in textile printing

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Summary
Evidence is presented to show that chitosan can be used successfully in the pigment printing of polyester and polyester-cotton blends. Studies of the colour fastness to washing, rubbing and light exposure gave results comparable with a commercial pigment printing composition. Drawbacks noted were lower colour yields and increased fabric stiffness.

Introduction

Given the properties of chitin and chitosan, a wide range of possible end-uses in medical, agricultural and industrial areas have been proposed [1]. Most recently, chitosan has been applied in textile processing to modify the dyeability of immature cotton neps in fabric dyeing [2,3], to improve the dyeability of wool [4], to enhance the dye-uptake of leather [5], to modify cotton fabrics [6] and to decolorize dye waste water [7]. No recent work on possible applications of chitosan in textile printing has been noted.

The production of printed fabric world-wide has shown a significant growth in the past few years, reaching an output of about 27 billion m^2 in 1996 [8].The regional breakdown of world textile printing production in 1994 is shown in figure 1 [9]. Of all printed fabrics produced in 1996, polyester and polyester-cellulose blends had a share of 30%, next in importance to cotton fabrics (figure 2) [8]. Because of the technical advantages of pigment printing and the simplicity of the process, pigment printing has become one of the most popular printing techniques in textile production. Pigment printing has about a 50% share of the total volume of printed fabric produced and it has been claimed that 70% of all printed fabric in the USA is pigment printed (figure 3) [8,10,11]. In pigment printing the pigment is fixed on the fabric in the desired pattern using a binding agent to adhere the pigment to the fabric. Pigment printing, in addition to using pigment, involves the use of such auxiliaries as softeners, binders, thickeners, cross-linking agents and other necessary chemicals.

Thickening agents are significant substances in printing paste compositions and are usually colourless. The thickeners which are mostly used in textile printing may be classified as polysaccharides, viscous emulsions (oil in water or water in oil), viscous foams and synthetic-polymer thickeners [12]. For many years natural thickeners were the only possible thickeners in printing. Natural thickeners had some limitations in textile printing which encouraged a search for alternatives. For pigment printing, natural thickeners were no longer required when emulsion systems were used. However, the popularity of this technique was short-lived in many countries because the use of organic solvents (white spirit or kerosene), led to problems. Much work has been done to substitute these solvent-based emulsions with more preferred materials [9, 13-17]. In the 1970s, the first synthetic thickener was introduced to the market

based on a very high molecular weight polyacrylic acid [13]. Now many products are available from different chemical companies.

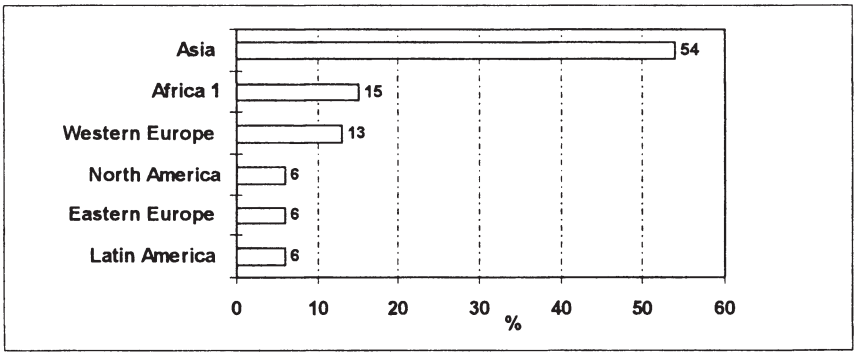

Figure 1. World-wide textile print production 1992, breakdown by region [9]

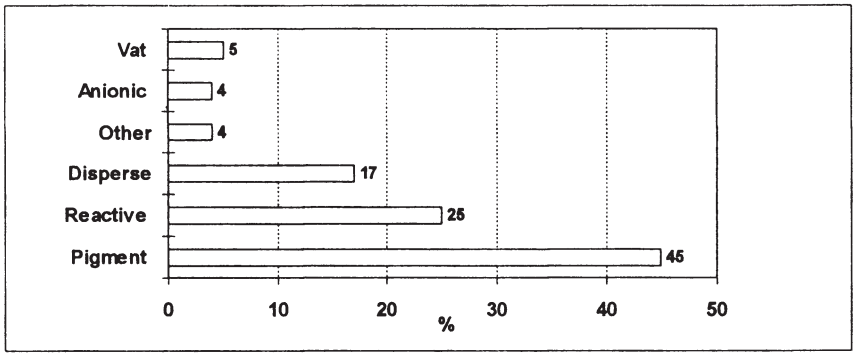

Figure 2. World-wide textile print production 1996, breakdown by dye class [8]

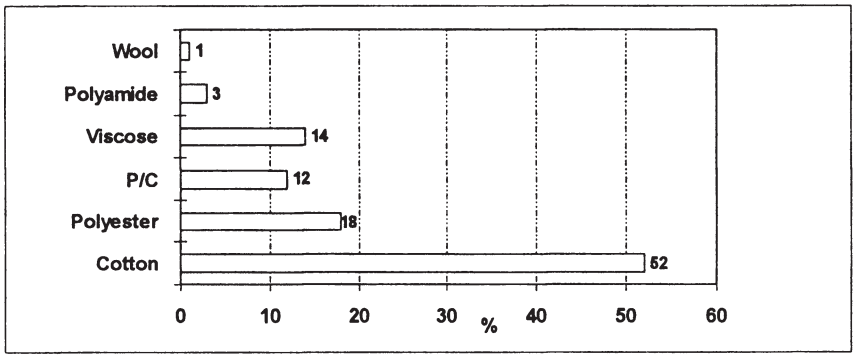

Figure 3. World-wide textile print production 1996, breakdown by fibre type [8].

In addition to thickener and pigment, the conventional system uses a binder which produces a three dimensional cross linked network binding the pigment to the fabric. Typical binders are based on styrene-butadiene, styrene-acrylate or vinyl acetate-acrylate copolymers and give a high rub fastness and a low stiffness [12]. In this work the quality and fastness of pigment printed polyester fabrics using chitosan as both binder and thickener were compared with those printed with a commercial pigment printing composition (Alcoprint).

Materials and Methods

Chitosan with medium molecular weight (171,000 - supplied by Pronova) was used in this study. Chitosan printing pastes were made up by dissolving chitosan in acetic acid solution (1% v/v) to obtain a solution of 2.5-3 % (w/w) chitosan. Then the appropriate amount of Helizarin pigments (supplied by BASF) were added to the system. Alcoprint pastes were made up, according to the instructions given by the supplier, using Alcoprint PBA, Alcoprint PSM, Alcoprint PHL and Alcoprint PT21. The Helizarin pigments were added to give the pigment content as required, matching the concentration used in the chitosan paste. Heat set polyester and polyester -cotton fabrics were separately printed using a flat screen printing system with a laboratory magnetic roll printing machine (J. Zimmer) and dried at room temperature. Chitosan-printed fabrics were cured at 150 °C for 6 minutes and Alcoprint-printed fabrics were cured at 140 °C for 3 minutes using a Werner Mathis AG laboratory oven.

To measure the fastness of the printed fabrics a solid blotch pattern was used. The colour fastness to rubbing, washing and light exposure were measured according to B.S. 1006: 1978 (X12), B.S. 1006: 1990 (C03) and B.S. 1006: 1990 (B01) respectively. The colour values of the prints were measured using a Colourgen spectrophotometer applying the Kubelka Munk equation. To evaluate the stiffness of the printed fabrics, the bending lengths of the samples were measured using the Fast-2 equipment.

Results and Discussion

The results for the colour fastness to dry and wet rubbing are shown in table 1. Chitosan prints show a very good colour fastness to dry rubbing, on occasions better than the Alcoprint prints. Both the Alcoprint and the chitosan prints show a fair colour fastness to wet rubbing. Chitosan-pigment prints showed a better colour fastness to wet rubbing on polyester-cotton fabric than polyester fabric. Moreover, colour fastness to wet rubbing of chitosan prints was better than Alcoprint prints on polyester-cotton but poorer on polyester fabric. On the whole, prints containing Helizarin Yellow G Conc. pigment showed better fastness to rubbing than Helizarin Red BN pigment.

The wash fastness results are shown in table 2. Chitosan-printed fabrics have an excellent colour fastness to washing and overall are better than the analogous Alcoprint samples. An excellent light fastness rating was obtained for both printing systems.

The ability of chitosan paste to print a pattern with a sharp border and very fine lines is shown in figure 4. The colour values for both printing systems are given in table 3. The colour values of the chitosan-printed samples are always lower than Alcoprint-printed samples, but the difference is marked with the Helizarin Red BN pigment. Pigments are small particles usually in the range of 0.1-3 microns which have little or no affinity for the fabrics. Most pigments (organic or inorganic) are used as dispersions, the dispersion usually consisting of dispersing agents, wetting agents, preservatives, thickeners as well as pigments [11]. The pigment dispersions are prepared generally for use under alkaline conditions as most binders cross-link in acidic media. It is thought that mixing pigment dispersions with chitosan solution with an

acidic pH value (e.g. pH 4), causes pigment aggregation leading to the lower colour value in pigment printed fabric. In fact, whilst the Helizarin Yellow G Conc. pigment could be dispersed uniformly in the chitosan solution, the dispersion of the Helizarin Red BN required a more complicated procedure.

The bending lengths of the printed fabrics are shown in table 4. Chitosan-printed fabrics have higher bending lengths than Alcoprint prints. Chitosan film, forming on the surface of the fabric, is very rigid whilst Alcoprint binder is based on an elastomeric polymer, hence it is not surprising that chitosan prints are stiffer. The increases in stiffness are more marked on polyester-cotton fabric than polyester fabric.

Table 1. Rub fastness test results (B.S. 1006; 1978 X12).

Printing system	Pigment	Fabric	Dry rubbing[1]		Wet rubbing[1]	
			warp	weft	warp	weft
Chitosan		P	3-4	3-4	2	2
Alcoprint	Helizarin Red BN	P	3	3	2-3	2-3
Chitosan	3 % (w/w)	P/C	3-4	4	3-4	3
Alcoprint		P/C	3-4	3-4	2	2
Chitosan		P	4	4	2-3	2-3
Alcoprint	Helizarin Yellow G	P	4-5	4-5	4	3-4
Chitosan	4% (w/w)	P/C	4	4	3-4	3-4
Alcoprint		P/C	4	3	3-4	3-4

1-Grey scale for assessing staining (ISO) (light source D65)
P: Polyester 100% , P/C: Polyester-cotton blend (67/33)

Table 2. Wash fastness test results on Multi-fibre Fabric DW (SDC).

Printing system	Pigment	Fabric	Secondary cellulose acetate[1]	Cotton bleached not-mercerised[1]	Nylon 6-6[1]	Polyester[1]	Acrylic[1]	Wool[1]	Change in colour[2]
Chitosan	Helizarin	P	5	5	5	5	5	5	4
Alcoprint	Red BN	P	5	5	5	5	5	5	4
Chitosan	3 %	P/C	5	5	5	5	5	5	4-5
Alcoprint		P/C	5	5	5	5	5	5	4-5
Chitosan	Helizarin	P	5	4-5	5	5	5	5	3
Alcoprint	Yellow G	P	5	3-4	4-5	5	5	5	2
Chitosan	4%	P/C	5	4-5	4	5	5	5	4-5
Alcoprint		P/C	5	3	4-5	5	5	5	3-4

1-Grey scale for assessing staining (ISO) (light source D65).
2- Grey scale for assessing colour change (ISO) (light source D65).
P: Polyester 100% , P/C: Polyester-cotton blend (67/33).

Table 3. Colour value and penetration for both Alcoprint and chitosan pastes.

Printing system	Pigment	Fabric	K/S values	
			Face	Back
Chitosan	Helizarin	P	5.2	3.2
Alcoprint	Red BN	P	11.0	4.4
Chitosan	3%	P/C	5.3	4.9
Alcoprint	(λ_{max}: 520 nm)	P/C	12.1	11.8
Chitosan	Helizarin	P	5.8	4.4
Alcoprint	Yellow G	P	8.8	8.6
Chitosan	4%	P/C	5.1	5.1
Alcoprint	(λ_{max}: 430 nm)	P/C	8.5	7.8

P: Polyester 100% , P/C: Polyester-cotton blend (67-33)

Table 4. Bending length and flexural rigidity of Alcoprint and chitosan-printed fabrics

Sample	Fabric	Bending length(mm)		Bending rigidity(μN.m)	
		warp	weft	warp	weft
Chitosan print	P	47.0	41.0	151	100
Alcoprint print	P	40.6	26.9	97	28
Original fabric	P	19.3	17.9	10	8
Chitosan print	P/C	47.6	40.6	91	56
Alcoprint print	P/C	25.0	19.7	13	6.4
Original fabric	P/C	17.0	14.7	4	2.7

Fabric Bending Rigidity = $wc^3 \times 9.81 \times 10^{-6}$, C =Bending length (mm)
W =Mass per unit area (g/m^2), P: Polyester 100% , P/C: Polyester-cotton blend (67-33)

Conclusions

This study has shown that satisfactory prints can be obtained using pigments dispersed in chitosan dissolved in acetic acid and the printed fabrics showed a very good colour fastness to washing and dry rubbing and a fair fastness to wet rubbing. All pigment printed fabrics showed a good colour fastness on exposure to light. Both chitosan- and Alcoprint-pigment prints caused increased fabric stiffness, but chitosan-printed fabrics are stiffer than Alcoprint-printed fabrics. However it may be possible to minimise the stiffness of the chitosan-printed fabric by adding a softener to the paste. It is also worth noting that the prints were made with 100% fabric coverage; if a pattern with small printed areas was chosen, the overall fabric stiffness would not be so noticeable.

The major problem with chitosan-pigment prints was the poor colour value. We suspect that the low colour value obtained on the fabrics is due to aggregation of the pigments in the chitosan pastes. The more the pigment aggregates, the lower the colour value. If this explanation for low colour values is correct, then it will be necessary either for pigment dispersions to be made which are stable at pH 4 or to employ suitable auxiliaries to obtain a better dispersion of pigment in the chitosan solution.

In conclusion, chitosan can be used as a thickener and binder in pigment printing with the following advantages:

a. Simple recipe

b. Can be applied on different kinds of fabrics such as polyester, polyester-cotton, cotton and possibly nylon.

c. The printing quality of chitosan prints such as sharpness of print and uniformity of the print are comparable with Alcoprint prints.

d. Easy application

e. Environmentally friendly

f. Good colour fastness to washing

g. Relatively good colour fastness to dry rubbing

h. Easily washed off the screen and other printing equipment

The main disadvantages of chitosan-pigment printing can be listed as follows:

a. Low colour value

b. Relatively poor colour fastness to wet rubbing

c. Increased fabric stiffness.

Further research and development work on the chitosan-pigment printing system should enable the process to be optimised, and the disadvantages ameliorated.

Figure 4. Chitosan-pigment printed fabric.

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Permanent modification of fibrous materials with biopolymers

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Summary
Novel methods are described for the **permanent** surface modification of fibrous materials with biopolymers. Such functionalized textiles show high biocompatibility and impart new functionality to the fibre surface. Thus long lasting effects like the regulation of micro climate between textile and human skin or wound healing ability of the modified textile may be expected.

Introduction
An increasing demand develops for imparting active agents to textile materials (fabrics and non-wovens) in order to create additional properties (**'functional textiles'**). With synthetic fibres this may create a better hydrophilic behaviour (water retention, sweat transport ...). On natural fibres this could mean the anchoring of bacteriostatic or odour binding agents and similar. An advantageous strategy is not to create new fibre types as is done exemplarily in [1], but to modify only the surface thus retaining the well known mechanical properties of the bulk fibre. In addition such a strategy imparts more flexibility to the textile finishing industry.

Biopolymers or their derivatives as surface modifiers can offer such properties because of their interesting physiological properties connected with the possibility for anchoring them in textile finishing steps onto the fabric. The friendliness of pure biopolymers from the carbohydrate family towards the skin or their wound-healing effect are well known [2,3]. By traditional use of such biopolymers within nutrition, within cosmetics or within ointments such properties are well acknowledged [4].

Materials and Methods
Textile materials used for surface modification of fibres with biopolymers have been conventional cotton webs. Permanent fixation of biopolymers mostly can be done by the application of cyanuric chloride onto solutions of the biopolymer at temperatures of 0-10 °C and subsequent using the resulting solutions without isolation of the modified biopolymer for application onto webs or non-wovens. Permanent fixation onto fibre surfaces follows usual receipes for reactive dyeing of cellulosic material. Other strategies uses biopolymer derivatives having hydrophobic chains for treatment of synthetic fibres (c.f. Fig. 2).

Results and Discussion
Expectations of effects by biopolymer-treated textiles: Selected properties which can be achieved by biopolymers on textiles are given in Figs. 1 and 2 (c.f. [5-7]):

Biopolymer (Derivative)	Property (Bulk)	Application on textile
- Dextrins	Hydrophily	Regulation of micro climate
- Chitosan	film formation Hydrophilie	Antibacterial- Anitfungal, Woundhealing
- Alginates	Gel, Film	Micro climate pH-value
- Pectin(s)	Gel, Film	Water retention

Figure 1. Selected properties of biopolymers usable for textile surface modification.

Strategies for permanent finish of textiles with biopolymers

So an important task for research and development lies in the evaluation of methods how to anchor biopolymers permanently onto fibre surfaces in a way that the biopolymers retain their beneficial properties of action. Anchoring of biopolymers requires - depending on the fibre used - different derivatization as is shown schematically in Fig. 2:

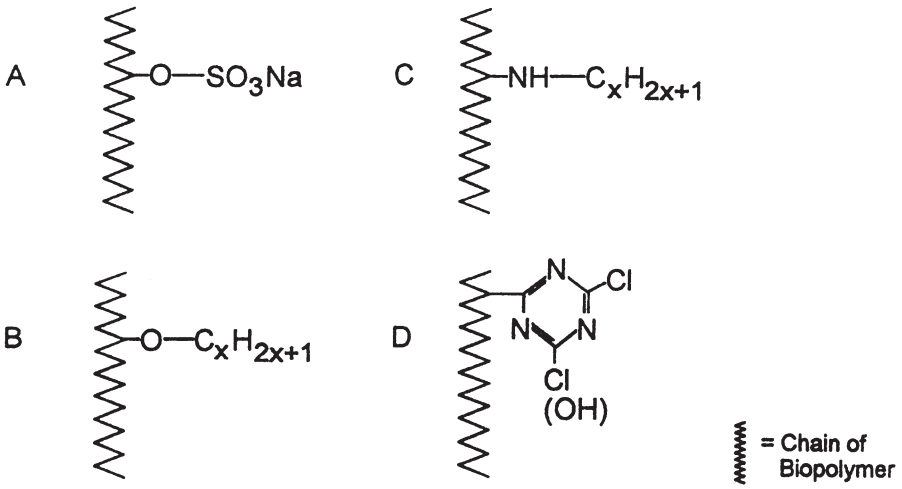

Figure 2. Scheme of anchoring strategies for biopolymers and their derivatives onto fibrous material acc. to different chemical functionalization.

Kind of Polymer-/ Biopolymer-Derivat-/ interaction	Fibre-Polymer				
	Cellulose Viscose Lyocell	Wool Silk	PA	PES PE/PP	PAN
ionic interaction	(+)	+	+	-	+
covalent boud	+	+	+	-	-
van-der-Waals-interaction	-	-	+	+	+

+ possible interaction
- impossible or weak interaction

Figure 3. Binding interaction possibilities of derivatized biopolymers with fibres.

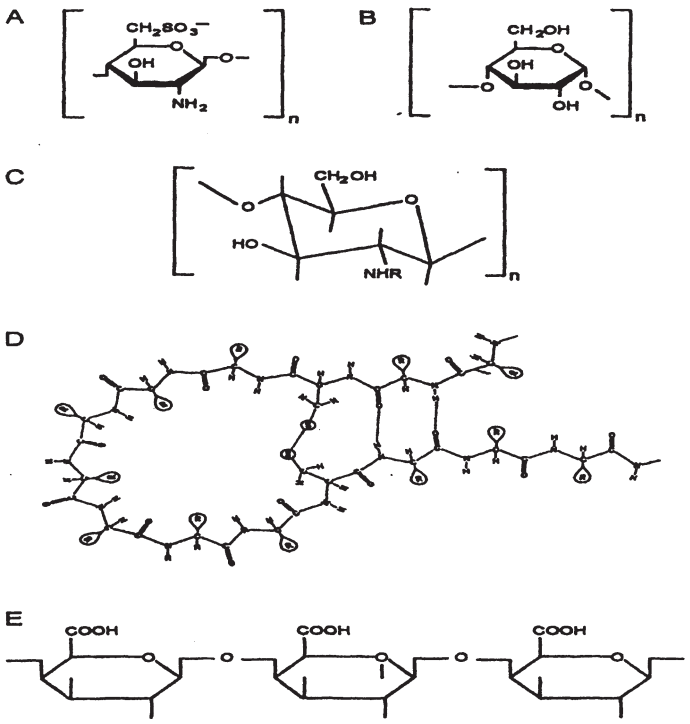

Figure 4. Chemical formulae of some biopolymers, which after derivatization have been bound permanently onto textiles. A Heparin and other sulfated carbohydrates, B Dextran(es), C Chitosan, D Protein (Gelatin), E Poly-Galacturonic acid (Alginates, Pectinates).

Method A: Such derivatization of a biopolymer increases the affinity of the substances to be fixed: An increasing amount of negatively charged groups (i.e. -SO3-Na+) of the derivatives favours the binding toward natural fibres giving an enhanced add-on on the fibre surfaces.

Methods B and C: Offering a fixation onto synthetic fibres like polyethyleneterephthalate by enhanced Van-der-Waals interaction between the long aliphatic chain of the derivative with fibre polymer chains (above glass transition temperature of the fibre).

Method D: Derivatization using reactive substituents like chlorotriazinyl moieties enables the formation of permanent chemical bonds between hydroxy- or amino-functions of natural fibres and the biopolymer.

As an example, in Fig. 5 a non-woven material is depicted which has been treated with reactive chitosan derivative resulting in a wash- and wear stable surface finish without interferring with a soft handle.

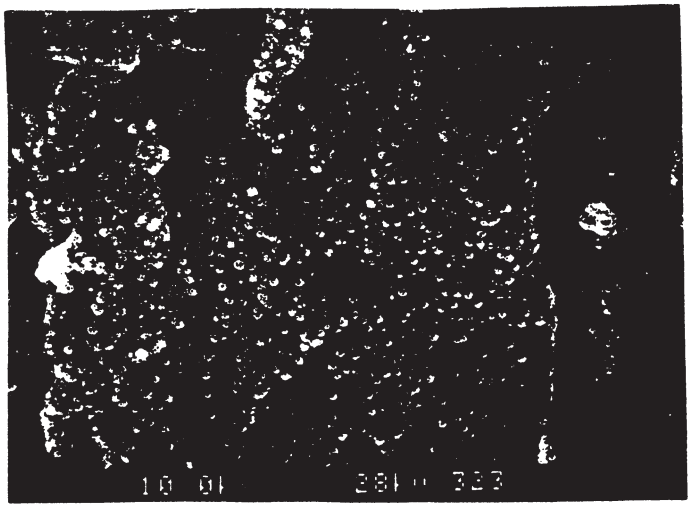

Figure 5. SEM-photograph of a chitosan treated non-woven (viscose).

The successful anchoring of the different biopolymers used may by qualitatively analyzed by colour staining methods, i.e. chitosans on fibres may be detected by use of ninhydrin reagents.

Aspects for the future: Research work has to be done on the degree of derivatization of the biopolymers - whose molecular mass has to be controlled too - in order to obtain a balance between sufficient chemical linkage points onto the base fabric for obtaining permanent fixation but simultaneously retaining a high polymer segment mobility of the biomolecule. By such means a high capability for swelling, for adsorption and similar of the bulk biopolymer and its physiological activity has to be maintained. Especially investigations regarding the biopolymer structural change like sol/gel formation of an anchored biopolymer layer are almost unknown and have to be evaluated.

Conclusions

Textile goods, fabrics or non-wovens, treated according to the strategies outlined above - having a permanent finish of biopolymers - may be useful as odour masking materials in fashion, in home textiles or for the automotive sector. Even more important will be the aspects of biopolymer-modified textiles for the protection clothes and for medical or hygienical applications.

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Ion exchanger from chitosan

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Summary
Chitosan beads and pellets with chitosan concentrations of about 3% and 20%, respectively, were prepared for selective adsorption of different heavy metals. The adsorption mechanism especially the adsorption kinetics depends on the adsorption material and on the preparation of the ion exchanger material. The diffusion and migration of the heavy metals to the active sites is related to the structure of the polymer chains.

Introduction
Chitosan has the great advantage to adsorb heavy metal ions as for instance Zn^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and not the alkaline and alkaline- earth metal-ions as Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} [1,2]. Therefore the Chitosan is very adopted to remove heavy metals from natural liquids. For special technical applications it is of great interest to adsorb certain heavy metals selectively (for instance in the galvanic industry).

However, for the technical application the chitosan has to be transferred into an ion exchanger material with the following properties:

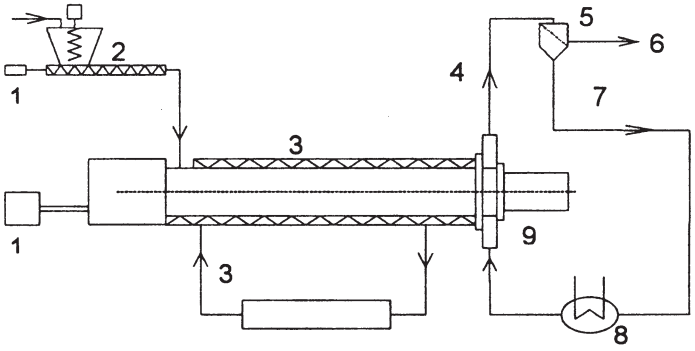
- high stability as:
  - mechanical stability (for reasonable flow rates of the liquid)
  - chemical stability, non soluble in acids (for regeneration)
- pH-dependence of the adsorption, to allow regeneration in strong acids
- high adsorption capacity and high adsorption kinetics
- selective adsorption for defined heavy metals.

Preparation of Ion Exchanger Material
Ion exchanger material from chitosan was produced by the known method [3] of dropping a chitosan solution of acetic acid in a NaOH-solution as beads and by extrusion of the chitosan solution into a NaOH-solution as pellets. The chitosan beads were prepared with a solution of about 2 w% of chitosan (deacetylated to about 85 %) in an aqueous solution with 2 w% acetic acid. The chitosan solution was dropped into an aqueous solution of NaOH (2.5 - 5 mol%). The beads thus obtained had a diameter of 1 to 3 mm and a specific surface up to 140 m^3/g. However the beads contain only about 3.5 % chitosan, the rest is water. To increase the content of chitosan in the ion exchanger material the original solution of chitosan in the acetic acid has to be increased, resulting in a higher viscosity of this solution. For this solution the dropping method is no more applicable and a new extrusion method was developed (Fig. 1).

The chitosan solution is pressed through a sieve and cut into small pellets by a rotating knife. The content of chitosan in the pellets is about 20 % compared to about 3.5 % in the beads. The specific surface in the pellets was about half the value as found for the beads. Electron scanning microscope pictures show that within the pellets there is a higher density of chitosan than in the beads [4].

To prevent the chitosan to be dissolved in acids, the chitosan polymer chains are crosslinked by glutardialdehyde (to about 20%) or by diaminopropanetetraacetic acid (to about 60%). The selectivity for the adsorption of different heavy metals is obtained by complexing the free NH_2-groups of the crosslinked chitosan chains with different molecule groups [5].

1) motor
2) pump
3) cooling device
4) product outlet
5) sieve separation
6) outlet of pellets
7) NaOH - solution
8) cooler
9) granulation device



Granulation device

1) granulation head
2) sieve
3) rotating knife
4) inlet and outlet
of a NaOH - solution

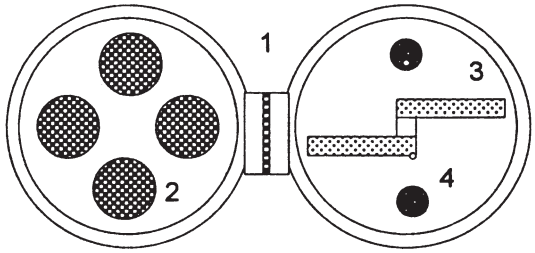


Figure 1: Preparation of pellets by extrusion (Firma Lihotzky)

Measurements

For the obtained ion exchanger material the following properties were measured:

- the adsorption capacity
- the selectivity
- the influence of the anions. - the pH dependence
- the adsorption kinetics
- the function in an adsorption column (breakthrough curve).

Results and Discussion

The adsorption capacity and the influence of the anions on the selective adsorption of different heavy metals are given in Fig. 2 for the derivative A (20 % crosslinked with glutardialdehyde) and for derivative B (60 % crosslinked with diaminopropanetetraacetic acid and to 40 % complexated with (R)-thiazolidine-4-carboxylic acid).

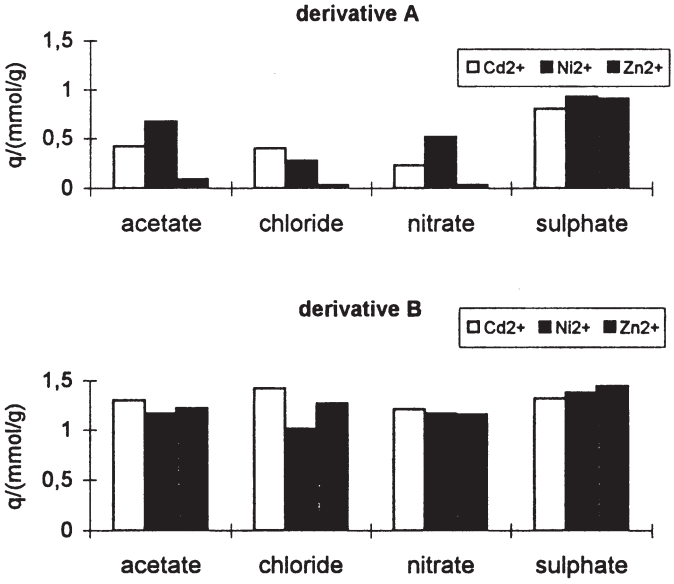


Figure 2. Capacity of the derivates A and B for Cd^{2+}-, Ni^{2+}- and Zn^{2+}-ions in dependance on the anion, pH 6.

The selectivity for the adsorption of different heavy metal ions depends on the molecular groups (complexing derivatives) attached to the NH-group, on the anion and on the pH-value of the liquid. For some derivatives the anion has an important influence on the adsorption of heavy metals, for others this influence is negligible [5]. In Fig. 3 the selectivity and the dependence on the pH-value are demonstrated for the derivatives A and B.

The adsorption isotherms and the adsorption kinetics for Cd^{2+} ions in a SO_4^{2-} aqueous solution are given in Fig. 4 and Fig. 5 (derivative A as beads and pellets). The kinetics is about 3 times slower in the pellets than in the beads. By energy dispersion x-ray analysis within an electron microscope it could be shown that there is an equal distribution of the adsorbed heavy metals within the pellets. So there are enough openings and macropores for the diffusion and migration of the liquid phase transporting the heavy metals into the inner part of the ion exchanger material. However the lower adsorption surface and the low adsorption kinetics found for the pellets compared to the beads indicate that there are less micropores in the pellets leading the heavy metals to the active adsorption sites. Furthermore the micropores only allow a slow diffusion to the active sites.

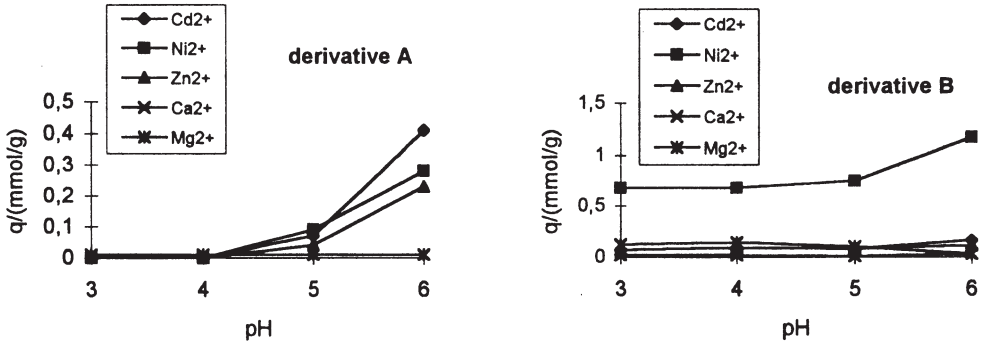


Figure 3. Selectivities of the derivates A and B at pH 6 for the ions Cd^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+} (each with a concentration of 0,02 mol/l as sulphate in the solution).

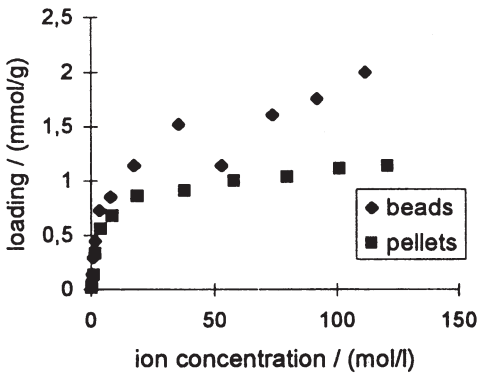


Figure 4. Adsorption isotherme for Cd^{2+} adsorbed at derivative A in a $CdSO_4$ solution at pH 6.

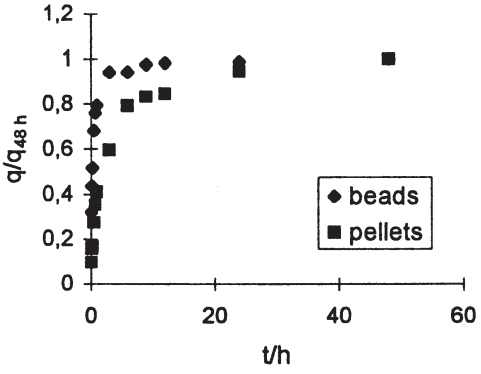


Figure 5. Adsorption kinetics for the adsorption of Cd^{2+} at derivative A in a 2.0 mol/l $CdSO_4$ solution, pH 6

The function for an ion exchanger adsorption column is demonstrated in Fig. 6 by break-through curves for the derivative A. The upper part of Fig. 6 gives the result for an aqueous liquid containing $CuSO_4$ (with two different concentrations: 0.1 mmol/l and 2.0 mmol/l). The breakthrough for the lower Cu^{2+} concentration in the liquid (0.15 mmol/l) is obtained after a larger volume of the liquid phase has passed through the column than for a higher ion concentration in the liquid (2.0 mmol/l). The lower part of Fig. 6 gives the break-through curves for different $CdSO_4$-concentrations in the liquid phase, demonstrating a totally different behaviour compared to the adsorption of the Cu^{2+}. For lower concentrations there seem to be less active places available than for higher concentrations. This is explained by a

model of bottle neck blocking. The first ions are adsorbed in the pores blocking further diffusion of the ions into the inner part of the pores. At higher concentrations of ions the concentration gradient is high enough to cause a diffusion through the bottle neck.

Comparing the breakthrough curves for Cu^{2+} and Cd^{2+} (Fig. 6) demonstrates a totally different behaviour for the two kinds of cations with low concentrations. This can be explained by assuming that the adsorption energy at the active sites is higher for Cd^{2+} than for Cu^{2+}. So the bottle neck effect is only essential for the Cd^{2+} cations.

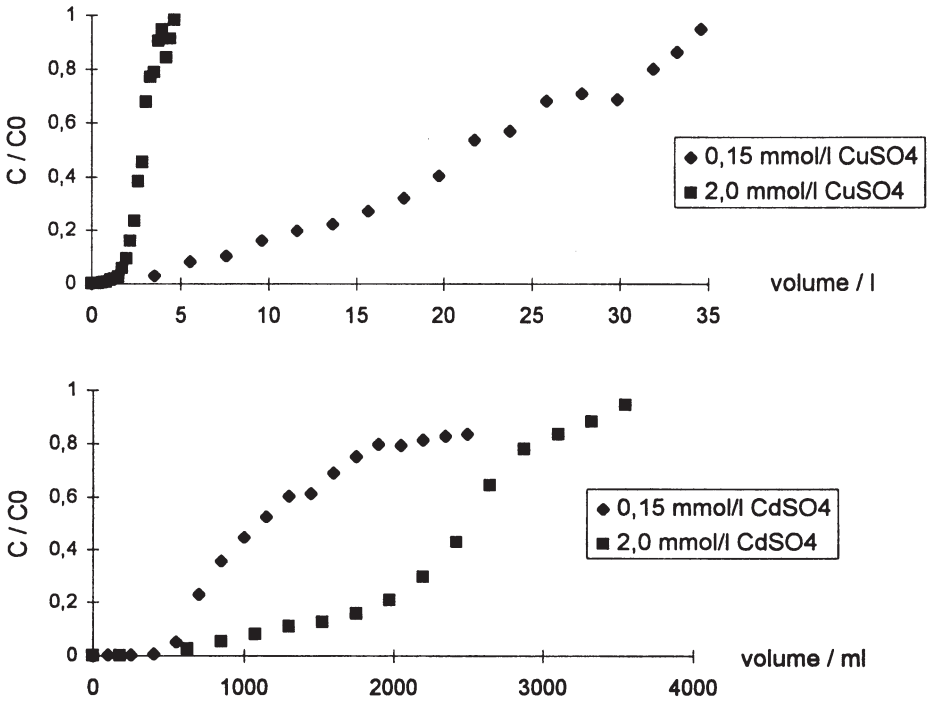


Figure 6. Breakthrough-curves for $CuSO_4$ and $CdSO_4$ solutions of two different concentrations co . c = concentration at the outlet of the adsorption column. Volume of the column: 380 ml, containing 190 g beads of derivative A. Flow rate of liquid through the column: 100 ml/h.

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Chitosan in wastewater treatment

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Summary
This article deals with the use of chitosan in municipal wastewater treatment. Long-term studies done in a laboratory sewage plant, where small amounts of a chitosan solution were supplied continuously to the aeration tank, resulted in a reduction of COD, total nitrogen and bacteria in the effluent as compared to operation of the plant without chitosan.

Introduction

Chitosan is well known as a flocculant for suspended solids, this property rendering it especially interesting for the treatment of wastewater. The early studies of Bough et al. focussed on effluents of food processing plants and showed the efficiency of chitosan in coagulating suspended solids (proteins, fats etc.). [1, 2]

This study deals with the use of chitosan in municipal wastewater treatment. Municipal sewage plants reduce the sewage load of raw wastewater so that the effluent can be returned into a natural waterway. The purification of municipal sewage is usually done by a combination of mechanical, physical and chemical procedures like filtration of coarse material, sedimentation of sand or heavy metal salts, flocculation, flotation of fats and by biological degradation of organic material. The most important step is the biological degradation which takes place in the aeration tank:

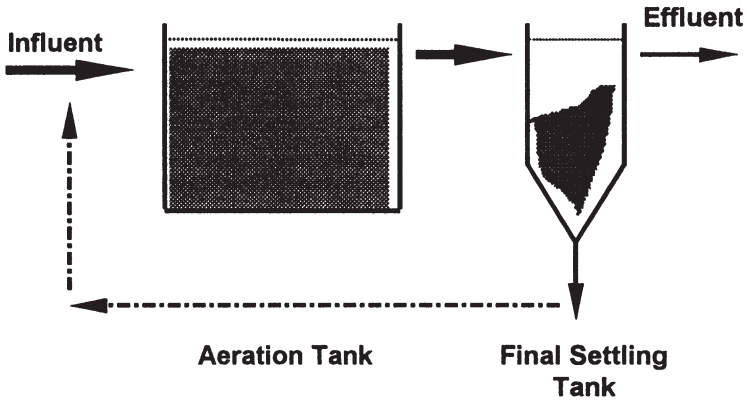

Figure 1. Scheme of a municipal sewage plant

Fig. 1 shows a scheme of a sewage plant consisting of an aeration tank and a final settling tank. First, wastewater is supplied to the aeration tank, which is filled with the activated sludge, a watery flaky sludge consisting of or settled with microorganisms, these microorganisms being responsible for the biochemical degradation of the organic matter of the wastewater. After passing through the aeration tank, the activated sludge enters the final settling tank and is divided into the effluent and a sludge component which is returned to the aeration tank.

The efficacy of the degradation reactions, namely the performance of the sewage plant, is usually characterized by measuring the following parameters in the influent and the effluent of the plant:

Table 1. Analytical parameters for wastewater

Parameter	Explanation	Max. amount in the effluent *
COD	Chemical oxygen demand	90 mg O_2 /l
BOD	Biochemical oxygen demand	20 mg O_2 /l
Total nitrogen	N-containing substances like proteins, urea, ammonium, nitrite, nitrate	18 mg/l
NO_3^-		
NH_4^+		10 mg NH_4-N /l
Total phosphate	P-containing substances like organic ortho- and polyphosphates, salts	2 mg/l

* Requirements of the Rahmen-Abwasser-VwV for municipal sewage plants of size 4 (20000 - 100000 inhabitants) [3]

Most important is the chemical oxygen demand COD. It presents the amount of oxygen needed for total chemical oxidation of a wastewater sample and gives an information about the amount of degradable (organic) matter. The biochemical oxygen demand BOD_5 measures the amount of oxygen consumed by microorganisms for the degradation of organic substrate in a period of five days. For municipal wastewater, the rate of COD : BOD_5 is in the range of 1.7, so for routine process control it is sufficient and usual to determine only COD. [4]

Materials and Methods

Operation of a laboratory sewage plant without and with chitosan: Laboratory sewage plant comprising a 50 l aeration tank and a 30 l final settling tank. The aeration tank is divided into two aerated zones for nitrification and three anoxic zones for denitrification.

- Wastewater: Double concentrated synthetic wastewater according to DIN 38412 - L24 (COD about 600 mg O_2/l, 30 g/d) or natural wastewater (influent to the aeration tank) of the municipal sewage plant in Emden (COD in the range of 250 - 350 mg O_2/l, that is 12.5 - 17.5 g/d) [5]
- Chitosan IFSO-03, fish contract GmbH (degree of deacetylation about 80 %, low viscosity)
- Chitosan stock solution: 1 % chitosan in 0,3 % acetic acid. Dilution with distilled water for use. Dosage: 3 % of COD of the influent (400 - 1000 mg chitosan /d)
- Analytical examinations of wastewater with the Nanocolor tests of Macherey-Nagel GmbH

To show the effects of chitosan in wastewater treatment, a laboratory sewage plant consisting mainly of an aeration tank and a final settling tank was operated under controlled

conditions for several months. The process was controlled by determining the COD, the total nitrogen content and the total phosphate content of both, the influent and the purified effluent of the plant. Then for a period of about eight weeks, small amounts of a chitosan solution were supplied continuously to the laboratory plant, this being the only change in operating conditions. Chitosan was added to the aeration tank and was spread all over the plant via the overflow to the final settling tank and the reflux of sludge. Again, analytical data of the influent and the effluent were taken.

Short-term laboratory tests

- Device for colony count (plate count agar, incubator)
- 1 % chitosan solution in 0,05 mol/l hydrochloric acid

Activated sludge of the sewage plant in Emden was mixed with a chitosan solution. After settling of the sludge, the number of microorganisms in the clear supernatant was counted.

Results

Operation of a sewage plant without and with chitosan - results: The results of these test series are summarized in the following schedule (Table 2):

Table 2: Effect of chitosan dosage on COD and total nitrogen TN in the effluent of the laboratory sewage plant

		COD in the effluent [mg O_2/l]	**Efficiency of COD reduction* %**	**TN in the effluent [mg/l]**	**Efficiency of TN reduction** %**
synthetic wastewater	without chitosan	57	90	72	22
	with chitosan	37	95	56	38
natural wastewater	without chitosan	49	80	23	38
	with chitosan	42	85	18	58

* Efficiency of COD reduction in % = $(COD_{influent} - COD_{effluent}) \times 100 / COD_{influent}$

** Efficiency of TN reduction in % = $(TN_{influent} - TN_{effluent}) \times 100 / TN_{influent}$

COD in the effluent of the plant operated with chitosan is maximum 35 % lower than in that without chitosan; the efficiency of COD reduction is increased from 80 % to 85 % in the tests with natural wastewater and from 90 % to 95 % in the tests with synthetic wastewater. [6].

The effect of total nitrogen reduction in the effluent of the "chitosan plant" is indicated in the test series using synthetic wastewater and becomes obvious in the tests with natural wastewater: The efficiency of N-reduction is increased from 38 % to 58 %! [6]

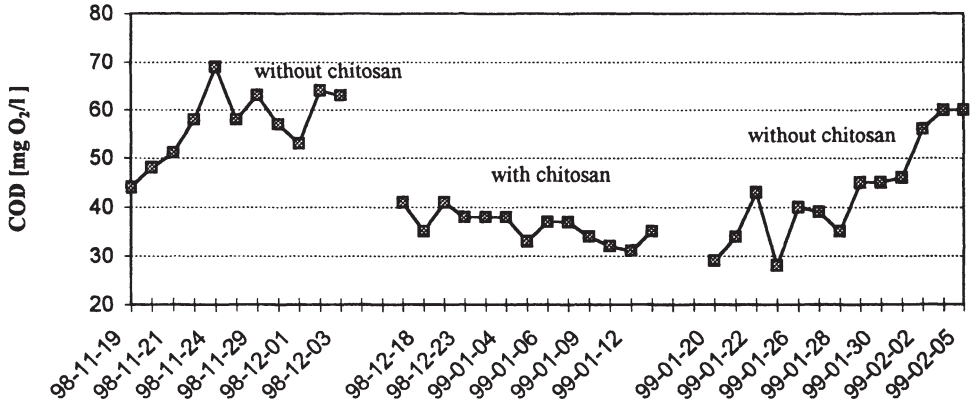

Figure 2. Reduction of COD in the effluent (synthetic wastewater)

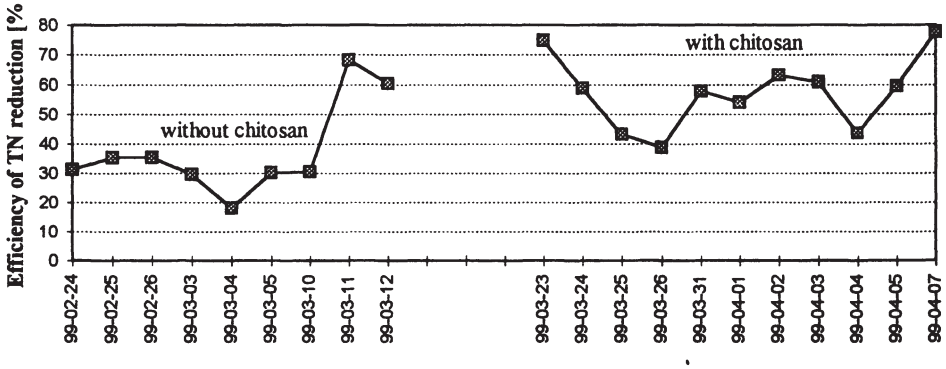

Figure 3. Efficiency of TN reduction in wastewater (natural wastewater)

The elimination of total phosphate was not affected, but the occasional examination of the number of bacteria in the effluent indicated a diminishing quantity.

Results of short-term laboratory tests: To confirm the reduction of bacteria in the effluent, activated sludge of the sewage plant in Emden was mixed with a chitosan solution. After settling of the sludge, the number of microorganisms in the clear supernatant was detected:

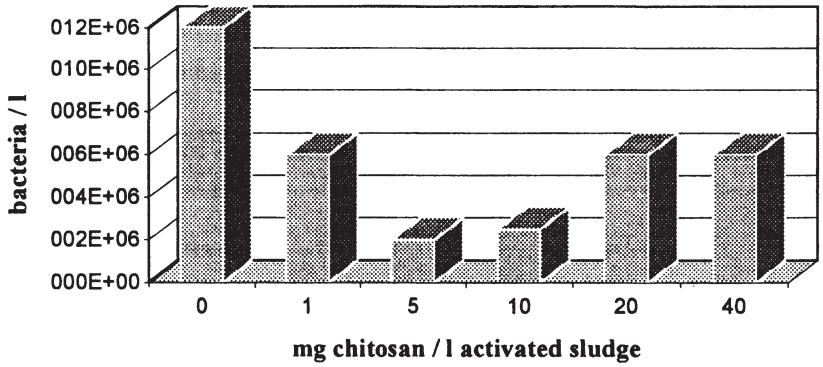

Figure 4. Reduction of bacteria in the effluent

Colony count of the samples treated with chitosan showed a lower number of living cells than the zero chemical regimes.

Discussion and outlook

It can be supposed that the effect on COD is due to the flocculating properties of chitosan.

The term total nitrogen comprises for example suspended proteins which are known to be coagulated by chitosan. Yet reduction of total nitrogen cannot be reproduced by precipitation tests done with natural wastewater from the aeration tank of the sewage plant in Emden. An explanation could be that the maximum amount of nitrogen containing matter in the natural wastewater is already degraded to ammonium before entering the aeration tank or will immediately undergo nitrification in the tank. So in this case, total nitrogen means ammonium and nitrate salts which are not precipitated by chitosan. As a consequence, the effect of chitosan cannot simply be explained by it's working as a flocculant. It can be assumed, that chitosan might also have an influence on the nitrogen degradation or on the microorganisms involved in the nitrogen degradation of the sewage plant. This assumption has still to be proved although some observations during the operation of our laboratory plant indicate an influence of chitosan on activated sludge and the bacteria and protozoa population of the sludge: A change in the structure of the activated sludge flakes with an increase of flake surface and a change in protozoa population could be observed.

Reduction of microorganisms in the effluent is due to adsorption of microorganisms by chitosan and flocculation of suspended bacteria flakes. A bactericidic effect of chitosan could not be noticed.

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The immobilization of tyrosinase on chitin and chitosan and its possible use in wastewater treatment

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Introduction

Chitin is the second most abundant natural biopolymer after cellulose. The chemical structure of chitin is similar to that of cellulose with 2-acetamido-2-deoxy-β-d-glucose (NAG) monomers attached via β(1-4))linkages. Chitosan is the form of chitin deacetylated in different degrees, which unlike chitin , is soluble in acidic solutions.

Chitin and its deacetylated derivative, chitosan, have been of interest in the past few decades due to their potential broad range of industrial applications, and some limited attention has been paid to wastewater treatment and potable water purification of these versatile biopolymers. Chitosan has been used as an effective flocculate and coagulating agent for organic matter.[1,2] .

Recently, enzymatic treatment has become the most interesting method for the removal of toxic chloroorganic compounds from industrial wastewater[3]. One of the effective enzymes used to remove toxic chemicals such as phenol and substituted phenol from an aqueous solutions is tyrosinase. Tyrosinase catalyzes hydroxylation of monophenols with molecular oxygen to form o-hydroquinones, and then, dehydrogenation of quinones occurs to form the corresponding o-benzoquinones. The benzoquinones undergo a nonenzymatic polymerization to yield water-insoluble substances. Peroxidase needs costly hydrogen peroxidase as oxidant, whereas tyrosinase use molecular oxygen.

The disadvantage in enzymatic treatment of phenol is the deactivation of the enzyme by the product (quinones) in the reaction media, not being possible its reuse. A combination of immobilized tyrosinase and chitosan is especially effective in removing toxic phenols from an aqueous solution [4].

Immobilized biocatalysts have the potential for future industrial and commercial use in many areas of food and fodder, pharmaceutical and chemical industries and chemical specialities in processes that have no current equivalents. For the numerous potential applications of immobilized enzymes the preparation technique should be easy and the cost low. In this regard chitin is considered to be an appropriate support for immobilization of enzymes, because it offers a high mechanical stability, appropriate density and a low solubility in most solvents and it is cheap. The literature abounds with examples of enzyme immobilization on chitin [5,6]

In this work, we use the properties of chitin and chitosan, chitin as a support in the immobilization reaction with tyrosinase and chitosan as an adsorbent for the application in removing phenolic compounds.

Materials and Methods

Chitin was obtained from shells of Cuban lobster (*Polinurus vulgaris)* and the chitosan used was from this chitin . Tyrosinase (EC 1.14.18.1), Lowry reactives, glutaraldehyde, sodium dihydrogen phosphate, sodium hydrogen phosphate and phenol were purchased from Sigma, while hydrochloric acid, acetone, sodium chloride, methanol and acetonitrile were supplied by Merck. All of the above chemicals reactive were of analitical reagent grade.

Isolation and physico-chemical characterization of chitin and chitosan: Chitin was isolated according to the method described elsewhere [7]. Chitosan was obtained by homogeneous hydrolysis from lobster chitin. The conditions are described in [8]

The degree of acetylation of chitosan was measured by ^1H NMR and the first derivative ultraviolet spectrophotometry method. ^1H NMR was performed on a AMX500 Brucker NMR spectrometer under a static magnetic field of 125 MHZ and 500.13 MHZ respectively at 70°C. For these measurements, 5 mg of sample were introduced into a 5 mm diameter NMR test tube, to wich 1 ml of 2 wt% DCl/D_2O solution was added.

*Immobilization of tyrosinase on chitin:*Chitin from lobster was used as a support. Glutaraldehyde was used as a crosslinking agent. The chitin (0.5 gr.) was added to a solution of phosphate buffer pH=7, 0.4ml of glutaraldyde of 25% of purity and 7000 units of tyrosinase were added, and all the reactives were stirred at 4° C overnight. Immobilized derivatives were washed with an aqueous solution, acetate sodium solution and finally they were stored on a sodium phosphate solution pH=7. The amount of immoblized tyrosinase in each case was calculated as the difference between the initial solution brought into contact with the support and that of the protein swept by washing , determined by the Lowry method.

*Measurement of Tyrosinase activity:*Tyrosinase activity, free and immoblized on chitin was determined from a change in optical density ($A_{265\ nm}$) in a reaction mixture containing phenol, and the disappearance of phenols was also monitored by high performance liquid chromatography using a Waters 625 LC system equipped with a photodiode array detector and millennium software. A reverse phase column Lichosphere 100 RP 18 5 μ was used.The mobile phase consisted of methanol and water, (50/50), the flow rate was 0.5 ml/min and a 50μl of reaction solution was injected.

Results and Discussion

Immobilization of tyrosinase was investigated in an effort to overcome the disadvantages in the enzymatic treatment of phenols by the contamination of enzyme remaining in the solution, producing the inactivity of the the enzyme, and thus, its reusability.

Immobilization of tyrosinase was carried out on chitin from lobster according to the method described before. The yield of tyrosinase immobilized was determined by the Lowry method on the washing. Four immobilizations were made, and the results in respect of coupling yield, activity and stability are shown in Table 1:

Table 1. Immobilized tyrosinase on chitin

% coupling yield	Activity	Stability
10%	50% (a)	30% (b)

(a) Phenol at 6 hours of reaction.

(b) Final activity, 50 days after immobilization.

As can be seen, we got an active derivative, with a mere 10 % of coupling yield, but very stable in time. All the measures of activity were determined by measuring the concentration of phenol (or its degradation), by HPLC at 265nm. This means that the products thus formed, although determined by U.V spectroscopy, are not as sure in its quantity determination as the phenol. This is the reason why all the results in this article are referred to quantity of phenol.

An interesting point with regard to this immobilized tyrosinase in chitin, is to consider the possible adsorption of phenol in chitin at the same time that the reaction occurs. In order to test that all the degraded phenol is due to reaction and not to adsorption, we made a comparation of the reaction with free tyrosinase and immobilized tyrosinase, in the same condition of reaction, and the [S]/[E]= 286. The result is shown in Fig. 1.

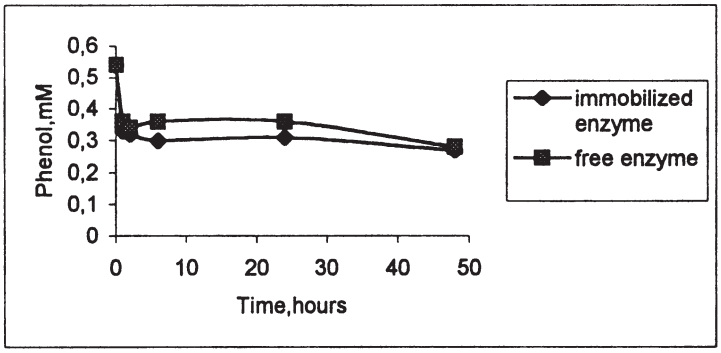

Figure 1. Degradation of Phenol by free and immobilized tyrosinase [E]/[S] = 286

In both experiments, the degradation of phenol was of 50% after 6 hours. Furthermore we confirmed the existence of adsorption of phenol on chitin putting in contact one dissolution of phenol with chitin for 24 hours. Figure. 2.

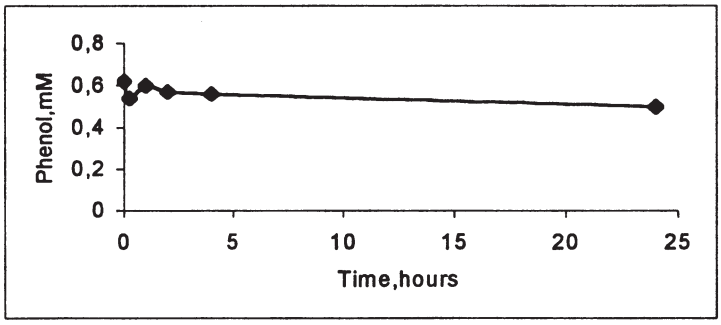

Figure 2. Adsorption of phenol on chitin

In fact 10% of phenol is adsorbed. This behaviour can be explained by the fact that when the tyrosinase is immobilized on chitin, all the active centers of chitin are occupied by the activator and /or the enzyme, and the chitin in these conditions is "saturated" for more adsorption.

The curves of adsorption of phenol on chitosan were also made, and the results are shown in the figure 3. In this case the adsorption values are 42% at 2 hours of reaction

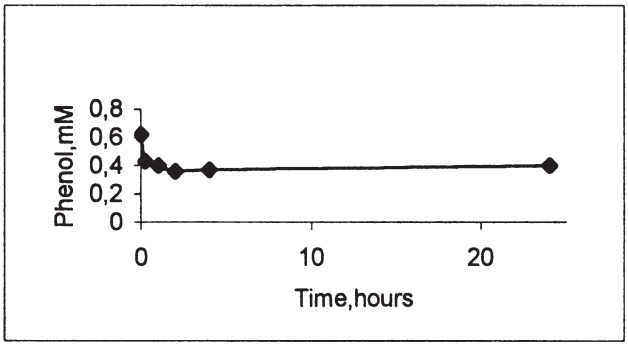

Figure 3. Adsorption of phenol on chitosan

As for the adsorption of reaction products we carried out two experiments: enzymatic reaction with free tyrosinase in presence of both chitin and chitosan. The results are in fig 4.

One can see an increase of Abs especially in the zone of 380 nm, due to the presence of quinones. The increase is higher with chitin in the medium than with chitosan. Chitosan adsorbs more quantity of quinones and these can react with it according to Pyne et al. [9].

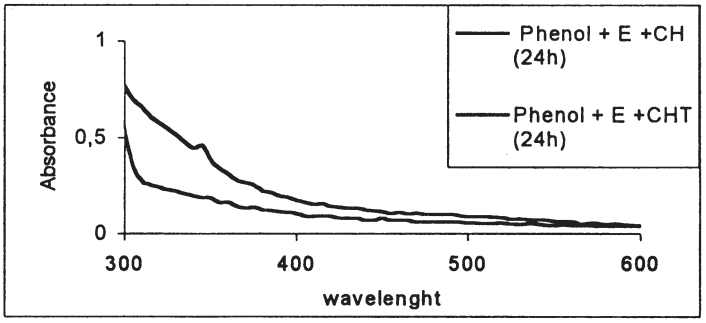

Figure 4. Enzymatic reaction with free Tyrosinase (E) in presence of Chitin (CH) and Chitosan (CHT). (At 24 hours of reaction)

From these previous experiments we designed a new complex one in three steps a) adsorption of the phenol in the initial solution on chitosan, b) reaction of immobilized tyrosinase on chitin with phenol in the filtered solution, and c) adsorption of the phenol left and reaction product on chitosan. The sequence and the results are shown in fig.4.As can be seen, with this experiment, we got a degradation and consequently an elimination of an 80% phenol and of a non-quantified amount of products. Figure 5 shows the results of this sequence in respect of the time.

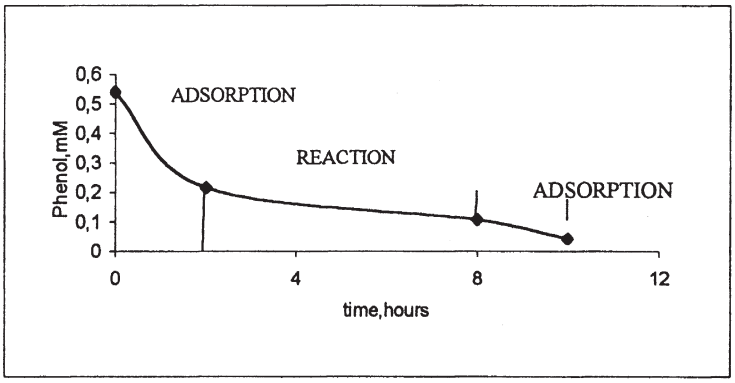

Figure 5. Results of the experimental sequence respect to the time

It is interesting to use the immobilized derivative and not only chitosan, because the derivative is stable for a long time (50 days) and it allows its reuse for some cycles, and finally adding the third step of the experiment (adsorption of residual phenol and product in chitosan), all the process is much more efficient (90%).

Conclusions

Chitin from lobster and chitosan from this chitin were obtained in our laboratory. Both pressent good qualities to be used in phenol degradation: Chitin as a support for the immobilization of tyrosinase and chitosan as an adsorbent of phenol and of the reaction product.

Immobilized tyrosinase was obtained. This immobilized derivative was active for 50 days, and it was able to make the degradation of phenol in a 50%, at 6 hours.

A sequential experiment was designed. It could reach 90% efficiency in phenol degradation. It was based on the properties of adsorption of chitosan and in the activity of immobilized tyrosinase in chitin.

This system can be useful in the treatment of wastewater to eliminate phenolic compounds.

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Utilization of modified chitosan in aqueous system treatment

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Summary
The feasibility of applying modified chitosan (η= 240 m Pa. s, degree of deacetylation= 82 % and calcium content = 48 %(w/w)) prepared from shrimps shells collected in Bahía Blanca estuary was performed for drinking water treatment. The coagulation- flocculation tests were developed by means of jar test. The optimal dosage of modified chitosan was 10,5 mg/L.

When analyzing the average of the obtained data before and after the flocculation treatment, we obtained decreases in turbidity of 99 %; COD of 75 % and total coliformes 96 %. No charge of conductivity was observed. Moreover, the total macro and micro algae elimination was verified.

These results confirm the excellent modified chitosan capacity for turbid, contaminated, high algae content water.

Introduction

Drinking water is produced by treating naturally occurring waters to reduce odor, taste, appearance, and sediment to acceptable levels. In general this involves removal of bacteria, viruses, algae, dissolved mineral, dissolved organic matter and suspended solids of the water.

The coagulation and flocculation processes promote the suspended solid agglomeration, thus allowing their effective separation.

The first phenomenon taking place in the process of flocculation is the neutralization of the net charge carried by each particle. Once charge neutralization takes place, several particles come together, which will result into coagulation. Flocculation is the stage whereby the destabilized particles are induced to collect into larger aggregates.[1]

Historically, inorganic coagulants based on aluminum, iron and calcium have been used for potable water treatment. Most common out of these is aluminum sulfate. [2] These operate by formation of aluminum hydroxide precipitate which sweeps down or co-precipitates the suspended matter. However, the use of such chemicals decreases the alkalinity of water, has a strong pH-dependence for effective coagulation, causes secondary pollution in the sludge disposal, and has raised public health concerns owing to the amount of aluminum remaining in treated water. [3-5]

Polyelectrolytes can replace in part or whole, the inorganic coagulant to meet the clarity norms at much reduced amounts (at ppm levels), thereby considerably reducing the sludge formation. [6-9]. A wide range of cationic polyelectrolytes are hence available depending on the cationic monomer present, the charge density and the molecular weight.

Lately, the coagulation of solids negatively charged by the action of certain cationic polymers has been suggested [7].

Chitosan possesses an enormous potential application in this area, being nowadays already in use by certain authors, such as Klopotek, 1994 and Struszczyk, 1994 [10]. Thus, it has great value as an industrial flocculent, and is approved as a potable water treatment substance by the U.S. Environmental Protection Agency.

This work is based on the study of this modified biopolymer utilization for the clarification of water from San Roque lake, Córdoba, which presents inorganic & organic material in solution and in suspension as well as high cyanophita algae content.

The presence of macroscopic plants causing blockage of intake screens and microscopic planktonic algae producing taste and odor must be investigated and controlled in order to protect the quality of water.

Material and Methods

Modified chitosan was produced, at our laboratory, from shrimp-shell waste collected in Bahía Blanca estuary. The procedure constitutes a pendent patent. Its characteristics were as follow: 82,0 % deacetylation degree, η = 240 m Pa s, and 48% w/w calcium content.

Working solutions: The tests were performed employing solution of modified chitosan 1% (w/v) in acetic acid 1% (v/v). Solution aluminum sulfate as coagulant aid was assayed at 10 ppm concentration.

Jar testing:The experiments were conducted in 1 litter jar test using a conventional jar test apparatus was according to ASTM Standards.[11] The stirring velocity was 80 rpm for 1 min.

Quality controls of the water before and after treatment with modified chitosan, aluminum sulfate and modified chitosan-aluminum sulfate optimal dosage were performed.
The measurements of turbidity, COD, BOD, QOD, solids and pH were made according to standards methods. [12]

The total macro and micro algae elimination was verified by ocular observation after flocculating.

Results and Discussion

Water samples

San Roque lake is a regulating reservoir and is used for water supply purposes.

In this study we worked with samples obtained from the outlet of San Antonio river after running through Carlos Paz city town.

The samples were got after flowering of cyanophita algae, so they had high content of them. The water sample parameter values are shown in Table 1.

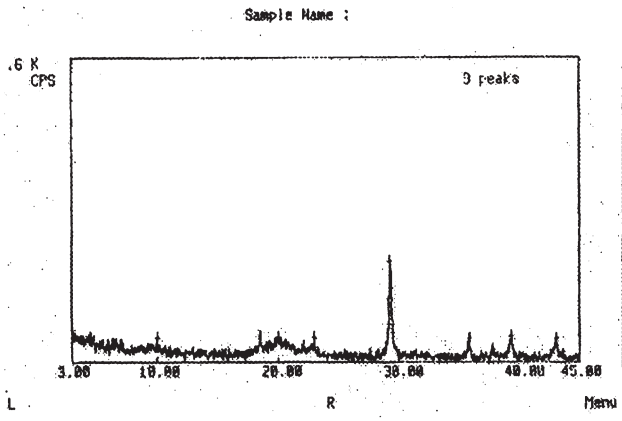
Modified chitosan

The modified chitosan used had a high mineral content (ash: 50 % w/w). The crystalline structure of this chitosan (fig. 1) was due to the presence of calcium, as calcium carbonate, in the matrix (48 % w/w). This presence accelerated the dissolution the chitosan in acetic acid because the salt in acidic medium produced CO_2 which "opens" the structure of the biopolymer increasing its solubility.

Table 1. Initial values of the measured parameters in San Roque lake water sample

Turbidity	NTU	4,5
Deposit solids 10 min	$mL.L^{-1}$	0,1
Deposit solids 2 h	$mL.L^{-1}$	0,2
pH		6,9
Floating algae		presence
Extract substance $HCCl_3$	mg. L^{-1}	170
COD	mg. L^{-1}	27
BOD	mg. L^{-1}	15,6
QOD	mg. L^{-1}	120
Total Coliformes	UFC. L^{-1}	15000

When the modified chitosan was precipitated from the acetic acid solution by adding sodium hydroxide, the formed flocculus had different aspect with respect to another chitosan obtained in our laboratory as is shown in the stereophotography obtained with stereomicroscope SZ- CTV Olympus with optical fiber (fig. 2).



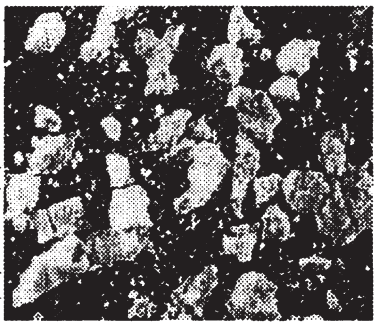
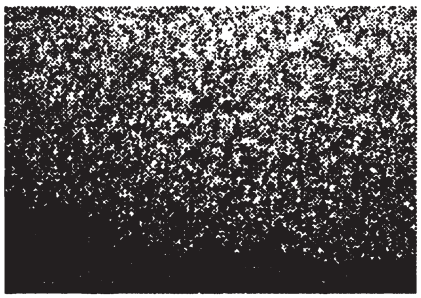


Figure 1. Crystalline structure of modified chitosan by X ray diffraction

Modified chitosan stereophotography obtained by stereomicroscope



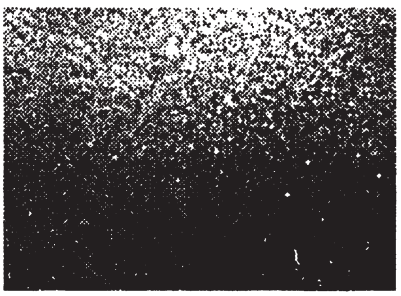


Figure 2. Modified chitosan flocculus obtained by stereomicroscope

Conventional chitosan flocculus obtained by stereomicroscope

Coagulation- flocculation study: Modified chitosan, aluminnium sulfate and modified chitosan-aluminnium sulfate were the flocculents used in this work.

Firstly the optimal dosages of them were determined by jar tests. The results are presented in Table 2.

Table 2

Flocculents	**Optimal dosage** $mg.L^{-1}$
Modified chitosan	10,5
Aluminum sulfate	11
Modified chitosan	3
Aluminum sulfate	11

Values of the measured parameters before (b/t) and after (a/t) modified chitosan treatment and percentage of disminution are given in Table 3.

Table 3. Initial and final values of the measured parameters and percentage of disminution after modified chitosan treatment.

		Modified chitosan	
		b/t	**a/t**
Turbidity	(NTU)	4,5	0,06
Deposit solids 10 min	($mL.L^{-1}$)	0,1	0,11
Deposit solids 2 h	($mL.L^{-1}$)	0,2	0,2
pH		7,4	6,4
Floating algae		presence	absence
Extract substance $HCCl_3$	($mg.L^{-1}$)	170	8
COD	(mg/L^{-1})	27	7,4
BOD	(mg/L^{-1})	15,6	14,6
QOD	(mg/L^{-1})	120	30
Total Coliformes	($UFC.L^{-1}$)	15000	500

Optimal dosage of modified chitosan: 10,5 mg. L^{-1}

Calcium, magnesium, nitrates, ammonia and fluorine were determined and no significative differences were found between the results b/t and a/t.

A comparative study between different flocculents was made and the parameter results are represented in table 4.

The three treatments were efficient in clarifying the water.

The use of aluminum sulfate as coagulant improve slightly the efficiency the of procedure and its utilization allowed lesser chitosan concentrations. Use of modified chitosan treatment had similar results with respect to classical treatment (aluminum sulfate), but the last one had a residual aluminum content over the maximum level allowed by Public Health Government Department.

Table 4. A comparative study between different flocculents

	Modified chitosan		$Al_2(SO_4)_3$		Modified chitosan-$Al_2(SO_4)_3$	
	b/t	a/t	b/t	a/t	b/t	a/t
Turbidity (NTU)	4,5	0,06	4,5	0,05	4,5	0,02
QOD (mg/L^{-1})	120	30	120	48	120	60
Floating algae	presence	absence	presence	absence	presence	absence
Total Coliformes ($UFC.L^{-1}$)	15000	500	15000	240	15000	300
Residual aluminum (ppm)				0,64		0,05
Optimal dosage (mg/L^{-1})		10,5		11,0	**Modified $Al_2(SO_4)_3$ chitosan**	
					3,0	11,0

This study demonstrates the feasibility of application of a modified chitosan in treatment of aqueous system to supply purposes.

This biopolymer is obtaining from shrimp shells, which may be the solution for waste disposal problems along the Bahía Blanca, Argentina, estuary. Moreover its obtention procedure is simpler and cheaper than the classical one.

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Chemical and preclinical studies on 6-oxychitin

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Introduction

This article collects the essential data obtained on preparation and applications of 6-oxychitin. Leading ideas, evaluations and perspectives are highlighted. This subject is being presented at an international conference for the first time. In consideration of the extension of the information and the little time and print space available, the reader is referred to the original articles for details of technical and methodological nature, as well as for the relevant bibliographies.

The sections treated here are: regiospecific oxidation of animal chitins; bone regeneration in the presence of 6-oxychitin; coating of prosthetic devices for enhanced bioactivity; polyelectrolyte complexes of chitosan and 6-oxychitin for drug delivery; upgrading of polysaccharides from fungal biomasses.

Regiospecific oxidation of animal chitins

Chitins of different origins, in the form of dry powders, were all found suitable for the regiospecific oxidation provided that they were preliminarily soaked in warm water. As an alternative, chitins regenerated with water from dimethylacetamide-LiCl solutions were most suitable. When chitins were isolated from fresh crustaceans, drying or heating were conveniently omitted, and the oxidation was carried out on the freshly isolated chitins.

The stable nitroxyl radical 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo®) was used as a catalyst, together with NaBr to regiospecifically oxidise chitin with 4 % NaOCl solutions. This reaction transformed the primary alcohol group at pH 10.8. Partial depolymerization took place. The 6-oxychitin Na salt, at the instrumental analysis showed:

- full solubility over the pH range 3-12;
- high yield, generally higher than 90 % (freeze-dried product, after dialysis);
- long shelf life: the white powder did not aggregate over a 2-year observation period;
- N/C ratio close to the calculated value of 0.146, most often 0.112 – 0.144;
- modest degree of crystallinity with weak diffraction bands corresponding to those of chitin;
- intense bands in the infrared spectrum at 1618 and 1415 for carboxylate and 1656 and 1552 cm^{-1} for chitin functions;
- large surface of an expanded and fragile material seen at the electron microscope;
- shift of the C-6 signal in the ^{14}C-NMR spectrum from 62 to 178 ppm;
- M_w values close to 10 kDa and M_n 3200 – 5200.

The reactivity of 6-oxychitin led to:

1. prompt reaction with polycations, such as chitosan, DEAE-dextran and polylysine;

2. coagulation of proteins, including papain, lipase and amylase;
3. chelation of metal ions;
4. ester formation with a number of simple alcohols.

The formation of polyelectrolyte complexes of chitosan and 6-oxychitin permitted an easy recovery of 6-oxychitin from the preparation medium, by simply adding equimolar chitosan salt solution at pH 2 to the alkaline NaOCl solution at the end of the 30-min oxidation process.

The 6-oxychitin-chitosan complex is the only example today of a complex made of a polycation and a polyanion from the same origin (i.e. chitin).

It was concluded that 6-oxychitin is simple to prepare (aqueous medium, room temperature, little labor, short time (30 min), common chemicals, and probably less environmental impact than in the case of the preparation of chitosan [1, 2].

Preparation of chitosan-oxychitin microspheres for drug delivery

It is known that hyaluronan can be extracted from fermentation solutions as a polyelectrolyte complex with chitosan acetate, and separated from the latter upon aqueous NaOH treatment and filtration. Chondroitin sulfate and chitosan were used to produce polyelectrolyte complexes, for the controlled release of diclofenac, and chitosan-pectin complexes were evaluated for specific drug delivery. Mucin-chitosan complexes were studied for the delivery of drugs to the oral cavity. Other polyanions made to react with chitosan include DNA: chitosan is useful as a non-viral vector for gene delivery, capable of protecting DNA from nuclease degradation, besides being non-toxic nor hemolytic. Absence of cytotoxicity and immunologic protection were demonstrated.

In this application of oxychitin, we took advantage of the capacity of chitosan to form polyelectrolyte complexes with polyanions.

Microcapsules were prepared by dropping through a needle the oxychitin aqueous solution (1 ml, 2 - 6 % w/v) into a chitosan aqueous solution (10 ml, 1, 2 or 4 % w/v) placed in a 25 ml Erlenmeyer flask and stirred gently.

Chitosans were dissolved in citric acid aqueous solution, whose molarity and pH were chosen, based on the polymer characteristics. The chitosan solutions contained $CaCl_2$ in different concentrations; $CaCl_2$ was also added to the oxychitin solution, or omitted. The microcapsules were allowed to settle for 1 hour, then the solution was removed and the microcapsules were rinsed twice with saline, and then freeze-dried at -40°C and 40 mbar.

The reaction between oxychitin sodium salt and chitosan acetate yielded a polyelectrolyte complex in the form of a spongy material, relatively rigid, highly hydrophilic, mucoadhesive, and easily amenable to a powder.

The coacervation process leading to microcapsule formation was immediate when the oxychitin solution was dropped into the chitosan solution. The microcapsule size mainly depended on the inner diameter of the needle (18G) used to drop oxychitin solution. While the preparations were carried out under gentle stirring, agitation did not affect the process. The formation of microcapsules took place only with chitosan glutamate or chloride salts; medium m.w. (400 kDa) and high m.w. (2 MDa) chitosans did not form microcapsules, while low m.w. (70 kDa) and regular chitosan (250 kDa) formed embrionic microcapsule suspension that collapsed during the freeze drying process.

Microcapsules did not form with chitosan chloride of low molecular weight leading to low viscosity solutions (25.6 mPa.s for 2 % conc.; 157.9 mPa.s for 4 % conc.); the chitosan solution viscosity had a certain relevance in improving the spherical shape of oxychitin drops.

Necessary conditions to obtain stable microcapsules were: chitosan concentration at 2 % w/v and $CaCl_2$ concentration at 1 % in the chitosan solution.

The reaction between chitosan and oxychitin was evaluated by colorimetric analysis with the anionic reactive dye Cibacron Brilliant Red [3]. The calibration curve was drawn for chitosan concentrations in the range 7 - 40 μg/ml. Absorbance was measured at 575 nm; the percentage of chitosan reacted was expressed as reaction index. The presence of $CaCl_2$ in the chitosan solution did not modify the amount of reacted chitosan. On the other hand, the $CaCl_2$ addition to oxychitin solution lowered the amount of reacted chitosan.

Oxychitin pH modifications at values below or above 6 increased the reaction index; lowering chitosan pH below 5 increased the reaction index as well. The best results (reaction indexes close to 100 %, meaning equimolarity) were obtained when mixing oxychitin at pH 10 and chitosan at pH 2, because the two polymers are completely ionized under these conditions.

The results obtained permit to conclude that microcapsules can be obtained by complex polyion coacervation between oxychitin sodium salt and chitosan chloride or glutamate salt. Their formation depends on the molecular weight of chitosan and viscosity of its solution, that should be higher than a determined value.

The use of oxychitin offers an alternative to alginate, having the advantage of producing microcapsules where both the anionic and cationic compounds have the same origin, i.e. chitin [4, 5].

Bone regeneration

Being similar to hyaluronan in nature and behaviour, 6-oxychitin was deemed interesting for a morphological study of bone regeneration. Hyaluronan shows in fact morphogenetic activity suitable for a correct bone architecture. Based on our previous experience with induction of bone formation, we have compared the bone healing capacities of N,N-dicarboxymethyl chitosan and 6-oxychitin. Surgical lesions in the rat condylus were medicated with one of these materials, either as such or coated with osteoblasts (1.5x103 cells/cm2).

Both materials, after 3 weeks produced a good histoarchitectural order in the newly formed bone tissue. There were no really evident differences, but 6-oxychitin gave a more ordered bone structure, based on trabecular bone volume, trabecular thickness and number of trabeculae per mm.

Control lesions showed the largest residual defect, the lowest trabecular bone volume and thickness. with a trabecular number higher than for the chitin derivatives. The addition of osteoblasts improved the results. With 6-oxychitin, healing was slower than with N,N-dicarboxymethyl chitosan, but the trabecular architecture was superior.

It was concluded that 6-oxychitin is perfectly tolerated for bone lesion medication, and is superior to other materials of chitinous origin so far tested in terms of bone reconstruction, even though healing is slower [6].

Coating of prosthetic materials

There is interest in producing prosthetic items capable of potentiation the biointegration particularly in aged or osteoporotic patients with altered bone metabolism. Titanium plates (Ti-6Al-4V alloy) were preliminarily coated with hydroxyapatite by plasma spray (80 μm coating), and their surface was characterized by x-ray diffraction, scanning electron microscopy, and FT infrared spectrometry.

Chitosan can be easily deposited on the hydroxyapatite coating, however, the chitosan films results too weak to sustain the mechanical stress to which it would be submitted during implantation. Therefore it was reacted with 6-oxychitin alone or with 6-oxychitin in the presence of carbodiimide. The layers of polyelectrolyte complexes, observed at the electron microscope, were uniform and smooth. No cracks or dishomogeneities were visible. Confirmation of their chemical nature was obtained by FTIR spectroscopy in the reflectance mode.

Similarly, the titanium alloy preliminarily coated with bioglass AP40 (10 μm thickness), were treated with chitosan and then with 6-oxychitin, according to the above mentioned protocols.

In vitro evaluation of the bioactivity of the chitosan-oxychitin-coated items, were carried out by using human fibroblasts and osteoblasts. In vivo studies were done on Sprague-Dawley rats submitted to bilateral ovariectomy to induce osteoporosis. Samples of coated titanium alloy were implanted in the femoral condyle.

Observations made 60 days after implantation indicated that both the ceramic and bioglass type chitosan-oxychitin-coated implants were able to exert bioactivity even in the presence of bone with altered turnover.

It was concluded that the chitosan coating with the aid of oxychitin can be obtained in a rapid and satisfactory way, and that it helps in promoting bone healing and integration of the prosthetic material [7].

Upgrading the polysaccharides from fungal biomasses

The biomasses of *Aspergillus niger, Trichoderma reesei* and *Saprolegnia* sp. were submitted to sodium dodecylsulfate washing, mechanical disruption of the mycelia, stirring at 60°C for 3 hr and protracted stirring overnight at room temperature. They were then oxidized regiospecifically at C-6 with NaOCl and NaBr for 30 min in the presence of Tempo® as a catalyst at room temperature.

The powders obtained were polyuronans in sodium salt form, fully soluble in water over the entire pH range. They were characterized by ^{1}H-NMR spectrometry and shown to contain 20 % and >75 % 6-oxychitin, for *A. niger* and *T. reesei*, respectively. The deconvolution of the FTIR spectra in the range 1500 – 1800 cm^{-1} confirmed the presence of a band at 1568 cm^{-1}, much more evident in the *T. reesei* than the *A. niger* material, assigned to chitin amide.

The fungi examined being representative of the three major types of cell walls, as well as of industrial activities, it is concluded that the process is of wide applicability, in consideration also of its simplicity. It would permit to upgrade the industrial spent biomasses and to exploit their polysaccharides [8].

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Diverse biological effects of fungal chitin-glucan complex

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Summary
Chitin-glucan (Ch-G) complex isolated from the fungal mycelium of the industrial strain of *Aspergillus niger* was solubilized using carboxymethylation and sulfoethylation. By means of subsequent ultrasonic treatment lower molecular weight water-soluble derivatives were obtained. Biological effect of the prepared compounds was evaluated in direct interaction on DNA *in vitro*. Monitoring of electrophoretic mobility of plasmid DNA implied that carboxymethyl chitin-glucan (CM-Ch-G) induced single and double strand breaks into supercoiled DNAin a concentration-dependent manner. On the other hand, sulfoethyl chitin-glucan (SE-Ch-G) alone did not induce any DNA breaks in plasmid DNA. However, process of DNA damaging induced by free-radical oxidation initiated with Fe^{2+} was inhibited, while the process of DNA breakage induced by H_2O_2 was increased in the presence of SE-Ch-G.

Introduction
In the recent years much attention has been paid to the research of the naturally occurring substances that are able to boost the immune defense mechanisms of the organism. High-molecular β-glucan is a common component of yeast and fungal cell walls. β-Glucans belong to the group of compounds known as biological response modifiers, which stimulate the immune system of the host and exert an amazing range of immunopharmacological activities that include protection against viral, bacterial, fungal and parasitic infections, antitumour effect and metastasis inhibition, radioprotection and stimulation of the hematopoiesis, *etc.* [1]. In the mycelia of the filamentous fungus *Aspergillus niger*, β-glucan is covalently associated with chitin [2] and presence of the two biologically active polysaccharides in the complex may enhance its pharmacological effect. Moreover, Ch-G can be easily prepared from the waste material left upon the industrial preparation of citric acid by *Aspergillus niger* and therefore represents a non-expensive natural source of potentially active non-toxic immunostimulatory drug.

Since β-glucan and Ch-G are water-insoluble, a special attention should be paid to the preparation of their water-soluble derivatives which would enable their parenteral administration, because intravenous or intraperitoneal application of insoluble microparticulate glucan can cause adverse side-effects such as microembolization and granulomatosis [3,4]. In our previous studies water-soluble derivatives of yeast β-glucan with pronounced antimutagenic protective activity have been prepared [5-7]. Since antigenotoxic activity of chitin and chitosan was previously described as well [8], it can be assumed that in

Ch-G both polysaccharide components could play role in its protective effect. Previously we have proved this assumption by demonstrating antimutagenic activity of high molecular weight CM-Ch-G against cyclophosphamide in mice [9]. The lower molecular weight fractions of CM-Ch-G obtained by means of ultrasonication of the original complex was protective not only at parenteral, but also at oral administration, which proved that decreased molecular weight helped CM-Ch-G molecule to pass into the bloodstream through the wall of gastrointestinal tract [10,11].

In the present paper we describe diverse activity of soluble derivatives of Ch-G preparation on DNA in the presence of DNA-damaging agents.

Materials and Methods

Isolation of the crude chitin-glucan complex: The crude, water-insoluble chitin-glucan complex was isolated from the cell walls of the industrial strain *Aspergillus niger* used for the commercial production of citric acid (Biopo, Leopoldov, Slovak Republic). The mycelium was subjected to a hot alkaline (1 M NaOH) digestion for 1 h. The alkali-insoluble sample was subsequently five times washed with distilled water, acetone, and finally with diethylether. The dry sample contained 2.24 % nitrogen which corresponded to a content of ca. 30 % chitin.

Preparation of the water-soluble chitin-glucan complexes: Carboxymethylation of Ch-G was performed using the modified procedure described by Sasaki *et al.* [12]. Sulfoethylation was performed as described by Chorvatovicová *et al.* [7]. In order to improve solubility of the obtained compounds, their ultrasonication was carried out using a procedure described by Chorvatovicová *et al.* [10]. The samples obtained upon ultrasonication were purified by gel-permeation chromatography and their molecular weight (MW) determined by HPLC as described in [10]. From the original high molecular samples with MW ca. 600 kDa, the following samples were prepared: CM-Ch-G with MW 57 kDa and SE-Ch-G with MW 40 kDa.

Chemicals and DNA: The DNA used was the plasmid pBR322 purchased from Advanced Biotechnologies Ltd. (U.K.) and was predominantly (>95%) in the supercoiled form (as judged by agarose-gel electrophoresis). Iron sulfate ($FeSO_4 .7H_2O$) was used as a source of Fe^{2+}, hydrogen peroxide (30% H_2O_2) and monosodium and disodium phosphates were analytical reagent grade and were commercially available.

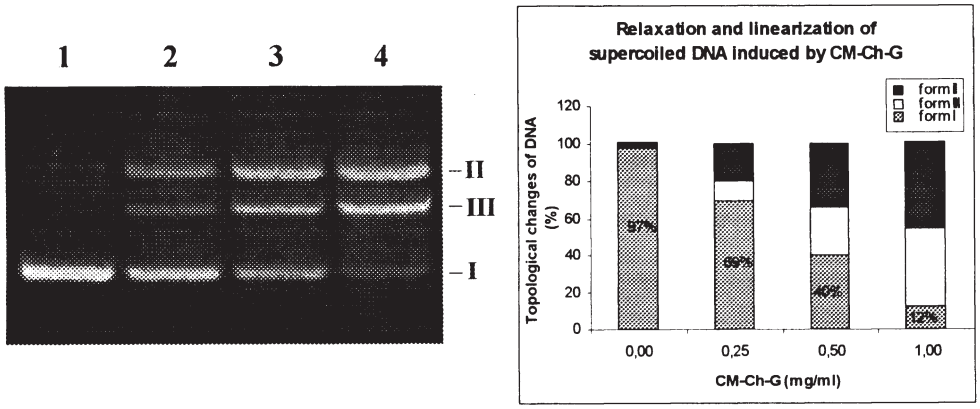
Reaction conditions for induction of DNA strand breakage: The reaction mixture (final volume 20 µl) contained 0.2 µg plasmid DNA (pBR322 30µM base-pairs) in 10 mM sodium phosphate buffer (pH 7.0) and either CM-Ch-G alone or SE-Ch-G in combinations with Fe^{2+} or H_2O_2. Specific details of incubation period and agent concentrations are given in the legends to Figures. The reaction was started by the addition of the inductor of DNA breakage to the reaction mixture and quenching of reaction was achieved by immediate loading and agarose-gel electrophoresis.

Analysis of DNA single/double strand breaks: Plasmid DNA isomers migrated as discrete bands in agarose-gel during electrophoresis (1.5% agarose, Sigma, USA; 60 min/ 60 V) in the minigel apparatus as described previously [13]. The DNA was made visible by staining with ethidium bromide (1µg/ml, Sigma, USA) and UV illumination (Ultra-Lum Electronic UV Transilluminator, USA). DNA single-strand breaks (ssb) were assayed by measuring the conversion of supercoiled DNA (form I) to relaxed circular DNA (form II), while double-strand breaks (dsb) were estimated basing on its conversion to linear DNA (form III). Percentages of supercoiled, relaxed, and linear DNA forms were calculated by a computer program (Uthsca, Image Tool for Windows, Version 1.27). Since supercoiled DNA

is restricted in its ability to bind ethidium bromide relative to relaxed circular and linear forms [14,15] it was necessary to correct values obtained for supercoiled DNA.

Results and Discussion

Using the conversion of the supercoiled pBR322 DNA to the relaxed and linear forms, we witnessed the capacity of CM-Ch-G to introduce single-strand and double-strand nicks into plasmid DNA in a concentration-dependent manner (Fig 1A,B). We observed the formation of relaxed and linear forms of DNA in approximately 1:1 proportion.



A B

Figure 1. The effect of CM-Ch-G on single and double strand break formation in pBR322 DNA. Samples contained DNA (200 ng) and the indicated concentration of CM-Ch-G (0.00; 0.25; 0.50; 1.00 mg/ml) in 10 mM sodium phosphate buffer (final volume 20 µl, final pH ≅ 7.0) were incubated at 37°C for 30 min. Cleavage of the supercoiled DNA (form I) to the relaxed (form II) and linear (form III) forms was assassed by electrophoresis (A) and determined by densitometry (B). Results are presented as the CM-Ch-G induced alteration in the ratio of the topological forms of plasmid DNA. Lanes in the Fig. A correspond to the respective bars in Fig. B.

We therefore concluded that DNA damage was caused not only by single-strand-breaks but by double-strand-breaks as well. CM-Ch-G presented itself in our experiments as a direct inductor of DNA damages, however, the mechanisms involved in the presented DNA damaging processes are not understood and need further investigation. On the other hand, SE-Ch-G alone did not induce any topological changes in supercoiled plasmid DNA (Fig.2A, lane 1). This characteristic is the important difference between SE-Ch-G and CM-Ch-G (Fig.1). Since both chitosan and β-glucan were demonstrated to possess radical-scavenging activities [16,17], it is quite improbable that the DNA damage was inflicted through the radical degradation.

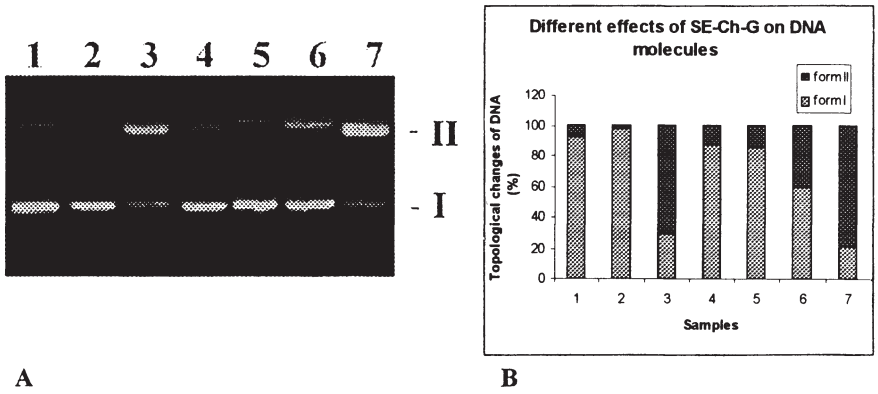


Figure 2. Effects of SE-Ch-G on DNA breakage induced by Fe^{2+} and H_2O_2 were assessed by agarose gel electrophoresis (A) and densitometry analysis (B). Plasmid DNA (200 ng) was incubated in 10 mM sodium phosphate buffer (samples 2; 3; 5; 6) containing 1mg SE-Ch-G /ml (samples 1; 4; 7) in the presence of either 10 μM Fe^{2+} (samples 3 and 4) or 100 μM H_2O_2 (samples 6 and 7). The samples (final volume 20 μl, final pH ≅ 7.0) were incubated at 37°C (samples 1-4) or 50°C (samples 5-7) for 5 min (samples 2-4) and 30 min (samples 1 and 5-7). Lanes in the Fig. A correspond to the respective bars in the Fig. B.

Furthermore, we have attempted to study the potential of SE-Ch-G in iron chelation and scavenging H_2O_2 in separate experiments. The ability of phosphate buffer to catalyze rapid Fe^{2+}oxidation to Fe^{3+} in the presence of DNA at low ionic strength is known [18,19]. In our experiments, the quantitative analysis of the different bands showed that addition of Fe^{2+} alone into the phosphate buffer with the supercoiled DNA caused conversion of supercoiled DNA to relaxed form DNA. (Fig.2A, lane 3). In the presence of SE-Ch-G, however, this process of DNA damaging induced by Fe^{2+}oxidation, was inhibited (Fig.2A, lane 4). In order to investigate the role of iron chelation or radical scavenging activity of SE-Ch-G in this DNA protective effect (since both these effects could account for the observed phenomenon), we used H_2O_2 as another inductor of DNA damaging conditions. In the previous paper, DNA nicking mediated with H_2O_2 has been examined in a temperature range from 20-50°C [20]. Now our experiments confirmed the induction of DNA breaks after exposition of the plasmid DNA to H_2O_2 at 50°C for 30 min. (Fig. 2A, lane 6). Interestingly, it was observed that SE-Ch-G was able to increase this effect of H_2O_2 on DNA nicking (Fig. 2A, lane 7). Thus, SE-Ch-G presented itself as a chelator of Fe^{2+} (inhibitor of Fe^{2+} induced DNA damage; sample 4), but also as an inductor of DNA breakage in the presence of H_2O_2 (sample 7).

Both activities of SE-Ch-G were verified in the dose-dependent experiments.

The results presented in Fig. 3 are consistent with our previous findings (Fig. 2) and demonstrate that DNA protective effect (experiment with Fe^{2+}) and the enhancing effect on DNA nicking (experiment with H_2O_2) are dose-dependent on concentrations of SE-Ch-G. These results also imply that SE-Ch-G probably does not function as a free radical scavenger. Its DNA protective activity can therefore be assigned to chelating of ferrous ions only. The observed difference between CM-Ch-G and SE-Ch-G can be probably ascribed to the role of

sulfoethyl substituent. The mechanism by which CM-Ch-G induced DNA damage needs further clarification. Further, since the observed effects took place at physiological temperature (37°C) and chitin-glucan is a common component of yeasts and fungi often occuring in the surrounding environment, our observations can therefore be of biological importance.

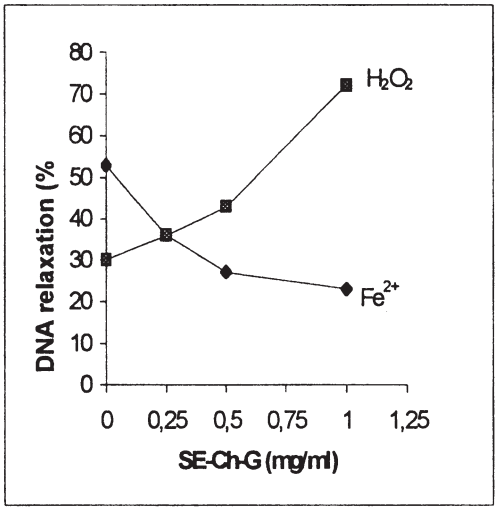


Figure 3. Effects of increasing SE-Ch-G concentrations (0.00; 0.25; 0.50; 1.00 mg/ml) on the nicking of supercoiled pBR322 DNA induced by Fe^{2+} (◆) or H_2O_2 (■). Plasmid DNA (200 ng) was incubated in 10 mM sodium phosphate buffer containing determined concentrations of SE-Ch-G plus 10 μM Fe^{2+} for 5 min/37°C or 100 μM H_2O_2 for 60 min/37°C (final volume 20 μl, final pH ≅ 7.0). DNA strand break formation was inhibited in an experiment with Fe^{2+} plus SE-Ch-G (DNA protective effect) and increased in an experiment with H_2O_2 plus SE-Ch-G (DNA damaging effect). The percentage of relaxed DNA (form II) was plotted against SE-Ch-G concentration. Results are the average from the triplicate samples (SD< 10%).

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Effect of concentration of neutralizing agent on chitosan membrane properties

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Summary
In a previous study we reported that the tensile strenght of membranes prepared by dissolving chitosan in an organic acid and then kneading with hydroxyapatite (HA) was influenced by the kind of chitosan used. The present study investigated the effect of concentration of sodium polyphosphate used as a neutralizing agent on membrane physical properties.

Introduction
Autogeneic bone transplantation has been generally employed for repairing bone defects. However, this method seems to exert a great burden both mentally and physically on patients when collecting the bone. For this reason, development of materials as a substitute for bone is required. We have been studying chitosan-bonded, self-hardening bone filling materials and chitosan membrane containing hydroxyapatite (HA).[1-7] In our previous study, we reported that the tensile strength of the membrane, which was prepared by dissolving chitosan with an organic acid and then kneaded with HA, was influenced by the kind of chitosan used.[8] Chitosan is obtained by deacetylating chitin. The properties of chitosan will differ depending on the degree of deacetylation. It has been suggested that a lower degree of acetylation might accelerate absorbtion in vivo.[9] It has also been reported that the molecular weight becomes smaller as the degree of deacetylation is elevated.[10,11] The molecular weight of chitosan affects the viscosity of chitosan solution and the tensile strength of the membrane. It is considered that absorbtion might be affected by the molecular weight as well as the degree of deacetylation. An ideal method for the repair and restoration of bone defects might be achieved when osteogenesis progresses with the absorption of chitosan membrane.

In the present study, the effect of the concentration of neutralizing material used for preparing chitosan membrane on the tensile strength and elongation of the chitosan membrane immersed in a physiological saline solution, used as a substitute for humor, was studied.

Materials and Methods
Preparation of Chitosan Membrane: 0.5g of malic acid (Nacalai Tesque) and malonic acid (Nacalai Tesque) each were dissolved on 10 ml of physiological saline in a room kept at a temperature of 24°C with a humidity of 60 %. The sol was made by dissolving 0.5 g of chitosan (Koyo Chemical) into these solutions. Two hours after dissolution, 2.0 g HA (Mitsui Toatsu, mean particle size 10 μm) was kneaded into the solution to make a paste. Each paste was syringed into a 70 cm diameter glass plate. The paste was neutralized with sodium

polyphosphate solution (Wako Chemical) at concentrations of 2, 5, 8 and 11%. The film was observed after 24 hours.

Changes in the neutralizing material pH value: The pH value of the solution neutralized in Item 1 was determined by using a pH meter (Toa Denpa) at the start of, 1 hour and 24 hours after neutralization. The value is expressed as the mean of three determinations.

Relation between the neutralizing material concentration, tensile strength and elongation: The chitosan membrane neutralized in Item 1 was sliced into 1.0 mm thick slices, 20 mm in length, 4 mm in width, and dried. After drying, the slices were immersed in a physiological saline at 37°C and stirred at 100 rpm per min. The samples were tested for tensile strength by using a universal testing machine (Imada Seisakusho) at a speed of 2.5 mm/min. At 1,14 and 28 days after immersing, elongation was determined as the distance of migration of the cross head. The value was expressed as the average of 5 samples for each condition. The results obtained in Items 2 and 3 were examined by analyzing the variance.

Results

Preparation of the chitosan film: Table 1 shows the methods of membrane preparation by dissolving chitosan using malic acid and malonic acid respectively, to which HAP was kneaded, and neutralized with sodium polyphosphate solution. According to the Table, the membrane should not be completed when chitosan sol was dissolved with malonic acid and neutralized by a 2 % sodium polyphosphate solution. However, membrane could be obtained under the other conditions.

Changes of the pH value of the neutralizing material: The pH value of the solution one hour after being neutralized by sodium polyphosphate at a concentration of 2 % was 6.55 +/--0.01, 7.74 +/--0.02 at a concentration of 5 %, 8.03 ± 0.01 at a concentration of 8 %, and 8.16 ± 0.02 at a concentration of 11 %. The pH value 24 hours after neutralization was 5.30 ± 0.01 for the 2 % concentration, 7.82 ± 0.01 for the 5 % concentration, 7.62 ± 0.01 for the 8 % concentration, and 7.82 ± 0.01 for the 11 % concentration. The pH value 24 hours after neutralization tended to be lower than 1 hour after neutralization (Fig. 1).

The value one hour after neutralization was 6.03 ± 0.01 at 2 %, 7.52 ± 0.02 at 5 %, 7.68 ± 0.02 at 8 %, and 7.87 ± 0.02 at 11 %. Meanwhile the value 24 hours after neutralization was 5.01 ± 0.01 at 2 %, 6.67 ± 0.02 at 5 %, 7.35 ± 0.02 at 8 %, and 7.58 ± 0.02 at 11%. The pH value after 24 hours was lower than that after 1 hour, as in the case of using malic acid for dissolving chitosan, although the pH value of the solution prepared by dissolving chitosan sol with malonic acid was lower (Fig. 2).

Relation between concentration, tensile strength and elongation: The tensile strenth of the test samples neutralized by using a 2 % sodium polyphosphate solution concentration for the dissolving and then immersed for one day in physiological saline was 21.2 ± 5.4 gf/mm^2. The test sample became too fragile to measure its strength after 14 days of immersion. When the membrane was neutralized with a 5 % concentration af sodium polyphosphate, the tensile strength was 26.7 ± 3.3 gf/mm^2, and could not be obtained after 14 days. When the membrane was neutralized with an 8 % concentration of sodium polyphosphate, the strength was 43.2 ± 1.6 gf/mm^2 one day after soaking and 8.4 ± 2.3 gf/mm^2 14 days after soaking. The strength could not be determined after 28 days. When the membrane was neutralized with an 11 % concentration of sodium polyphosphate, the strength after one day of immersion was 61.1 ± 3.3 gf/mm^2 and 14 days later it was 33.5 ± 5.5 gf/mm^2. The tensile strength tended to increase

Table 1 Preparation of chitosan membrane

Concentration %	Malic acid	Malonic acid
2	○	×
5	○	○
8	○	○
11	○	○

○ good × poor

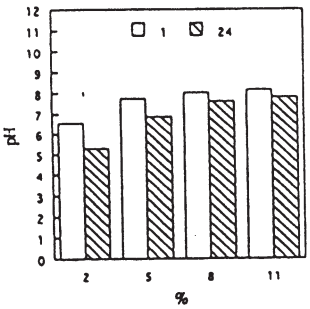

Fig.1 Result of pH value
(Dissolving chitosan with malic acid)

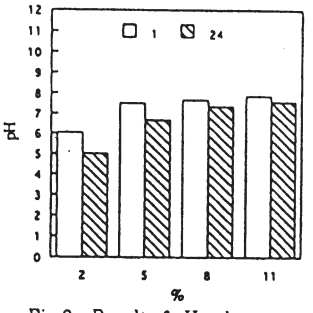

Fig.2 Result of pH value
(Dissolving chitosan with malonic acid)

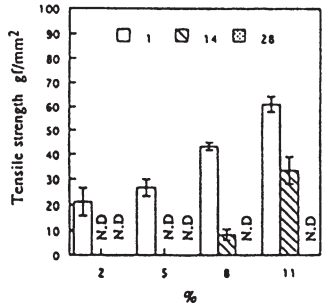

Fig.3 Result of Tensile strength
(Dissolving chitosan with malic acid)

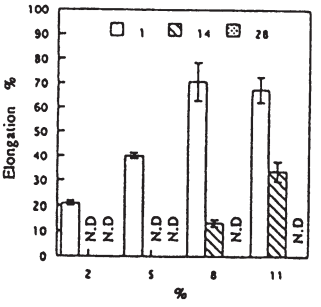

Fig.4 Result of Elongation

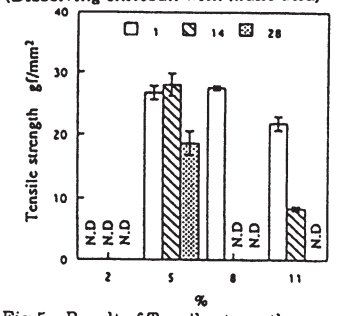

Fig.5 Result of Tensile strength
(Dissolving chitosan with malonic acid)

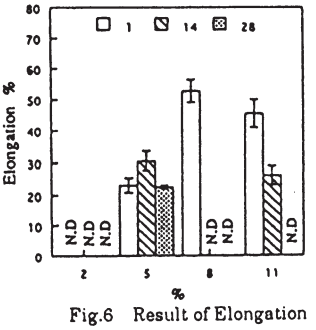

Fig.6 Result of Elongation

as the concentration of the neutralizing material became higher. 28 days after soaking the tensile strength could not be measured for any of the test samples (Fig. 3). When the membrane was neutralized with 2 % sodium polyphosphate, the elogation was 20.9 ± 1.0 %. The elongation could not be determined after 14 days. When neutralized with 5 % sodium polyphosphate, the elongation was 40.4 ± 1.3 % after one day of soaking, but could not be obtained after 14 days. When neutralized with 8 % sodium polyphosphate, the elongation was 70.7 ± 7.9 % after one day of soaking and 13.3 ± 1.5 % after 14 days. When using an 11 % conentration of neutralizing material, the elongation was 67.4 ± 5.2 % after one day of soaking and 33.6 ± 4.1 % after 14 days. The elongation values became larger with higher concentrations of neutralizing material. Elongation could not be determined after 28 days of soaking for any of the test samples (Fig. 4).

When using a 2 % concentration of neutralizing material, chitosan film could not be obtained, so determination was not possible. When neutralized with a 5 % concentration, the tensile strength of the membrane was 26.7 ± 1.1 gf/mm^2 after one day of immersion, 28.0 ± 1.8 gf/mm^2 after 14 days, and 18.7 ± 1.9 gf/mm^2 after 28 days. The tensile strength measurement after 14 days was the largest of all. The tensile strength of the membrane neutralized with 8 % material was 27.5 ± 0.4 gf/mm^2 after one day of soaking but could not be determined after 14 days. Meanwhile, when neutralized with an 11 % concentration of material, after one day the strength was 21.8 ± 1.1 gf/mm^2, and 8.4 ± 0.4 gf/mm^2 after 14 days. The chitosan membrane neutralized with a 5 % concentration of material showed the smallest reduction in tensile strength, even when the immersion time became longer (fig.5). The elongation of the chitosan membrane neutralized with a 5 % concentration of material was 23.1 ± 2.3 % after one day of soaking 30.6 ± 3.0 % after 14 days, and 22.6 ± 0.7 % after 28 days. The elongation of the membrane neutralized with an 8 % concentration of material was 52.7 ± 3.78 after one day of soaking but could not be obtained after 14 days. Meanwhile, the elongation of the membrane neutralized with 11 % concentration of material was 45.5+/--4.6 % after one day of soaking 26.1 ± 2.1 % after 14 days, but could not be determined after 28 days. The elongation results of the membrane neutralized with 8 % concentration of material were the langest. Moreover, the reduction in elongation was the smallest for the membrane neutralized with the 5 % concentration of material (Fig. 6).

Discussion

The effect of the concentration of sodium polyphosphate solution for neutralization on chitosan films prepared by dissolving chitosan with malonic acid was investigated.

When the chitosan sol dissolved in malonic acidwas neutralized with 2 % concentration of sodium polyphosphate, the membrane could not be obtained. This result was considered likely to happen as the pH value of the malonic acid was low, at 2.8, while the malic acid's pH value was 3.4 and the pH value of the sodium polyphosphate was about 8.5. [6] A low value possibly exerts an influence on the condition of the membrane preparation.

The changes in pH value of the neutralizing materials after neutralization were monitored. The pH of the solution prepared by dissolving chitosan sol with malic acid and neutralizing it with 2 % material after 24 hours was 5.3, while that of the solution in which chitosan sol was dissolved with malonic acid was 5.0. The value was higher than 6.67 for the solution prepared by dissolving chitosan sol with malonic acid and neutralizing it. Accordingly, it was considered that the membrane could be prepared as long as the pH value of neutralizing

material was higher than 5.3, using the processing method of neutralizing the chitosan sol that was prepared by dissolving chitosan with malonic acid.

The tensile strength results of the film prepared by using chitosan sol dissolved with malic acid tended to be larger as the concentration of sodium polyphosphate became higher. The strength after 14 days of soaking could not be measured for the membranes which had concentrations of sodium polyphosphate at 2 and 5 %. Therefore, it was considered that the concentration should be more than 8 % for the purpose of maintaining a certain strength on vivo for a lengthy period of time. Meanwhile, the elongation of the membranes which were neutralized with concentrations of 8 or 11 % sodium polyphosphate after one day of soaking did not show a great difference.

The tensile strength of the chitosan membranes prepared by dissolving chitosan with malonic acid and neutralizing the sol with sodium polyphosphate at 4 different concentrations, did not step up in proportion to the concentration of the neutralizing materials, contrary to the membranes prepared by using malic acid as the neutralizing material. The strength of the membrane neutralized at the concentration of 5 % tended to be greater at 14 days of soaking than that at one and 28 days of soaking. The tensile strength after one day of immersion did not show any difference between the neutralizing solutions of 5 % and 8 %, but was less for the 11 % concentration.

The elongation was the least for the membrane neutralized with 5 % material after one day of soaking followed by that neutralized with the 11 % concentration. The elongation results of the memebrane neutralized with 8 % material tended to be the greatest. In the future study, we would like to review the response in vivo.

Conclusions

Chitosan is a material which is decomposed and absorbed in vivo. Chitosan sol is made by dissolving chitosan with malic or malonic acid. The chitosan sol is then kneaded with HAP and neutralized with sodium polyphosphate. The chitosan membrane is then completed by gelling the sol. In the present study, we discussed how various concentrations of sodium polyphosphate used as a neutralizing agent affect the tensile strength and elongation of chitosan membrane.

Our conclusion are the follows:

At a 2 % concentration of sodium polyphosphate, membrane could not be prepared by using the chitosan sol dissolved with malonic acid.

The pH value of the neutralized membrane became smaller as the concentration of sodium polyphosphate became lower.

The tensile strength and elongation results of the membrane became greater when the concentration of sodium polyphosphate which neutralized the chitosan sol dissolved with malic acid was higher.

The tensile strength and elongation of the membrane prepared by neutralizing the chitosan sol dissolved with malic acid reduced as the soaking time became longer.

No relationshipwas obtained between the tensile strength and elongation of the membrane, when prepared by neutralizing the chitosan sol dissolved with malonic acid, and the concentration of the neutralizing material.

The strength in the physiological saline was maintained when the membrane was prepared by neutralizing the chitosan sol dissolved with malonic acid by sodium polyphosphate at a low concentration. Meanwhile, the chitosan dissolved with malic acid maintained its strength when the membrane was neutralized with the material at a high concentration.

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Preliminary Investigation of the Compatibility of a Chitosan-Based Peritoneal Dialysis Solution

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Summary

A preliminary in vivo study was performed to test a chitosan-based dialysis solution aimed at improving peritoneal membrane function and avoid the side-effects of glucose. Intraperitoneal toxicity test and morphostructural evaluation of the peritoneum were performed in 5 rats up to 72 hours after intraperitoneal injection of normal saline (control animals) or of a solution containing partially hydrolysed modified chitosan (experimental animals). A peritoneal biopsy was performed for morphological analysis 72 hours after the injection. The chitosan-based solution did not determine toxic effects and abdominal inflammatory or fibrotic reactions were not observed.

Introduction

In peritoneal dialysis (PD), osmosis is the physical principle utilised for the transport of water and solutes through the peritoneal membrane.
The most important advantage of PD over haemodialysis is that it obviates the need for machines and synthetic filters [1]. At present, PD is performed on paediatric or aged patients, in cases where vascular accesses are not available, and on patients with haemocoagulative disorders. However, patients on haemodyalisis for renal failure requiring hospitalisation may be admitted to wards that are not equipped for renal-replacement therapy. For example, renal osteodystrophy is responsible for orthopaedic admissions due to: pathological fractures [2], spontaneous tendon ruptures [3] and others [4]. Those requiring surgical [5,6] or orthopaedic treatment or rehabilitation and must later be transferred to wards equipped for renal-replacement therapy. Acute renal failure can sometimes develop in multiple-trauma patients [7,8] or after rhabdomyolysis [9] and intraoperative myonecrosis [10]. In all these cases, PD offers considerable advantages.

Glucose is the most frequently used osmotic agent in PD solutions in spite of the fact that its excessive absorption may lead to obesity, hyperinsulinaemia and hyperlipidaemia; dextrose is also widely used [11]. However, the exposure of the peritoneum to hypertonic solutions may induce several side-effects on mesothelial cells, such as local inflammation, fibrosis, hyperplasia, toxicity and apoptosis [12-15].

For these reasons, a variety of agents (albumin, glycerol, amino acids, glucose polymers, polyanions) have been proposed as alternative osmotic agents for peritoneal-dialysis solutions [16-18]. Among them chitosan, already utilised for various medical applications, has

been the subject of a study of the dialytic treatment of patients with renal failure [19]. Chitosan could be used either as a component of the ultrafiltration liquid for PD or as an element of the ultrafiltration haemodyalisis membrane. Chitosan-polyethylene-oxide blend membranes have recently been found to improve the permeability of toxic metabolites and to reduce the thrombogenicity of haemodialysis [20]. Furthermore, chitosan membranes modified by polyelectrolyte complex formation with dextran sulphate seemed to improve blood compatibility in haemodialysis. Surface modifications with anionic polysaccharides prevent complement activation, and platelet adhesion and activation [21]. Modified chitosans may also be suitable for peritoneal dialysis owing to: 1) their high water-adsorbance capacity; 2) the absence of toxicity when administered orally, topically or intravenously; 3) their full compatibility with inorganic and organic ions found in commercial dialysis solutions; 4) their reparative action on wounded tissue [22] and 5) their bacteriostatic/bactericidal effect [23].

The peritoneal corion is a tissue whose cellular kinetics shows distinctive characteristics with regard to senescence processes. Senescence has common features in different cell lineages, but its rate of progresssion differs markedly among them. In cultures of human fibroblasts, senescence is due to a proportion of the cells becoming senescent at each passage, rather than to all cells entering senescence simultaneously at the and of their life-span. Mesothelial cells enter senescence significantly faster than fibroblasts [24]. One reason for the utilisation of chitosan in the dialytic liquid is its angiogenetic activity [25], which could improve the vascularisation of the *tunica propria* of the peritoneum. This is an important factor, since peritoneal fibrosis and senescence cause the loss of dialytic function and are closely related to the proliferation of peritoneal fibroblasts and the deposition of extracellular matrix.

In this study, a morphostructural evaluation of the biocompatiblity of a solution containing partially hydrolysed chitosans for peritoneal dialysis was performed in an experimental model.

Materials and Methods

Chitosan was depolymerised with sodium nitrite [26] and then 30 g (degree of acetylation 0.27] was suspended in water (270 ml) and glacial acetic acid (30 g). After 30 min $NaNO_2$ (26 ml, 10%) was added. After 4 hours of continuous stirring, NH_4OH (130 ml, 10%) was added to reach pH 7.6, together with $NaBH_4$ (4g, 5%). Overnight the pH was brought to 7.2 with HCl (6 ml, 6N) and the solution was filtered. The low-molecular weight (MW) chitosan was insolubilised with CH_3OH and filtered under vacuum. Wet chitosan was dissolved again in HCl (1 ml, 0.5 M) and brought to 100 ml. Its concentration (5.6 g/100ml) was determined by freeze-drying 10 ml. The following solution was prepared: low-MW chitosan 3.86 g; NaCl 0.583 g; $CaCl_2$ 0.025 g; $MgCl_2$ 0.005 g; sodium lactate 0.448 g; water to 100 g. This partially depolymerised chitosan did not appreciably increase the viscosity of the solution.

Five adult male Wistar rats (500±50 g b/w) were divided into a control group of 2 and an experimental group of 3 animals. They were stabled in a metal cage, fed a standard pellet diet and water *ad libitum*. Twenty ml of sterile normal saline or of sterile hyperosmotic chitosan solution were administered intraperitoneally (i.p.) to the control and the experimental group, respectively. Animals were observed immediately after the injection and 4, 24 and 72 hours later for symptoms of slight, moderate or marked toxicity, or for death. Observations were recorded together with the body weights of all animals at the same times (ISO 10993-11). A peritoneal biopsy was also performed 72 hours after the injection under general

anaesthesia (87 mg/kg ketamine and 2 mg/kg xylazine) for morphological analysis.

For light microscopy, specimens were fixed in 4% buffered formalin, embedded in paraffin and cut in sections 5-6 microns thick which were then stained with haematoxylin-eosin and Masson and Gomori's thrichromic stain.

Results

During the 72-hour observation period, none of the experimental animals showed biological reactions differing from those of controls. All animals gained 20 g immediately after the injection and no significant differences were observed between the two groups. At laparotomy no liquids, abdominal adhesions or peritoneal fibrosis were observed in either group.

In the longitudinal sections that make up the peritoneal lamina with epithelial and corionic components, the peritoneum appeared regularly structured. The squamous epithelium was continuous and showed well-aligned cells. Numerous small vessels were homogeneously distributed in the underlying corion, without anomalies in the walls (Fig. 1).
The physiological compatibility of chitosan upon injection was demonstrated by the absence of immunological infiltration (Fig. 2).

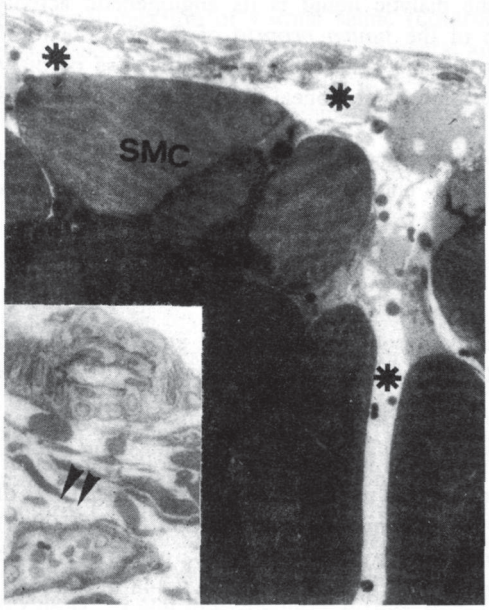

Figure 1. Semithin section of peritoneal barrier after physiological-solution injection (SMC = smooth muscular cell). Intracellular spaces are moderately enlarged (**). Inset: Increase of intercellular spaces in chitosan-treated peritoneum (mesenchymal cells ▼▼)

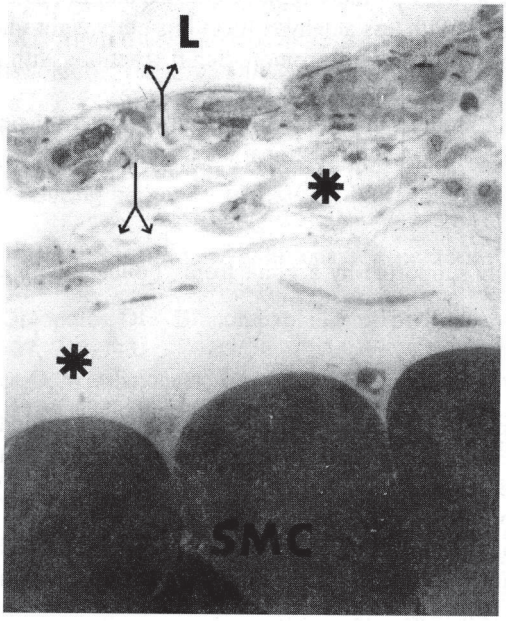

Figure 2. Semithin section of the peritoneal barrier after chitosan-solution injection (↙ ↘ barrier transit). Intercellular spaces (**) are enlarged but cellular morphology is correct (L = lumen, SMC= smooth muscular cell).

Discussion

Peritoneal inflammation, a common complication of abdominal surgery and peritoneal dialysis, results in intraperitoneal adhesions with fibroblast proliferation and formation of fibrovascular adhesions, which are the main causes of intestinal obstruction [27]. In human peritoneal mesothelial cells, the lack of induction of antioxidant enzymes by inflammatory cytokines and the high superoxide dismutase activity, accompanied by insufficient glutathione peroxidase and catalase activities, may contribute to the susceptibility of these cells to oxidative damage [28].

In the light of these considerations, and given the tendency of mesothelial cells to undergo senescence precociously [24], the use of chitosan in the dialysis liquid represents a strategically important choice not only in terms of the qualitative improvement of the dialytic process, but also because of the trophic effect that chitosan exerts on tissues of mesenchymal origin, i.e. favouring angiogenesis and exhibiting the capability to link growth factors, for example FGF [29]. Chitosan-based dialysis solutions have been administered to rats with no apparent effects. Chitosan exerted its known tissue-repair action, at the same time avoiding repeated traumatisms to the peritoneum and limiting the subsequent inflammatory response.

While the biocompatibility of conventional PD solutions is debated [30,31], the

chitosan-based solution used in this study showed good biocompatibility. Low-molecular-weight chitosan could at least partially replace glucose and act as a protective compound for the peritoneal membrane. With less aldehyde functions per weight unit, chitosan is less reactive than glucose and is thus less likely to promote Schiff reactions with tissue proteins. Due to its polymeric nature it is also less rapidly adsorbed.

Our group in a model simulating continuous ambulatory peritoneal dialysis [32] will further study the morphological and functional effects of chitosan.

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Chitosan influence on the growth of several cellular lines.

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Summary

Several cell lines were growth over chitosan films (12.5 μm thickness) treated with different chemicals. Assays were performed in order to follow up the time course of different parameters (Protein content, DNA content and phosphatase activity). It is showed that cellular growth depends on the chemical treatment of the film and also on the kind of cell line chosen.

Introduction

Chitosan is a naturally occurred biopolymer with wide industrial applications. Its mechanical properties, along with its biocompatibility and biodegradability allows its successful use on Medical applications. It is also well documented its osteoinductive properties, playing a stimulating effect on the process involved in the bone defect repair [1,2]. Its ability to form film make it an attractive biomaterial to covering prosthesis. Nevertheless there is only few studies in the Bibliography on the effect of chitosan itself on the proliferation and cellular survival [3-6], being focused almost exclusively on cellular growth in chitosan blended hydrogels.

In the present study we analyze the effect produced by chitosan on the growth characteristics of several established cellular lines from different sources when they are cultured on chitosan films.

We have choose among the different cell lines those that are more frequently used in the assay of osteoinductive properties for different chemical compounds (C3H10T1/2, clone 8, from mouse embryo; C2C12, muscle myoblast from mouse, and ROS17/2.8 from rat osteosarcome), and one related with the recommended for the *in vitro* citotoxicity assays by the EN-ISO (Swiss 3T3, fibroblastic, from mouse embryo) and we have studied the influence of the chitosan film on the viability, adherence and cellular proliferation.

We have used chitosan obtained from prawn (*Pleoticus müllari*), with a deacetylation degree of 84% determined by N.M.R. and the second derivative method. It has been used different chemical approaches in order to stabilize the film obtained, comparing the results obtained for the parameters which characterize the cellular proliferation in these conditions with those obtained using commercially available multiwell plates.

Materials and methods

Chitosan films: Chitosan was obtained from prawn shells (*Pleoticus Müllari)* by heterogeneous hydrolysis. The deacetylation degree was 84% and the M.W 223.872. 200 µl of a 1% chitosan solution in acetic acid previously filtered through at sterile 0.22 µm filter was layered over each plate well (surface 1cm^2) . Solvent was allowed to evaporate in the open plate in a sterile laminar flow hood overnight at room temperature. The thickness of the film obtained was 12.5 µm. Previously to the cells seed, films were treated in several ways in order to stabilize them. Treatment A: buffer phosphate 0.25M pH 7.0; Treatment B: Sodium hydroxide 0.5 M; Treatment C: a solution of sodium hydroxide (0.5M) and glutaraldehyde (0.0625%) and treatment D: Glutaraldehyde 0.0625%. 400 µl of each solution was layered over the wells and allowed to stand over 30 minutes. After this time the medium was removed and extensively washed with PBS.

Cell culture: C2C12 and C3H10T1/2 cell lines were obtained from the American Type Culture Collection (Manassas, Virginia). ROS 17/2.8 and Swiss 3T3 cell lines were obtained from the Hospital La Princesa (Madrid, Spain) and the Centro de Investigaciones Biológicas (Madrid, Spain), respectively. All the cell lines were maintained on 25 cm2 flasks with DMEM containing 10% Fetal Bovine Serum plus antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin sulfate). Previously to the experiment the cells were grown in 175 cm^2 flask in order to obtain enough cell quantity for all the plates.

Cell experimental cultures were achieved on 48-well plates with chitosan films previously formed. Trypsinized cells were seeded in the growing medium previously described at 4000, 8000 and 16000 cells/cm^2.

Protein, viability and DNA content assays: For protein assays the culture medium from 48-well plate was removed and wells carefully washed once with Phosphate Buffer Saline. 200 µl of Bradford reagent and 800 µl of distilled water were added, mixed and incubated at room temperature for 30 minutes. Colorimetric measurements were done at 590 nm in a Biotek FL-600 Microplate Fluorescence Reader.

The viability assay was performed trough the measurement of phosphatase activity of the intact cell with Calcein AM. The DNA content was assayed with Etidium homodimer. Calcein AM and Etidium homodimer assays were performed using the Live/Dead kit from Molecular Probes. Briefly described, medium from 48-well plate cultures was removed and 100 µl of a mixture of 2 µM calcein AM and 8 µM Etidium homodimer in PBS was added. Plates were stored one hour in the dark and measurement was done at 530 nm after exciting at 490 nm. After that time cells were killed upon addition of methanol until a 70% final concentration, and after 30 more minutes fluorescence measurements were done at 645 nm (λ_{exc} 530 nm).

Controls were performed doing measurements over the same cells line seeded at the same initial density over untreated 48-well plates and also over formed films without cells.

All assays were performed every 24 hours.

Results and discussion

Table 1 summarizes the results obtained in the measurements of the total protein content, DNA content and phosphatase activity at day 1 and 5, for the four cell lines employed seeded at a initial density of 16000 cells/cm^2. All the determinations were performed in triplicate. Figure 1 shows the time evolution of the values obtained for one of the cell lines at the three different initial densities employed in the work.

Table 1. Results obtained for the protein, DNA and viability assays performed at days 1 and 5 after the initial seeding of the different cell lines used in this work.

Cell line	Chitosan treatment	A_{590} (Protein content)		F_{645} (DNA content)		F_{530}(Phosphatase act.)	
		day 1	day 5	day 1	day 5	day 1	day 5
C2C12	A	0.2	0.09	384	716	3386	5819
	B	0.28	0.99	313	1348	3873	9481
	C	0.24	1.02	308	2644	4166	7207
	D	0.37	0.23	18333	9572	6063	4468
ROS	A	0.17	0.42	385	1333	3835	6581
	B	0.18	1.01	389	1546	3816	6844
	C	0.27	1.17	775	2791	5776	7776
	D	0.35	0.22	11788	13087	7419	5079
C3H10T 1/2	A	0.06	0.22	660	1140	3566	3847
	B	0.20	0.33	795	739	3529	6065
	C	0.19	0.89	260	1180	6029	5568
	D	0.33	0.14	9587	4364	8619	9338
Swiss 3T3	A	0.22	0.19	395	603	3989	3624
	B	0.31	0.12	449	736	6239	3918
	C	0.4	0.25	550	814	9123	2899
	D	0.14	0.27	6782	6651	4027	3424

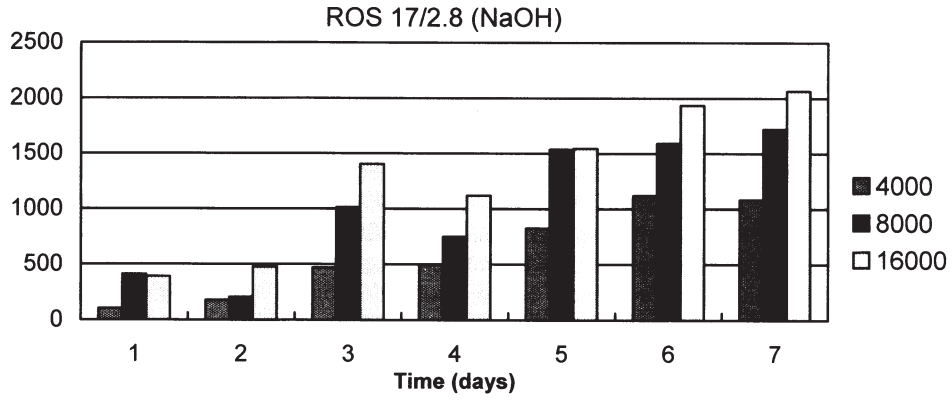

Figure 1. Time evolution of fluorescence intensity at 645 nm for ROS 17/2.8 seeded at 4000, 8000 and 16000 cells/cm^2 over chitosan with treatment B.

The values offered before are only a semiquantitative estimation of the proliferation and viability phenomena that take place within each cell line, standing these values, in our opinion, as a lower limit to the real one. These are very difficult to obtain due to the low cellular adhesion to the chitosan films, compared to the untreated plastic. This is clearly showed in Figure 2, were the A section shows the time evolution for the A_{590} (cell line C2C12) performing a change in the growth medium at the fourth day, while in the section B

the same experiment is performed without doing it. There is another additional problem in the case of chitosan treated with glutaraldehyde (Treatment D), due to the very high background of these samples. These data show a much higher dispersion that the other samples.

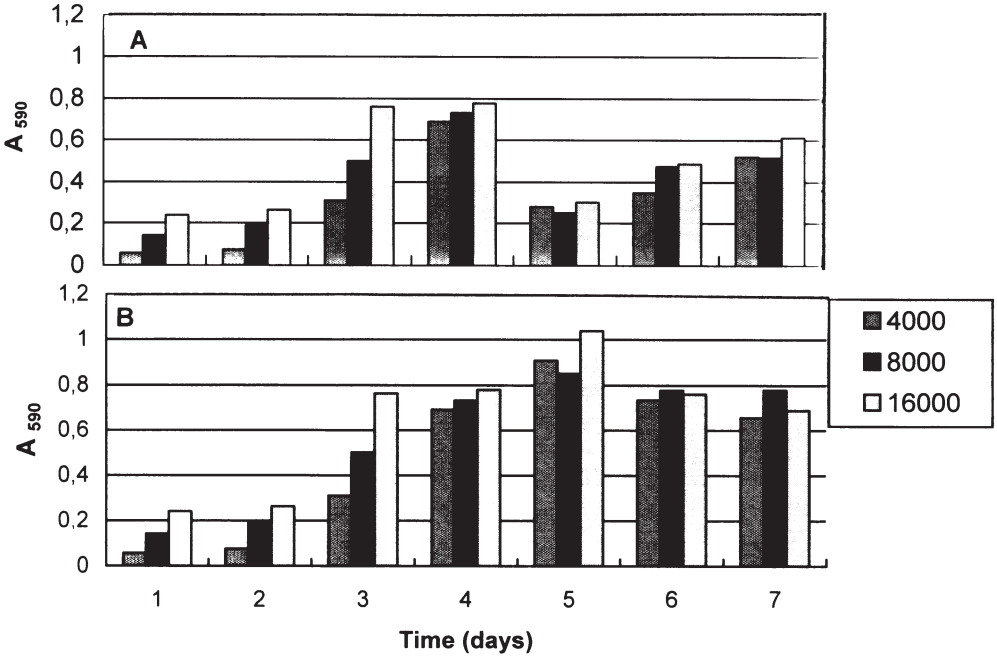

Figure 2. Time course evolution for the A_{590} obtained for C2C12 cell line with (A) or without (B) change of the growth medium at the fourth day

However it is possible to draw some general conclusions of the data here offered. The cellular growth is better supported on chitosan films that suffered treatment C (sodium hydroxide and glutaraldehyde mixed) and B (sodium hydroxide alone), but there are also differences between cell lines. The lowest proliferation level is shown by the Swiss 3T3 (fibroblast) while the highest values stand for the ROS (rat osteogenic sarcome) and C2C12 cell lines (mouse muscle myoblast, that can differentiate into osteoblast like cells upon the addition of BMP-2). It is attractive to speculate if this different response of the cellular lines can be related with the well-known chitosan stimulation of the reparation of bone lesions [1,2].

The levels obtained for the different parameters at day 5 are much lower that those showed by the untreated controls. This can be due to several facts. First, the inhibition of cellular proliferation. Another possibility lies in the lowest cellular adhesion to the biomaterial, that can introduce a delay on the onset of the cellular proliferation due to a lower real initial cellular density. Finally, the chitosan films clearly affect the cytoarchitecture of the different cell lines, showing these a wide range of shapes depending on the film treatment.

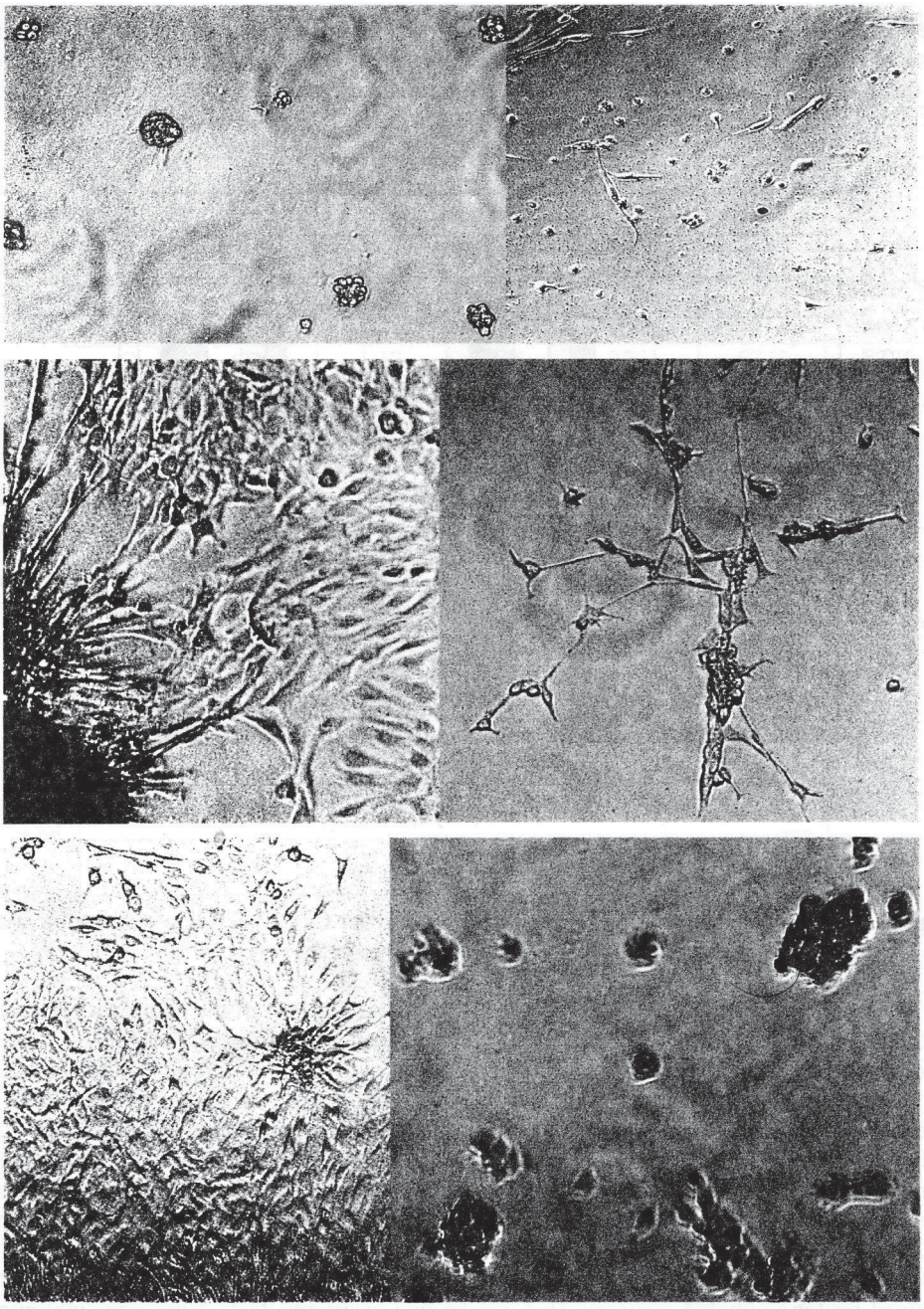

Figure 3. Microphotographs (x10) taken directly over chitosan cultured CC12 cell . Upper section: Treatment A (Left) and treatment D (Right). Middle section: Treatment C. Lower section: Treatment B (ROS cells in this case).

This last fact can be seen in the Figure 3, where it is showed microphotographs (10x) obtained directly from the multiwell plates before performing the assays over them. It can be observed several cellular forms, depending on the chitosan film treatment. Namely, rounded cells when the film has been treated with Phosphate or glutaraldehyde; spindle form cells on chitosan with the C treatment, alterning in this case with some strange formations of grouped cells, flower shaped and from where they spread over the film in a monolayer like form. Finally, in those sodium treated films there is small groups of rounded cells, normally in the center of the well, and typical cell monolayer formations. The high number of cells present in those rounded formations can explain the sensitivity of the assays to the washing step, because in those groups there are only few cells directly attached to the film.

All these cellular shapes should have its counterpart in a different cellular metabolism that can account also for the slower growth rate in respect of the normal conditions.

Anyway the data here presented show the chitosan film ability to support cellular growth over it, being the sodium hydroxide and the mixture of sodium hydroxide and glutaraldehyde the best treatments in order to achieve maximum cell growth, being also these treatments the ones that better preserve the morphological characteristics of the cells. Perhaps this fact is due to the neutralization carried out by the basic medium, that diminishes the number of NH_3^+ groups presents initially in the film.

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A new chitosan containing phosphonic group with chelating properties

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Summary
A novel water-soluble chitosan derivative carrying phosphonic groups, the N-methylene phosphonic chitosan (NMPC) was briefly described.The structure was partly N-monophosphonomethylated (0.24) and N,N-diphosphonomethylated (0.14) and N-acetylated (0.16) without modification of the initial degree of acetylation.

Taking advantage of the knew chelating ability of the phosphonic groups, specially for calcium, this was verify for NMPC. Thus, the results obtained by atomic absorption spectrometry and elemental analysis gave Ca(II) 0.89 % in the complex NMPC-Ca. The NMPC-Ca complex was characterized by IR and X-ray.

This new derivative opens interesting perspectives in front of the posibility to quelate others bivalents metals such as Cu (II), Cd (II), Zn (II) etc and additionally, with potential biomedical applications.

Introduction

Chitosan and many of its derivatives have chelating ability towards transition metals ions but do not exhibit the same ability for alkali metals ions[1]; for instance, chitosans carrying carboxyl groups might chelate calcium [2][3][4].

Since the report by Schwarzenbach et al.[5] of the high affinity of the phosphonic anion for the Ca(II) ion , it appeared that the phosphonate group might be as effective or even more than the carboxylic group in contributing to the chelating tendencies of polyfunctional amino acids.

The presence of an amine group such as the case of chitosan into the molecule to obtain NH_2-CH_2-PO_3^{2-} combines its strong donor effect with a monodentate ligand as -PO_3^{2-}; increasing the metal-binding abilities [6][7].The stability constant values for different cations reported by Westerback et al.[7], Bassegio and Grassi [8] and Grassi et al.[9] show a high affinity of phosphonic acids for bivalent metal cations. These aminoalkylphosphonic ligands have a tendency to form ring structures with metal ions resulting in different spatial conformations [10].

Chelating properties of the phosphonic group in rigid and aromatic systems had been described, Acebal et al [11].

The purpose of the present report is to describe a new derivative: N-methylene phosphonic chitosan (NMPC), as well as the chelating properties of Ca(II) ion regarding its potential biomedical applications.

Materials and Methods

X-ray diffraction spectrometry : The material in the powder form was submitted to X-ray diffraction spectrometry by using a vertical powder diffractometer ; the source was a rotating anode generator Rigaku Denki RU-300 and Ni filtered Cu K_α radiation (λ=0.154 nm).

NMR spectroscopy: ^{13}C and ^{1}H NMR measurements were performed on a AMX500 Brukker NMR spectrometer under a static magnetic field of 125 MHZ and 500.13 MHZ respectily at 70°C. For those measurements, 10 mg of sample was introduced into a 5mm ϕ NMR test tube, to which 0.5 mL of 2 % (w/w) DCl/D_2O solution was added, and finally the tube was kept at 70°C to dissolve the polymer in solution.

Solubility test: The sample (10 mg) and 5 mL of solvent were placed in a test tube and stored at 20 °C for 7 days.

Film casting: NMPC powder (1.0 g) was dissolved in 100 mL of water. Films could be cast by evaporation from 10 mL of this solution in polystyrene Petri dishes at 37°C (overnight). The thickness of the film was measured using a micrometer.

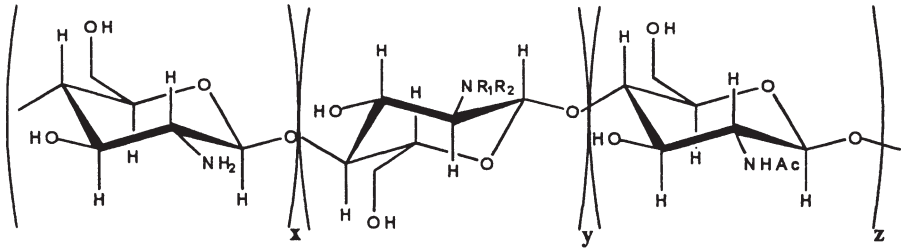
IR spectroscopy: NMPC and NMPC-Ca powders samples have been obtained between 800 - 4000 cm^{-1} on a Nicolet 20-SX FT-IR spectrometer (DTGS detector) equipped with a Spectra Tech. Multiple Internal Reflectance (DRIFT) accessory and it was filled with a mixture of a small amount of sample grounded with anhydrous KBr.

Scanning electron microscopy: A Philips SEM 505 scanning electron microscope was used to characterize the surface of NMPC and NMPC-Ca particles. The samples were prepared by gold coating.

Atomic absorption spectrophotometry: The inorganic elements were determined using a Sequential Plasma Spectrometer Shimadzu ICPS 1000 III.

Results and Discussion

The introduction of phosphonic acid function in the chitosan macromolecule via the Moedritzer and Irani [12] reaction modified by us with phosphorous acid and formaldehyde yielded a derivative (Fig. 1) with different properties from chitosan [13].



$R_1 = H$, $R_2 = CH_2PO_3H_2$

$R_1 = R_2 = CH_2\text{-}PO_3H_2$

Figure 1. Chemical structure of N-methylene phosphonic chitosan

The two forms (I and II) was estimated to be 64 % and 36 % respectively and the structures were distinguishable by ^{1}H NMR spectroscopy; confirmed by ^{13}C NMR spectrum and mainly solved using $^{1}H - ^{13}C$ correlations.

Chitosan-NH-CH_2-PO_3H_2 (I)

Chitosan- N (-CH_2-PO_3H_2)$_2$ (II)

In addition to these results, elemental analysis made on chitosan derivative gave an indication that the degree of acetylation was 0.16, the degree of substitution was 0.38 (N-monosubstitution 0.24 and N,N-disubstitution 0.14), the rest of the amino groups being in the free form (0.37) [13].

In order to verify the most suitable conditions for the formation of NMPC-Ca complex some preparations was carried out:

(A): 2.7 g NMPC + 0.29 g Ca(II), the latter corresponding to $Ca(CH_3COO)_2$ (70mL, 2% w/v)
(B): 3.0 g NMPC + 0.66 g Ca(II), the latter corresponding to 3.2 g $Ca(CH_3COO)_2$
(C): 3.0 g NMPC + 0.99 g Ca(II), the latter corresponding to 4.8 g $Ca(CH_3COO)_2$

The elements were determined by atomic absorption spectrometry and the capacity to quelate Ca(II) for the polymer in terms of g metal per 100 g polymer were calculated. The results can be summarized as follows:

(A): Ca %, 0.018; P%, 3.03
(B): Ca %, 0.62; P%, 3.35
(C): Ca %, 0.89; P%, 3.53

These results were confirmed by the elemental analysis (%) and were as follows:
Chitosan: C, 39.48; O, 46.31; H, 6.93; N, 7.29; N/C = 0.185
NMPC: C, 32,66 ; O, 49.81; H, 6.79 ; N, 5.42 ; P, 5.3 N/C = 0.166
(B): C, 37.37 ; O, 47.31; H, 7.02 ; N, 5.44 ; P, 2.75; Ca, 0.55 N/C = 0.145
(C): C, 37.20 ; O, 46.81; H, 6.95 ; N, 5.37 ; P, 2.85; Ca, 0.82 N/C = 0.144

The chemical derivatization introduce carbon, oxygen and phosphorous into the polysaccharide with a resulting N/C ratio for NMPC of 0.166 below the N/C ratio for chitosan (0.185). In the complex NMPC-Ca the N/C ratio dropped to 0.144. The sum N+C+H for NMPC 45 % while for chitosan it was 54%.

The chitosan derivative and the NMPC-Ca complex show a big solubility with respect to chitosan, specially in aqueous media over an extended pH range.

In the spongy freeze-dried NMPC, the tendency to form films is quite evident when the material is examined at the electron microscope. It shows a relatively homogeneous aspect with a tightly packed structure (Fig. 2). The films obtained were translucent, brillant and mechanically resistent and had homogeneous aspect. On the contrary, the NMPC-Ca complex (Fig. 3) does not keep the filmogenic properties of the parent NMPC. The material in the dry state is fragile and can be crushed easily.

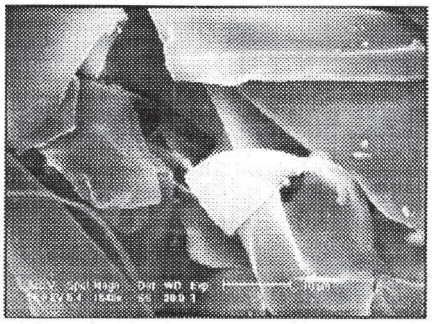


Figure 2. Electron micrograph for NMPC (x 198)

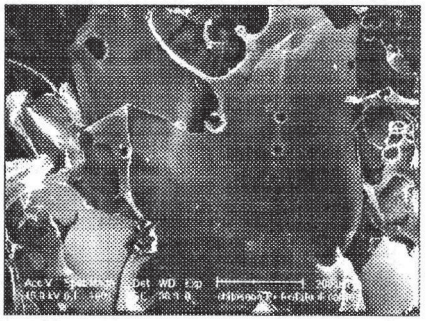


Figure 3. Electron micrograph for NMPC-Ca (x 100)

At the X-ray analysis, the water-soluble derivative (Fig. 4) and the NMPC-Ca complex (Fig.5) were amorphous; most of the diffraction bands were depressed or absent.

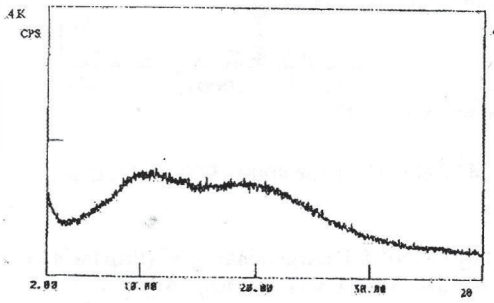


Figure 4. X-ray diffraction spectrum of NMPC

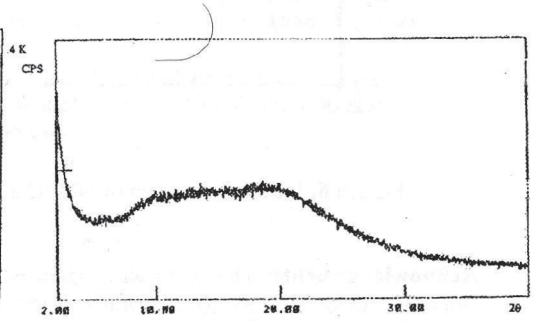


Figure 5. X-ray diffraction spectrum of NMPC-Ca

The FTIR spectra of NMPC and NMPC-Ca show the characteristic peaks at 2500 - 3500 cm^{-1} (P- OH), 1141 cm^{-1} (P=O), 1065 cm^{-1} (P-OH) and 904 cm^{-1} (P-O). The infrared spectra of the two compounds, between 2000-800 cm^{-1} show the running of the amine bands at 1558 and at 1654 cm^{-1} and the increased intensity of the characteristic absorption at 1060 cm^{-1} in the Ca-complex in contrast with the values of NMPC (Figure 6) .

The results obtained show the capacity of the derivative NMPC to chelate calcium and opens new perspectives as biomedical material.

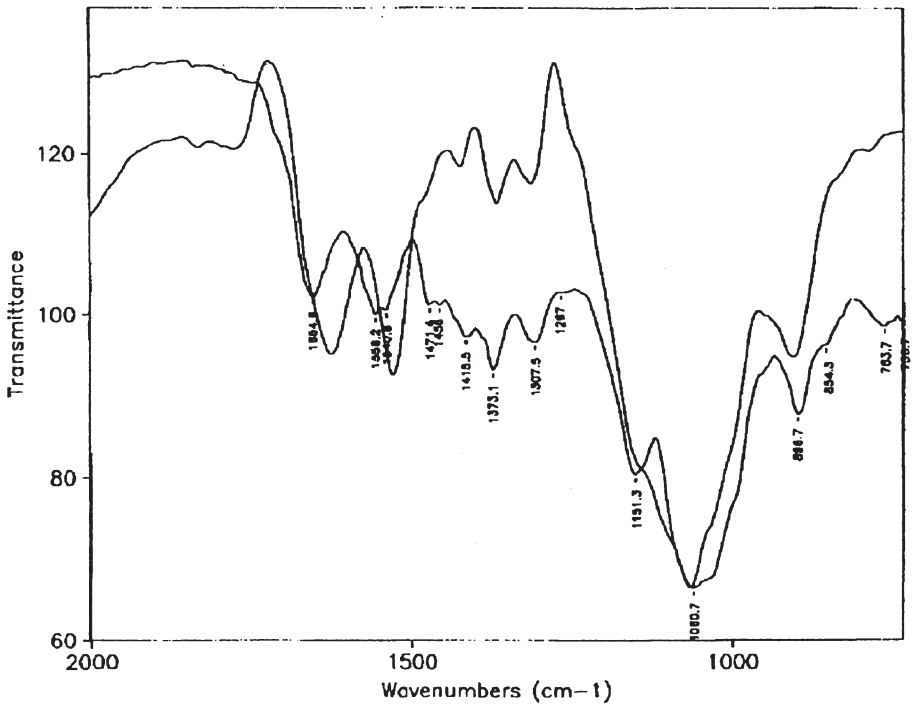


Figure 6. Infrared spectrum of NMPC and NMPC-Ca in the 4000 - 900 cm^{-1} region.

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Biocompatibility of chitin materials using cell culture method

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Summary
Chitin and its derivatives have various biomedical applications such as acceleration of wound healing[1] and as carrier in drug delivery systems[2]. Therefore the absence of cytotoxicity activity is crucial for ultimate clinical applications in humans. Chitin beads and several chitin derivatives were evaluated *in vitro* for their cytotoxic potential. Mouse L929 fibroblast (ATCC CCL 1) and human IMR-90 fibroblast (ATCC CCL 186) cell lines were used for this acute cytotoxic test. The effects of these chitin materials on the proliferation of these cells were quantitiated using Tetrazolium-based colorimetric assay (MTT) [3]. This method is based on the cleavage of a yellow tetrazolium salt to purple formazan crystals by mitochondrial enzymes of metabolically active cells. The results reported in this study were compared with various recommended reference controls.

Introduction

Chitin and its derivatives have wide potential in biomedical applications[4,5] such as wound healing and dressings[1], drug delivery[2], anti-cholesterolemic agent, blood anticoagulants, antitumor agents and immunoadjuvants. Therefore it is crucial for the chitin materials to be non-cytotoxic and compatible with the functions of the specific cells in close contact with.

Biocompatibility has been defined as "the ability of a biomaterial to perform with an appropriate host response in a specific application"[6]. It consists of two components i.e. cytotoxicity and cytocompatibility. Evaluation of cytotoxicity involves acute cytotoxicity tests while further tests on specific cell functions are required to confirm cytocompatibility. Fibroblastic cell lines can be and are commonly used to assess functions common to all cells i.e. membrane integrity, adhesion to surfaces, replication etc in the cytotoxicity phase.

Viability of cells can be quantified using the Tetrazolium-based rapid colorimetric assay (MTT) in which the mitochondria of metabolically active cells will cleave the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals. This MTT assay was first developed by Mosmann[7] to detect cell growth and cell death; it was subsequently applied to the detection of lymphotoxin[8], growth factors[9], interleukins[10] and interferons. The MTT assay has also been adopted for the *in-vitro* evaluation of biomaterial cytotoxicity[11,12,13], apart from the neutral red vital stain uptake and trypan blue exclusion test.

This work was carried out to determine the impact of chemical modifications and purification on the biocompatibility of chitin and chitosan materials from various sources with fibroblastic cell line CCL 1 from mouse and CCL 186 from human.

Materials and Methods

Samples

Fungal chitin was extracted from *Aspergillus niger* 0576, *Aspergillus n*iger 0307 and fungal chitosan from *Gongronella butleri* USDB 0201. Commercial chitin were from Polyscience (Chitin P.(R) =as received, Chitin P.(P) =purified) and Sigma (Chitin S.(R) =as received, Chitin S.(P) =purified). Commercial chitosan was from Kasei (Chitosan (R)=as received, Chitosan (P)=purified). Carboxymethylated chitin beads were B5, B10 and B15; D=6-carboxyethyl chitin, L=6-sulfonylethyl chitin. Poly-L-Lysine (AV. Mol. Wt. 30,000-70,000) and latex rubber were used as the positive controls. Dextran, (clinical grade; Av. Mol. Wt. 60,000-90,000) from Sigma and high-density polyethylene from U.S. Pharmacopeia grade were used as the negative controls.

Materials

3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tetrazolium bromide (MTT) was obtained from BDH. MTT was dissolved in phosphate buffered saline (pH 7.4) at a concentration of 5mg/ml and filtered through 0.22μm filter to remove any blue formazan crystals and stored for no more than two weeks in the dark at 4°C. Dimethyl Sulfoxide (DMSO), GC grade, was obtained from Fluka.

Cell lines

NCTC Clone 929 (ATCC CCL-1) mouse fibroblast and IMR-90 (ATCC CCL-186) human fibroblast cell lines, from American Type Culture Collection (ATCC), were used in this study. Both lines were grown and maintained in Eagle's Minimum Essential Medium (EMEM) with 1.5g/L sodium bicarbonate and Earle's BSS adjusted to contain 2mM L-glutamine, 0.1mM non-essential amino acids, 1.0mM sodium pyruvate and 10% fetal bovine serum, at 37°C in 5% CO_2 atmosphere. All medium supplements were from Gibco.

MTT assay

The MTT assay was carried out with slight modification [3]. 1×10^4 cells were inoculated into 96 well plate in 100μl EMEM medium with 10% fetal bovine serum. Sample was added to each well (eight replicates per sample) and incubated for 24, 48, 72 and 96 hours. After the specified exposure time, 20μl of MTT was added to each well and incubated for 3 hour at 37°C. After the incubation, the medium was removed and the cells were washed very gently with phosphate buffered saline (PBS-pH7.5) to remove any untransformed MTT and residues of samples. Subsequently, 150μl of DMSO was added to each well to dissolve the MTT formazan purple crystals. The dissolution of the purple crystals was hastened by agitation of the solution with suction in and out of the solution using an 8-channel pipette. Absorbance was measured at 540nm wavelength using a microplate spectrophotometer (CERES UV 900C, Bio-Tek Instruments, INC.). Results are reported as percentage of absorbance relative to a control without exposure to any samples. Degree of MTT reduction is proportional to the percentage of control.

Results and Discussion

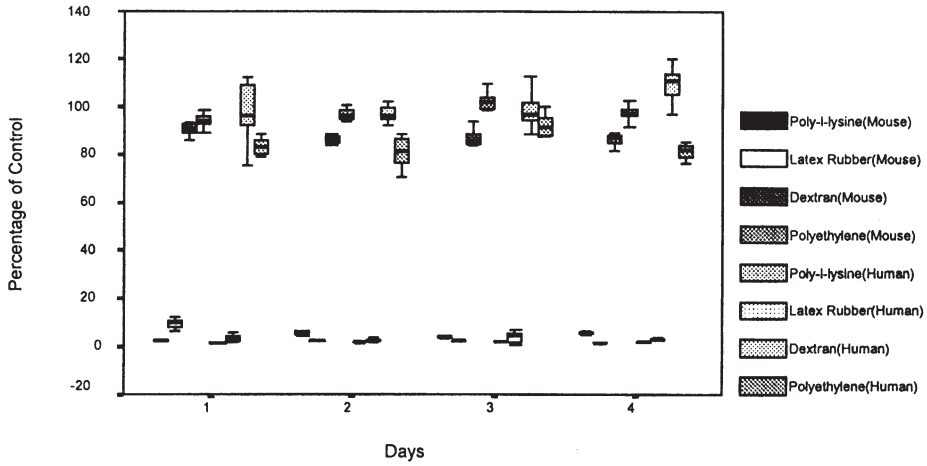

Figure 1: Effect of soluble and insoluble positive and negative controls on MTT reduction by both mouse and human fibroblast cells

Figure 1 shows that both mouse CCL 1 and human CCL 186 fibroblast cells were able to respond to the presence or absence of toxic moieties from either soluble or insoluble positive and negative controls respectively. Hence they were further used as the indicator system in the cytotoxicity evaluation of various chitin materials.

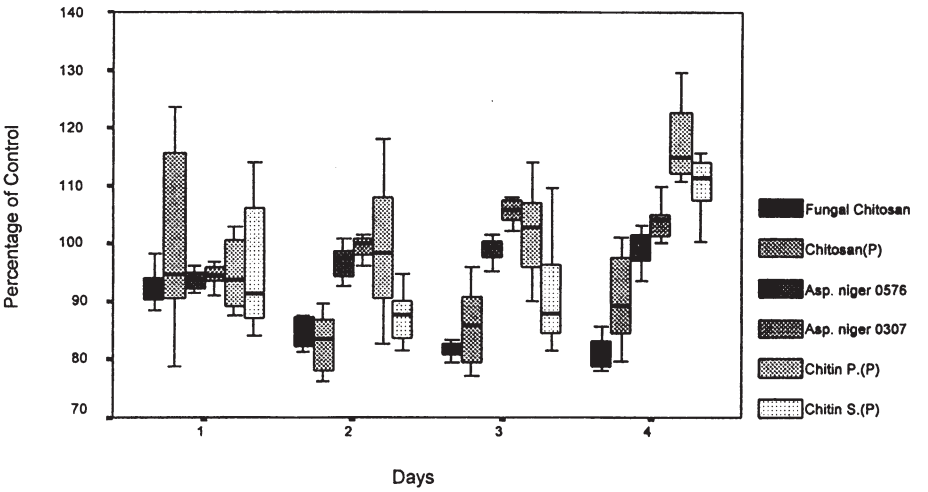

Figure 2: Comparison of MTT reduction by mouse fibroblast cells between commercial and fungal chitin and chitosan

Both chitin and chitosan extracted from fungus were significantly ($P<0.05$) lower in MTT reductions by mouse CCL 1 fibroblast cells compared with purified commercial chitin and chitosan on day 4 (Figure 2). The same trend was observed for human CCL 186 fibroblast cells from day 2 onwards (Figure 3). Human CCL 186 fibroblast cells was thus more sensitive to fungal chitin and chitosan compared to those from commercial sources, which were of crustacean origin. Purified chitin from Polyscience generally had higher MTT reductions by both mouse and human fibroblast cells compared to that from Sigma. Thus for the development of chitin into biomedical useful products, the source of chitin is critical for optimum biocompatibility with the host cells.

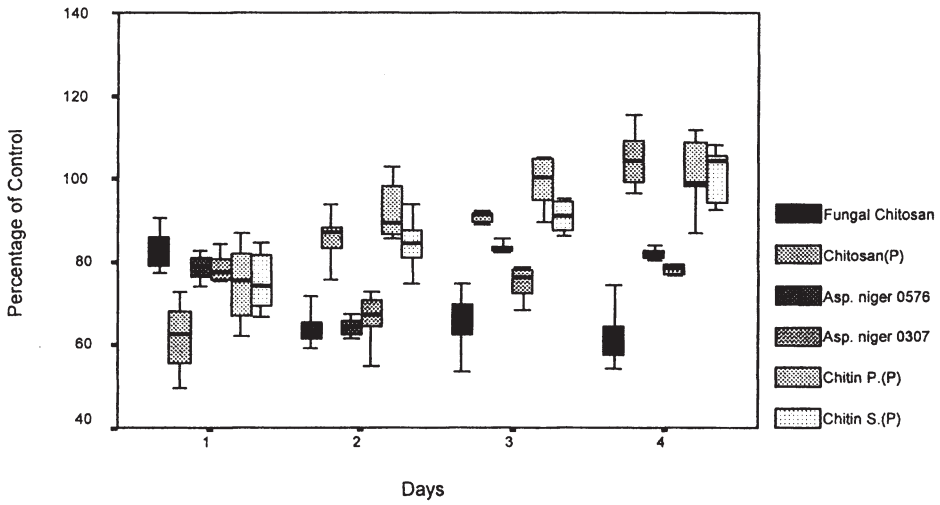

Figure 3: Comparison of MTT reduction by human fibroblast cells between commercial and fungal chitin and chitosan

Generally, chitosan samples were found to have lower MTT reductions compared with chitin samples in both mouse CCL 1 and human CCL 186 fibroblast cells. The polycationic chitosan may bind the serum proteins leaving less protein available for cell proliferation. Alternatively, it may have strong interaction with the negatively charged cell surface resulting in membrane damage and cell death[14].

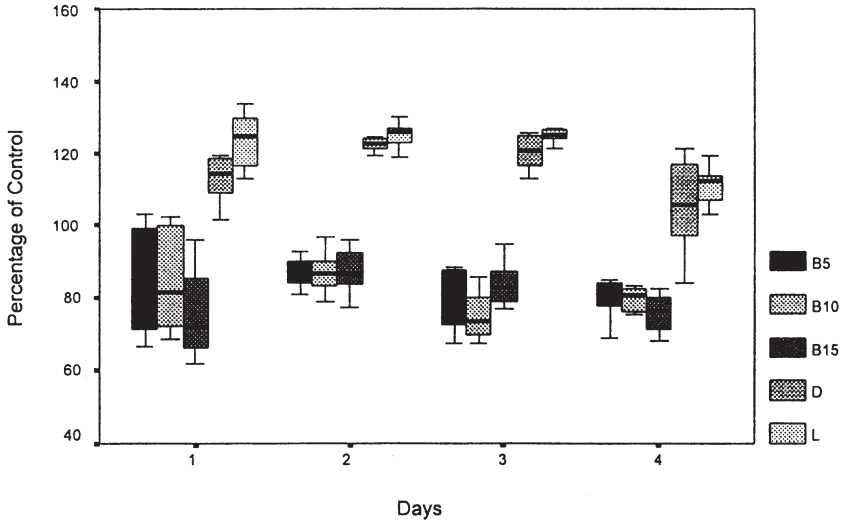

Figure 4: Effect of chitin derivatives on mouse fibroblast CCL 1 cells. B5-B15 are chitin beads with increasing degree of carboxymethylation; D is 6-carboxyethyl chitin and L is 6-sulfonylethyl chitin derivatives.

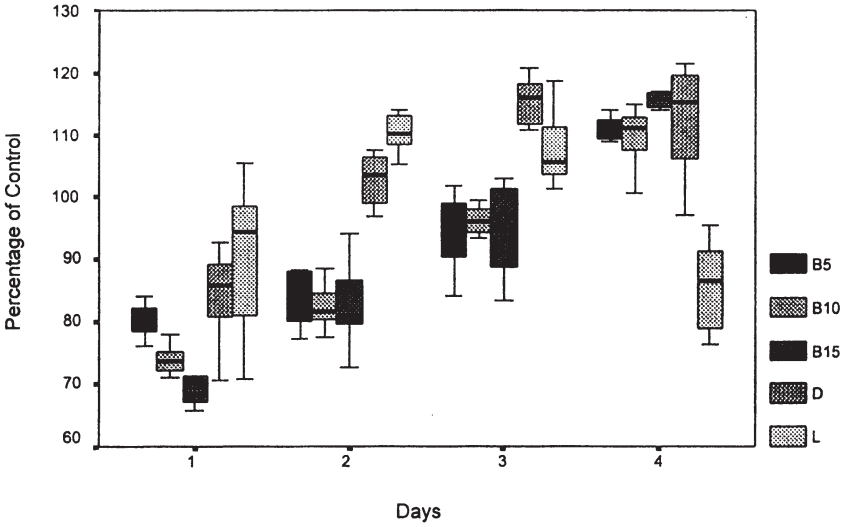

Figure 5: Effect of Chitin derivatives on human fibroblast CCL 186 cells. B5-B15 are chitin beads with increasing degree of carboxymethylation; D is 6-carboxyethyl chitin and L is 6-sulfonylethyl chitin derivatives.

Increased degree of carboxymethylation of chitin beads from B5 to B15 did not significantly reduce the viability of mouse CCL 1 and human CCL 186 fibroblast cells, except on only day 1 for the CCL 186 (Figure 4 and 5). Then 6-carboxyethyl and 6-sulfonylethyl chitin derivatives respectively had higher overall MTT reductions than the carboxymethylated chitin beads in both mouse CCL 1 and human CCL 186 fibroblast cells. Different chemical derivatives of chitin developed for various applications, had different impact on cell viability but were generally not cytotoxic towards mouse CCL 1 and human CCL 186 fibroblast cells.

In conclusion, chitin and its derivatives tested in this study did not affect the growth and surface attachment of fibroblast cells. The cells continue to proliferate even upon the addition of the chitin sample. Cell death was not observed after prolonged (> 24 hours <30days) exposure to the samples. The sources of chitin and chitosan, presence of impurities and specific chemical modifications have to be considered with respect to biocompatibility with host cells. Since chitosan has wide potential in biomedical applications, the mechanisms that cause it to hinder fibroblast cells proliferation should be thoroughly investigated.

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Recent results in the oral administration of chitosan

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Summary

This review takes into consideration the oral administration of chitosan in view of its hypocholesterolemic action, its use in body weight control, the prevention of osteoarthrosis, the delivery of drugs to the colon and the oral vaccination.

Hypocholesterolemic action of chitosan

Cholesterol, the most abundant sterol in mammalian cell, is required for normal cell growth and proper membrane structure and function. The brain contains 10 % of cholesterol (dry weight), most of which is incorporated into myelin. The liver is the key organ in the maintenance of cholesterol omeostasis in the body. Adrenals, gonads and placenta transform cholesterol into hormones.

The most important elimination pathway for water-insoluble cholesterol is the conversion to water-soluble bile acids. The flux of cholesterol and bile acids regulates the activity of the following enzymes:

- cholesterol:acyl coenzyme A transferase (ACAT);
- cholesterol 7-alpha-hydroxylase (a monooxygenase located in the endoplasmic reticulum);
- 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Bile acids are efficient suppressors of the HMG-CoA-R in vivo.

Unregulated accumulation of cholesterol is cytotoxic and failure to maintain omeostasis of the sterol results in a number of pathologies, such as gallstone disease, atherosclerosis, corneal crystalline dystrophy and tumour proliferation. There is therefore much interest in keeping under control the cholesterol level and in limiting its ingestion.

The following three processes promote absorption of the water-insoluble lipids into intestinal epithelial cells (enterocytes):

- hydrolysis of triacylglycerols and phospholipids by pancreatic lipase;
- lipids and hydrolysis products are dispersed in the lumen of the small intestine by bile salts yielding micelles and vesicles, which transport lipids via Brownian diffusion to the surface of the small intestine.
- The lipids are transferred into the brush border membrane of enterocytes, by protein-mediated collision-induced transfer.

The surface and core components of lipid carriers are crucial for absorption of dietary cholesterol in the gastrointestinal tract. Cellular uptake of cholesterol from emulsions with a phospholipid / triacylglycerol molar ratio < 0.3 could be stimulated by pancreatic lipase and colipase hydrolysis of the core neutral lipids. However, the enzyme was ineffective in emulsions with phospholipid / triacylglycerol molar ratio > 0.3.

Chitosan prevents cholesterol absorption by disturbing the finely tuned physical chemistry of the digestive process:

- Sequestering bile acids
- Forming lipid aggregates
- Altering the ratios of various compounds
- Acting as an alternative substrate for lipases
- Precipitating at the intestinal pH value

In general, the chitosan administration is associated to a diet.

Reportedly, chitosan exhibits anticholesterolemic, antiulcer, antiarthritic and antiuricemic properties [1-8]. These properties are related to the capacity to bind bile acids, with consequent reduction of their enterohepatic recycling, phospholipids, and uric acid.

Clinical trials for hypocholesterolemic action

Fat assimilation was reduced by 13 - 25 % by chitosan (glycerol tri[1-^{14}C]oleate test), serum cholesterol and triglycerides were reduced in volunteers from 7.4 to 5.2 mmol/l and 1.6 to 1.3 mmol/l, respectively, over a 5 week period. Weight reduction of 6.9 kg (2.5 kg in placebo group) and blood pressure reductions of 8.6 (vs. 3.9 placebo) and 19.6 (vs. 11.5 placebo) for diastolic and systolic values, respectively, were also reported in a randomised double blind study (0.96 g chitosan each meal, 1000 kcal/day diet).

The results on humans show a favourable effect with a very low dose within a short period. Chitosan was administered to adult males in the form of biscuit over a study period of 4 weeks. When chitosan was given in the diet (3-6 g/day), the serum total cholesterol level significantly decreased, while the serum HDL-cholesterol level significantly increased when compared with the level for each of them before ingestion.

Clinical trials for overweight reduction

While the clinical trials with chitosan were directed to the abatement of cholesterol levels, later on it appeared that chitosan could be used as a diet integrator for the more general purpose of overweight control.

For obese patients, the orally administered chitosan leads to overweight reduction after a few week treatment. For example, according to Lassus & Abelin the weight reduction is 4.4 kg better than that for placebo groups.

For overweight subjects, the meta-analysis of the data obtained by six Italian teams indicates that the mean difference between chitosan and placebo was 3.28 kg of weight reduction (95 % confidence interval: 1.5 – 5.1).

Antiarthritic action

Orally administered chitosan increases serum concentration of glucosamine. Glucosamine is an anti-inflammatory compound, on the market since long. The pharmacokinetics of glucosamine sulfate has been investigated after intravenous, intramuscular and oral administration in dogs, rats and humans.

Upon oral administration, the concentrations are five times lower than after intravenous administration, probably because of a first pass effect in the liver, where glucosamine is metabolized to carbon dioxide, water and urea. Glucosamine diffuses very

rapidly in most tissues and organs, even after oral administration, and it accumulates in the articular tissue and in the bone.

Glucosamine stimulates proteoglycan biosynthesis: the newly synthesized proteoglycans may stabilize cell membranes, resulting in an anti-inflammatory effect. Glucosamine also reduces the generation of superoxide radicals by macrophages and inhibits lysosomal enzymes. Deal & Moskovitz (1999) list the numerous clinical trials (over 20) where glucosamine has been tested recently on over 3000 patients. One of the most recent clinical trials was conducted by Leffler et al. (1999) on 34 males from the U.S. Navy diving and special warfare community, with chronic pain and radiographic evidence of knee or low back diseases. Knee osteoarthritis symptoms were relieved as demonstrated by various assessments including physical examination score (-43.3 %), thus it was concluded that the therapy relieves symptoms of knee osteoarthritis, while a larger set of data was deemed necessary for spinal diseases.

Drug delivery to the colon

The specific drug delivery to the colon is considered an important alternative for the treatment of serious local diseases such as Crohn's disease, ulcerative colitis, carcinomas and infections. On the other hand, specific systemic adsorption in the colonic region offers interesting possibilities for the treatment of diseases susceptible to the diurnal rhythm, such as asthma, arthritis or inflammation.

There is an increasing interest in the development of safe, efficient and reliable mucosal delivery system especially for poorly absorbable drugs such as peptides and proteins. Recent studies prove that chitosan has mucoadhesive properties and enhances the penetration of macromolecules across the intestinal and nasal barrier and open new perspectives in formulating bioadhesive dosage forms for mucosal administration (ocular, nasal, buccal, gastro-enteric and vaginal-uterine therapy).

The chitosan mucoadhesive properties seem to be mediated by ionic interaction between sialic acid residues in mucus and the polymer amino groups. They are òost reòarkable in chitosan, coòpared to other biopolyòers, and enable chitosan to retain an drug in the target absorption site.

Chitosans couple their specific degradability in the colon with their good adhesiveness to the gastrointestinal mucosa favouring the specific and persistent delivery of the local drug. The only inconvenience of these polymers is their high solubility in the gastrointestinal fluids: this implies the need for crosslinking.

A multiparticulate system based on chitosan core microspheres coated with enteric polymers (Eudragit) was developed to overcome the problem due to the high solubility of chitosan in the gastric cavity, while avoiding chemical cross-linking with aldehydes.

In the case of insulin delivery to colon, there are several factors, which may affect the release of the drug:

(a) the pH-dependent solubility of the Eudragit coating;
(b) the size and swelling behaviour of chitosan microcores;
(c) the core/coat ratio;
(d) the microcore-coating interaction;
(e) drug solubility and diffusion through the chitosan gel;
(f) chitosan degradation in the colonic region.

The colon specific delivery of insulin from chitosan capsules in rats brought about improvement of insulin absorption from the rat colon.

Delivery of vaccines, oral immunization and oral vaccination with chitosan

A wide range of proteins such as vaccines, cytokines, enzymes, hormones and growth factors are now commercially available in large quantity, but several problems are associated with the therapeutic use of protein drugs. Among them are the short in vivo half-lives and the side effects attributable to the multiple and high-dose injections. An interesting approach to maintain therapeutic levels is to deliver the proteins with the aid of biodegradable polymers.

Oral immunization induces a vigorous immune response in the mucosal surfaces of various organs, the most common site of entry of infection agents. Numerous experimental systems have demonstrated the ability of oral immunization to induce antibody secretion into the mucus which bathes these surfaces. These mucosal antibodies are restricted almost entirely to the secretory form of IgA, an antibody type that is not effectively induced through conventional intramuscular or subcutaneous immunization.

Microencapsulation systems, in which antigens are incorporated into liposomes or polymer microspheres, were tested as the most plausible systems for oral vaccination. The microparticles were taken up by Peyer's patches (lymphoid aggregates in small intestine) where antigens were gradually released from the particles to induce immune response.

Conclusions

The prescribed dose of chitosan is always much lower than that used in animal tests, and chitosan is recognized as a safe compound, being non-toxic and deprived of activity on certain human enzymes involved in the cholesterol synthesis, as a point of difference from certain drugs. Immunopotentiating and anticancer / antimetastatic actions have also been documented. Therefore, there is no risk of overdose, no side effect, no stimulant action: chitosan is not a medicine, is easy for the overweight to use and is a non-addictive substance. The mucoadhesive properties of chitosan qualify it for drug delivery to the colon; it is also useful for oral delivery of vaccines mainly because it induces antibody secretion in the òucosal surfaces, site of entry of infection.

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Reduction of absorption of dietary lipids and cholesterol by chitosan, its derivatives and special formulations

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Summary

The hypocholesterolemic and lipid lowering effects of chitosan in different species are well recognized but their molecular mechanism is not fully understood. The previously proposed mechanisms are categorized and evaluated. More comprehensive mechanism describing chitosan interaction with lipids and cholesterol is proposed. It appears that there may not be a single mechanism which exists in all species. Approaches to magnify cholesterol and lipid binding by chitosan in humans are reviewed.

Introduction

In spite of the continuous progress in prevention and treatment of coronary heart disease and obesity, these diseases remain the major health problems in the affluent societies. Traditionally, the treatments of these diseases include reduction of serum cholesterol and reduced intake of dietary lipids.

While the hypocholesterolemic effects of chitosan in experimental animals were first observed by Sugano et al. [1,2], Nagyvary et al. [3], and Kobayashi et al. [4], its lipid binding properties in vitro and in vivo were originally described by Furda [5,6], Nauss et al. [7] and Vahouny et al. [8].

Later, numerous additional studies involving rats, Aoyama et al. [9], Jennings et al. [10], Gordon et al. [11], Ebihara et al. [12], Kanauchi et al. [13], Deuchi et al. [14], rabbits, hens and broilers, Hirano et al. [15,16], broiler chickens, Razdan et al. [17,18,19], mice, Miura et el. [20] and few others confirmed the initially observed results.

Ormrod et al. [21], reported that chitosan inhibited hypocholesterolemia and atherogenesis, specifically reduced aortic plaque in the whole aorta and the arch in the apolipoprotein E-deficient mouse model.

Most of these observations were confirmed recently in humans. Significant serum cholesterol reduction and increase in HDL-cholesterol was achieved in healthy males who consumed 3-6 grams of chitosan per day, Maezaki et al. [22]. The weight, blood pressure and cholesterol reductions were observed in double blind studies in obese subjects who took chitosan integrator and hypocaloric diet for one month, [23, 24, 25, 26,27,49]. Abelin & Lasus [28] reported significant weight and blood pressure reductions with chitosan lipid binder in

moderately obese subjects. In the absence of dietary alterations involving obese subjects, two grams of chitosan per day did not affect the body weight and total cholesterol after four weeks [29]. Ingestion of less than 2g/day of chitosan significantly reduced total serum cholesterol in patients with chronic renal failure after eight and twelve weeks of ingestion [30].

Mechanism of hypocholesterolemic and lipid lowering effects of chitosan

The mechanisms which have been thought that could explain hypocholesterolemic and lipid lowering effects of chitosan are primarily [31]:

-Viscosity effect
-Ionic interaction
-Entrapment of mixed micelles and bile acids and fatty acids
-Entrapment of triglycerides
-Inhibition of pancreatic lipase

Viscosity effect

Effect on cholesterol absorption

The fact that the large number of studies with animals or humans demonstrated significant hypocholesterolemic effects of chitosan in which different preparation of chitosan with different viscosities were used suggests that this parameter may not be of critical importance for achieving significant effects [32]. Since chitosan is viscous only under gastric conditions of low pH values, and in the small intestine at neutral pH it precipitates loosing its viscous character, it should not function as a physical barrier for cholesterol and lipid absorption.

Based on number of studies with rats by Sugano et al. [33] and Ikeda et al. [34], only chitosan hydrolysates with extremely low viscosity of less than 1.3 cP proved to be not active. This viscosity corresponded to average molecular weight of less than $5x10^3$. Chitosan hydrolysates with low viscosity of 1.6 - 1.9 cP (Mw $10x10^3$ - $20x10^3$) were at least as effective as high viscosity chitosans or slightly better. Similar results were obtained by LeHoux et al. [35], who observed slightly greater plasma cholesterol reduction and greater reduction of activity of HMG-CoA reductase in rats fed 5% chitosan and 1% cholesterol containing diet with chitosans having low and medium molecular weight (70 kDa and 750 kDa) rather than the high one (2,000 kDa).

Another illustration of superiority of low (100 mPa.s) and medium viscosity (200mPa.s) over high viscosity of chitosans (400 mPa.s) on serum cholesterol lowering in rats was reported [36]. It appears that low and medium viscosity chitosans are generally somewhat more effective serum cholesterol reducers in rats than preparations having very high viscosity.

Effect on dietary lipids absorption

While the effective serum cholesterol reduction in rats favors chitosans with low and medium viscosity , it appears that reduction of lipid absorption favors preparations with higher viscosity. The increased reduction of fat digestibility by more viscous chitosans was observed by Deuchi et al. [37].

It is possible that the precipitation of chitosan in the small intestine (duodenum) of rats due to the gradual pH increase is not necessarily spontaneous but rather gradual, and that chitosan remains viscous in the initial segments of the duodenum. This apparently affects fat absorption mechanism in rats more than cholesterol absorption.

Increased reduction of ileal fat absorption in broiler chickens by chitosan having highest viscosity was reported by Razdan et al. [18]. In lymph cannulated rats, the absorption of oleic acid and triglycerides was reduced by high viscosity chitosan [8,38], but only delayed by low viscosity chitosan hydrolysates [34].

Ionic interaction

Chitosan, being a polycationic polymer of pKa = 6.5 can attract and ionically bind anions such as bile acids and fatty acids, or entire mixed micelles containing additional lipids namely monoglycerides, cholesterol and phospholipids [5, 6, 7]. Most of the early studies describing hypocholesterolemic effects in rats fed chitosan containing diets [1, 2, 3] proposed that ionic interaction of this type could be responsible for the cholesterol lowering. Bound bile and fatty acids or mixed micelles by ionic linkages to protonated amino groups of chitosan would be co-excreted with chitosan rather than absorbed from the gastrointestinal tract.

In the gastric environment (pH 1-3), chitosan is fully protonated and as such it could bind free fatty acids released from dietary triglycerides by gastric lipase. The amount of bound fatty acids, however, would be extremely small due to the low activity of gastric lipase, and to suppressed ionization of released fatty acids which would be entirely solubilized in triglycerides [39].

It had been suggested that binding of fatty acids, bile acids and mixed micelles which occurs to any appreciable degree takes place in the small intestine (pH 6-7), namely in duodenum and possibly jejunum [1, 2, 3, 4, 5]. There, the partially protonated chitosan (approx. 30%) can attract those negatively charged entities which are mainly the product of lipid hydrolysis. The result of this attraction can lead to the attachment of the entire micelle to the chitosan chains, or to the disruption of the micelle due to the removal of bile acids and/or fatty acids from the micelle. In either case, the components of mixed micelles would be malabsorbed. Based on their relative pK values, it could be expected that the bile acids, which are fully ionized at neutral pH, would be bound more extensively than the fatty acids which are only partially ionized. The disruption of mixed micelles, however, is not very likely since even the powerful bile acid binders such as cholestyramine usually do not cause significant malabsorption of dietary lipids.

The main concern with the ionic attachment of mixed micelles and binding of fatty acids and also bile acids is the extent of such binding, considering only partial ionization of chitosan as well as of fatty acids (pK approx. 6.5) [40] at the pH of small intestine, and the rough process of digestion. Another weakness of this theory is that the chitosan hydrolysates of the average molecular weight of $\leq$2,000 did not show serum cholesterol reductions or enhancement of excretion of acidic or neutral steroids in rats [32, 33, 34]. They should had been efficacious since they posses protonated amino groups similar to unhydrolyzed chitosan resulting in equal bile acid binding capacity [34].

Therefore, this mechanism alone may not explain fully the hypocholesterolemic and hypolipidemic properties of chitosan. It could, however, represent the initial phase of binding, followed by precipitation of chitosan leading to the lipid and cholesterol entrapment.

Entrapment of mixed micelles, bile acids and fatty acids

Entrapment of these entities can be explained by the unique solubility profile of chitosan. After ingestion, chitosan is partially or fully solubilized by gastric hydrochloric acid. As it enters duodenum, being insoluble at neutral pH it starts to precipitate provided its average molecular weight is greater than $5x10^3$ [34]. The precipitation is likely gradual because of the pH gradient in duodenum ranging from approx. pH 5.5-7.5. It is therefore likely that the previously attached mixed micelles or bound bile and fatty acids by ionic forces to chitosan chains will become entrapped during chitosan aggregation and precipitation. This is so called "polar entrapment" mechanism proposed by Furda [6]. Being entrapped, they all will escape absorption from the small intestine. This type of immobilization of lipids and their components between chitosan chains is firmer binding which could withstand the process of intraluminal digestion.

Entrapment of dietary triglycerides

Kanauchi et al. [13] proposed a mechanism for inhibition of fat digestion in rats that starts in gastric environment. In the stomach stage, solubilized chitosan by gastric acid emulsifies fat droplets by mixing with them and surrounding them. After the "rough" emulsion reaches small intestine (intestine stage), the solubilized chitosan changes into insoluble gel form, and firmly entrapped fat droplets can not be attacked by pancreatic or intestinal enzymes. Some fat droplets which are emulsified by casein partly digested by pepsin and are not sufficiently emulsified by chitosan are expected to be digested in the intestine.

This mechanism was proposed after artificially stained dietary oil was microscopically observed in the ileal contents of rats which were fed diet containing 20% of oil and 5% chitosan. The authors concluded that the observed colored oil droplets were dietary triglycerides and not fatty acids which could have been formed and could retain the dye.

The key requirement for excretion of dietary triglycerides in Kanauchi's model is *effective emulsification and engulfing of lipid droplets by chitosan in the gastric environment*,

which due to the tendency of lipids to form a separate layer in the stomach could be difficult [40], and the precipitation of chitosan prior enzymatic hydrolysis of triglycerides. Knorr [41] reported that chitosan was not effective emulsifier for the neutral oil in vitro.

In spite of these concerns, it is possible that under certain conditions and in certain species the entrapped and excreted lipids are primarily or exclusively triglycerides as proposed by Kanauchi et al.

Inhibition of pancreatic lipase

There is no evidence that chitosan inhibits the activity of human pancreatic lipase per se which, if it existed, would result in reduced lipid absorption. It was reported, however, that chitosan is partially hydrolyzed in vitro by different lipases, and slightly by human salivary amylase [42]. The authors implied that if chitosan is a substrate for human lipase, it might be possible that relatively large amount of chitosan in diet would in part prevent lipases from hydrolyzing the lipids. Whether lipases from gastrointestinal tract of humans can effectively hydrolyze chitosan has not been shown.

Mechanistic alternatives

The Fig.1 illustrates a more comprehensive mechanistic alternatives which may explain hypocholesterolemic and lipid lowering action of chitosan.

The three major pathways differ in a few specifics.

The Pathway 1 which is essentially Kanauchi model is based on the assumption that dietary lipids are emulsified and surrounded by chitosan chains in the stomach, and immediately after they enter intestine entrapped by the same chains which solidify at neutral pH.

The Pathway 2A assumes that dietary lipids are not emulsified and necessarily surrounded by chitosan chains in the stomach, but they are coated by trace amount of fatty acids released from triglycerides by gastric lipase. As the lipids enter duodenum, the surface fatty acids become partially ionized which will initiate polar attraction between partially protonated chitosan chains and lipid droplets. The rapid precipitation of chitosan precedes the intestinal emulsification of lipids and the attack by pancreatic lipase resulting in the entrapment of attracted lipid droplets without undergoing enzymatic hydrolysis.

The end products of Pathway 1 and Pathway 2A are the same.

The Pathway 2B is identical with Pathway 2A in the gastric stage. In the intestine, however, the enzymatic hydrolysis of lipids precedes the precipitation of chitosan. The formed mixed micelles are attracted by partially protonated chitosan chains and either immediately entrapped by precipitating chitosan (polar entrapment) or disintegrated prior their individual components become also entrapped by precipitating chitosan chains.

Under the Pathways 1 and 2A which exclude the possibility of enzymatic hydrolysis prior the lipid entrapment, one can expect mainly the entrapment and excretion of dietary triglycerides

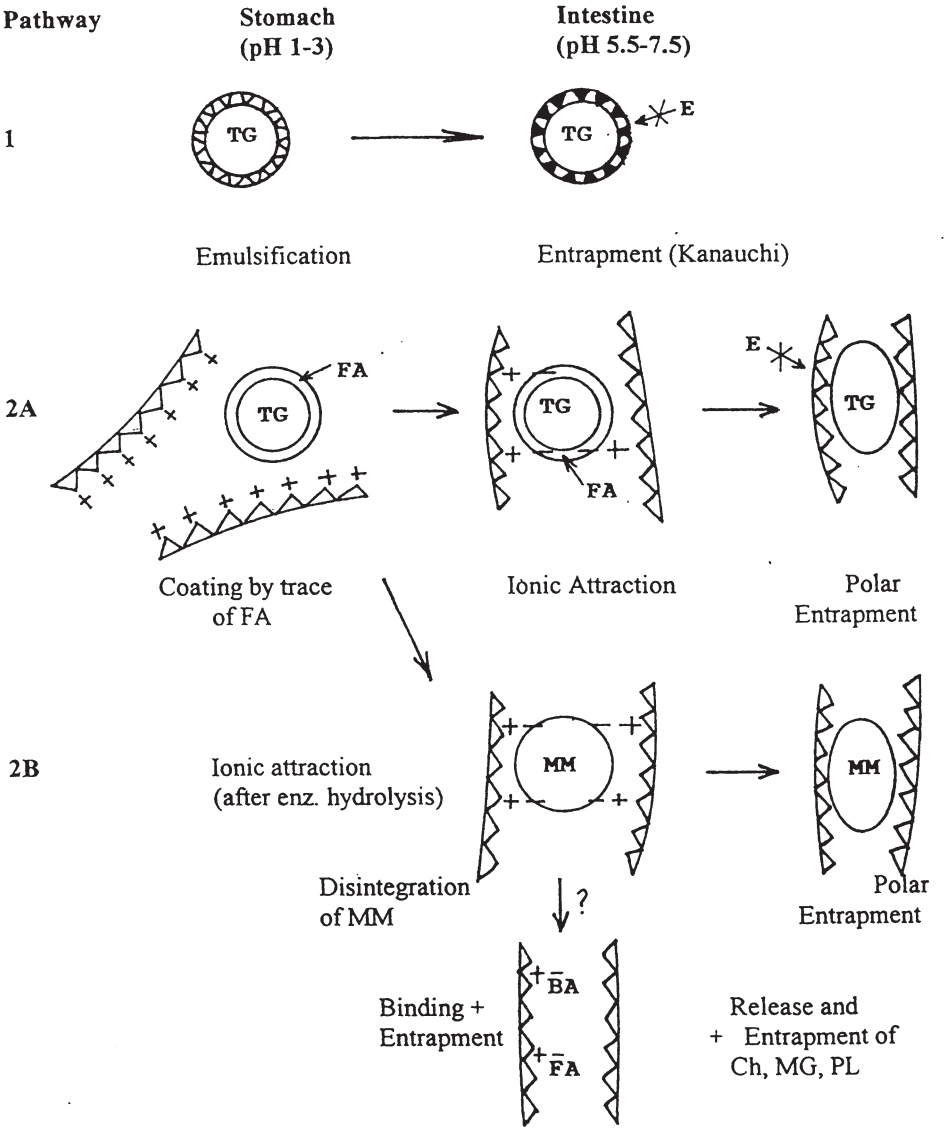

Fig.1. Mechanistic alternatives for binding of triglycerides (TG) and mixed micelles (MM) by chitosan chains (ΔΔΔΔ).
(BA bile acids, FA fatty acids, Ch cholesterol, PL phospholipids, MG monoglycerides, E enzyme).

and cholesterol, since protonated amino groups of chitosan are likely involved in polar interaction with surface fatty acids which makes them less available for other ions.

Under the Pathway 2B, primarily acid steroids (bile acids) and also fatty acids should be excreted.

Sugano et al. [1,2], have shown that when rats were fed chitosan and cholesterol containing diets, the fecal output of neutral sterols, but not acid steroids, was increased. Fukada et al. [43], observed that the excretion of bile acids was not increased, but only their compositional profile was changed when male rats were fed chitosan containing diet for three weeks. Kanauchi et al. [13], after using dietary oil stained with red dye and observing the red oily droplets in the ileal contents of rats fed chitosan and oil containing diets concluded that chitosan entrapped the droplets of dietary oil. They did not indicate or show entrapment, binding or excretion of bile acids. On the other hand, Ebihara et al. [12], had shown significantly increased concentration of bile acids in the intestinal contents relative to aqueous phase in rats fed test meal containing 5% chitosan compared to cellulose. Since excretion analysis was not conducted, it is not clear whether the bile acids were co-excreted with chitosan or reabsorbed. It is possible that some bile acids are weakly bound to chitosan in the upper intestine, which could destabilize mixed micelles, but they are later released and reabsorbed from the lower intestine.

From these examples it appears that in rats, the hypocholesterolemic and lipid lowering effects of chitosan may not be due to increased excretion of bile acids. If there is any elevation of excretion of bile acids in rats fed chitosan hydrolysates in cholesterol containing diets, it is very minor, Ikeda et al. [34]. Rather the increased excretion of neutral sterols and possibly undigested dietary oil, Kanauchi et al. [13], could explain these effects. Consequently the mechanistic Pathways 1 and 2A may be more appropriate for rats.

Contrary to the studies with rats, significantly increased binding and excretion of bile acids was observed in other species. In rabbits fed a cholesterol enriched diet containing chitosan, Hirano et al. [16], the excretion of bile acids was increased by 40% and cholic acid by 500%. The excretion of neutral sterols was also increased by 18%.

In broiler chicken, Razdan et al. [19], the bile acid concentration in duodenum was reduced by 55% after feeding diet containing 3% chitosan, indicating strong bile acid binding by chitosan.

In man, Maezaki et al. [22], the diet containing 3-6 g/day of chitosan reduced serum cholesterol by 6.5% while increasing the excretion of total bile acids by 8-19%. When compared to control period after feeding, the excretion of bile acids was increased by chitosan diet by as much as 62%. The excretion of cholic acid was increased by almost three fold. There was no increase in fecal excretion of neutral sterols.

Based on these examples, in species like rabbits, chickens or men, the hypocholesterolemic effect of chitosan appears to be achieved by binding and promoting the excretion of bile acids which results in interruption of enterohepatic bile acid circulation and requirement for hepatic cholesterol oxidation to bile acids to compensate for their loses. Therefore, the mechanistic Pathway 2B, describing the interaction of chitosan with dietary lipids, could be the appropriate model for those species.

Naturally, additional studies in variety of species, but mainly in humans are required, before the effects of chitosan on cholesterol and lipid absorption will become fully understood.

Approaches to increase lipid and cholesterol lowering effects of chitosan

Ascorbic acid and sodium ascorbate

Fat digestibility in the presence of chitosan can be further reduced by adding ascorbic acid or sodium ascorbate to chitosan [13,14]. The synergistic effect of these two compounds with chitosan was demonstrated in vivo, specifically in rats which were fed high fat diet containing 5% chitosan and 1.5% of ascorbic acid or sodium ascorbate. In a study which lasted two weeks, the amount of fecal lipids was on average 10% larger in group which consumed diet containing chitosan with ascorbic acid than in the group which consumed chitosan alone. Weight of the gastrointestinal contents, fat digestibility as well as the weight of epididymal fat pads, all demonstrated greater effect of the diet containing ascorbic acid or its sodium salt.

The authors explained the synergistic effect of ascorbic acid with chitosan as acid-independent, and specific to ascorbic acid, by reduced viscosity in the stomach, greater oil holding capacity of chitosan gel, and greater flexibility of chitosan-fat gel. The reduced gastric viscosity is believed to mediate effectively the emulsifying process of lipid-soluble chitosan mixture in the stomach. Surprisingly, in the separate experiment the same authors found that greater the in vitro viscosity of chitosan was, the lower the fat digestibility in rats was [37].

The key assumptions of this approach are that the dietary lipids are emulsified and engulfed in the stomach of rats by chitosan, which emulsification is enhanced by ascorbic acid or sodium ascorbate, and that the precipitation/solidification of chitosan ring around the lipid droplets in duodenum is immediate and impenetrable by pancreatic lipase so there is no enzymatic hydrolysis of surrounded lipid droplets.

Chitosan - nicotinic acid (chitosan nicotinate)

A compound which has been proposed to enhance serum cholesterol lowering and elevation of HDL-cholesterol by chitosan is nicotinic acid [45]. Nicotinic acid alone is used routinely for serum cholesterol reduction and augmentation of HDL-cholesterol at the therapeutic level which is three grams or more per day. At these levels it has few side effects, especially flushing. Combining chitosan with niacin, either as chitosan-nicotinate or as a mixture, results in a product which components complement each other, one delivers its hypocholesterolemic effects by systemic mechanism, the other one by nonsystemic one. This product enables to use low levels of nicotinic acid which minimize flushing and other side effects typical for nicotinic acid.

Since the nicotinic acid, either in the mixture with chitosan or as the chitosan-nicotinate, increases the solubility and thus surface area of chitosan in the gastric environment and in the upper part of duodenum, and reduces the pH, chitosan can bind more bile and fatty acids due to

the greater number of available binding sites. Only one percent of nicotinic acid in the mixture with chitosan increases in vitro binding of oleic acid by about ten percent. This synergistic effect is reflected in increased binding of dietary lipids and cholesterol ultimately leading to enhanced reduction of serum cholesterol and lipid absorption.

Quaternary ammonium salts derived from chitosan

In order to maximize the positive charge on chitosan's amino group which at the neutral pH of small intestine is limited, quaternary ammonium salts of chitosan were prepared [46]. Quaternary ammonium salt of chitosan would provide maximum number of binding sites under all pH values of gastrointestinal tract for anions such as bile acids and fatty acids. This will result in increased excretion of bile acids which will be reflected in reduced serum cholesterol.

Mixtures of chitosan with compounds having complementary physiological activities

These were described in detail in Italian Patent [47]. The compounds which were added to chitosan included guar meal, ascorbic acid, Garcinia cambogia extract, chromium and other micronutrients. As stated earlier, this new "chitosan dietary integrator" was tested in several double blind clinical studies with obese subjects and showed few beneficial physiological effects [23,24,25,26,27,49]. The desirable effects included significant reduction in body weight, body mass index, body fat, blood pressure, plasma cholesterol and triglycerides. The levels of HDL-cholesterol were significantly increased in all studies confirming earlier results [22].

It is not entirely surprising that the "chitosan dietary integrator" was highly effective since all of the added substances were previously reported to posses some beneficial effects. For example guar is known to have mild hypocholesterolemic effect, ascorbic acid enhances fat binding by chitosan, Garcinia cambogia extract inhibits fat production and helps curb appetite, etc. What is noteworthy about these studies is that the desirable effects were achieved by relatively low intake of chitosan integrator (less than 2 grams/day) and that only transient or no side effects were reported in all trials which lasted one month.

Ernst et al. [48] proposed that the clinical effectiveness of chitosan in these studies as a means of weight reduction needs to be confirmed by independent rigorous trials since all studies were conducted in one country within two year period.

Conclusions

1. Some previously proposed mechanisms for explaining hypocholesterolemic and lipid lowering effects of chitosan appear to be more substantiated than the others.

2. One which is largely substantiated is based on entrapment of lipids and cholesterol by chitosan during its aggregation and precipitation in the small intestine.

3. The entrapment phase may be preceded by:
   a) emulsification of lipids by chitosan in the stomach, or
   b) their ionic attraction to chitosan in small intestine (duodenum), or
   c) their enzymatic hydrolysis and formation of mixed micelles in small intestine

4. Experimental data indicate that while in rats the hypocholesterolemic and lipid lowering effects of chitosan used at moderate levels ($\leq$ 5%) are not necessarily achieved through increased bile acids excretion, in other species such as chicken, rabbits or man this mechanism apparently plays a major role.

5. Attempts to increase hypocholesterolemic and lipid lowering effects of chitosan have been based on formation of specific salts of chitosan, either prior their use or in situ, on preparation of chemical derivatives of chitosan providing greater number of binding sites, and on administering chitosan mixed with compounds possessing similar or complementary effects.

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Chitosan in weight reduction: results from a large scale consumer study

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Introduction

In the industrialised world overweight has during the recent years developed with an alarming pace in most populations [1]. A number of diseases is linked to the increase in body weight of which three are of main concern for the health authorities in most countries; hypertension, hyper-lipedimia and diabetes II. Health authorities are very concerned about the present development, as it will mean impaired public health and big spendings on the health budgets for pharmacological treatments of these «life-style» diseases. This trend has already started. WHO recently reported on the world wide development of obesity and a task force has been established with the aim of proposing how the present development of obesity can be stopped and reversed [1].

The pharmaceutical industry has also started an intensive research in order to find pharmacological tools that can be used for the treatment of obesity. New agents are already launched internationally and more are to come. The business possibilities are enormous, as in some nations 25-30% of the populations are obese.

Sedentary life and a diet containing too much energy normally in form of fat are probably the main factors for the present development of obesity. Several health authorities have launched national programmes urging their inhabitants to increase their physical activities and to reduce the fat content in their diets. The fat content in the diet should ideally be less than 30%, meaning that less than 30% of the daily energy intake should be coming from fat. The source of fat should also be concentrated on unsaturated fat.

These recommendations have in most countries been without effect, and the obesity problems have continued to develop. A combination of tools, that be pharmacological or natural substances together with increase in physical exercise and change in diets, will probably in the future show to be a very common way to solve the obesity problem for individuals and for populations. Effective and well tolerated tools are therefore needed.

We have during the recent years looked at the possibility for utilising a Biopolymer L 112 chitosan in reducing the absorption of fat in the human body and thus indirectly having a tool for treatment of obesity.

Materials and Methods

Biopolymer L 112 is a chitosan substance developed in Norway. Chitosan is a complex polysaccharide found in shrimps and crabs. It has been shown in several experiments that chitosan has the possibility for trapping fat through electrostatic binding. The idea was then born that if it was well tolerated by humans, it could be used for binding fat in the gastrointestinal tract and then prevent fat from being absorbed through the mucosa on its

transport through the digestive system. In order to investigate the efficacy and tolerability of the Biopolymer L 112, a clinical study in form of an open consumer trial where a large number of persons (>1000) were invited to participate taking the product for a period of 12 weeks, was initiated. The participants to this study were recruited through an add in a local newspaper.

Moderate overweight persons, but otherwise healthy (Body Mass Index – BMI - ≥27.5 kg/m^2 of both genders above 18 years were included in the study. The participants were instructed not to change their life-style during the 12 weeks they participated in the study. The study was based on self-reporting of effect and tolerability data after 4, 8 and 12 weeks. The dosage of L 112 was 3 capsules tid (breakfast, lunch and dinner). L 112 was taken together with food and swallowed with 200 ml of water on each occasion. Each capsule contained 250 mg L 112.

Results

332 subjects (266&, 66%) concluded the study according to the study protocol. The average BMI was 29.3 kg/m^2 and the average age 42.1 years. The demographic data for the participants are given in Table 1. The average weight reduction for the total population was 2.3 kg.

Table 1. Demographic data for the participants. SD in brackets.

N	GENDER	AGE (yrs.)	HEIGHT (cm)	WEIGHT AT START
332	266& 66%	42.1 (11.4)	168.8 (8.1)	83.9 (14.5)

The development of the weight reduction during the study period is shown in Table 2. The weight reduction is statistically significant ($p<0.01$, t-test). A closer analysis of the population shows, however, that the population can be split in two – responders and non-responders to the treatment. Of the total population 111 (30%) can be classified as non-responders. This group has on average a weight increase of 1.3 kg during the period taking L 112. On the other hand the responder group had an average weight reduction of 4.1 kg. The development of the body weights in the two sub-populations is shown in Figure 1 and in Table 3

Table 2. Weight reduction in kg during the study. SD in brackets.

N	INITIALLY	WEEK 4	WEEK 8	WEEK 12	DIFF. Initially – Week 12
332	83.9 (14.5)	82.5 (14.2)	81.8 (14.1)	81.6 (14.7)	2.3 (4.7)

Compliance to the dosage scheme was carefully checked by counting returned capsules. Only participants taking 80% or more of the recommended dose are included in the statistical analysis.

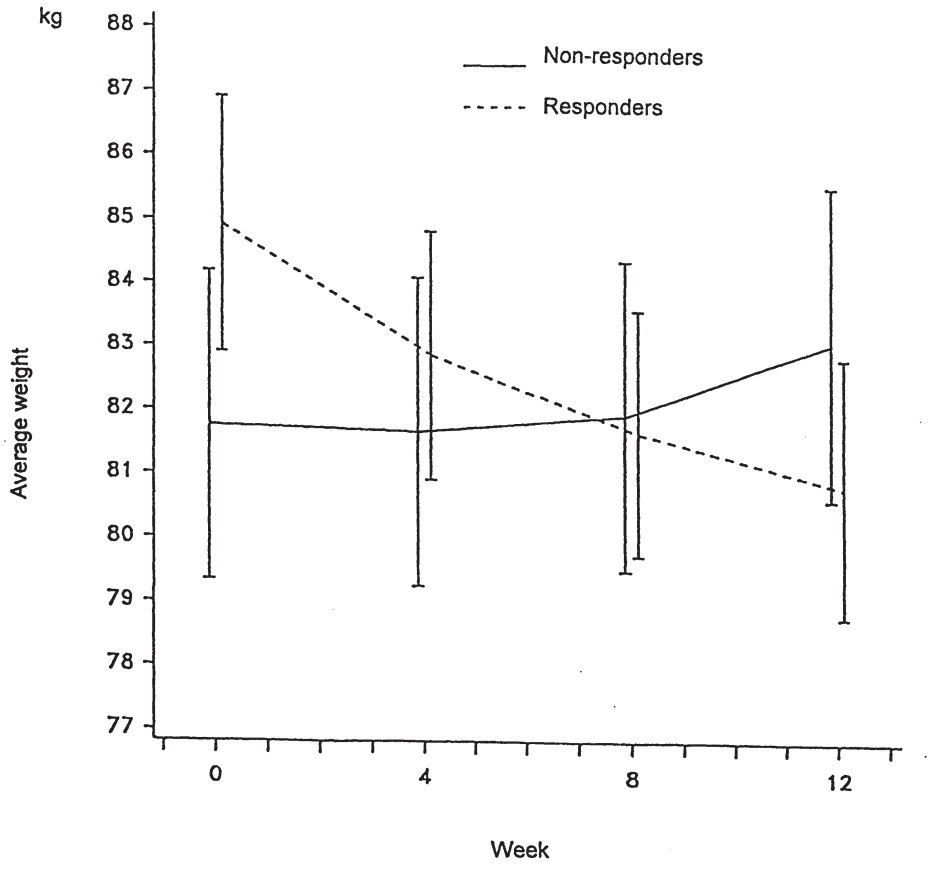

Figure 1. The weight reduction in the responder and non-responder groups during the study.

Table 3. Weight reduction development in the responder and non- responder groups respectively. SD in brackets.

GROUP	N	INITIALLY	AFTER 12 WEEKS	DIFF. Initially – Week 12
Responder	221	84.9 (15.2)	80.8 (15.4)	-4.1 (4.6)
Non-responder	111	81.8 (12.9)	83.1 (13.1)	1.3 (1.6)

Gastrointestinal problems in form of obstipation were the most frequently reported nuisance of the treatment. A number of the participants reported this as an initial problem. However, these problems declined on continuous use and by adequate water intake. None of the patients stopped the treatment because of gastrointestinal problems. 2-3% of the participants reported skin-rash linked to the treatment.

Discussion

Of the total population of 332 concluding the study 111 persons did not respond to the treatment (had no weight reduction) while 222 persons had on average a significant weight reduction of 4.1 kgs during the 12 week observation period, giving on average a weight reduction of 350 g per week. Considering that the participants were asked not to make any substantial changes in their activity level or diet when participating in the study, the observed weight reduction must be considered as satisfactory. The weight reduction in our study is not as pronounced as the ones reported by others [2,3]. However, in these studies the intake of chitosan was combined with a low calorie diet.

As stressed above it would be interesting and valuable trying to characterised responders vs non-responders. The material we have so far does not enable us to carry out such characterisation as the materials either are too limited (the fat absorption study) or not having enough person details about the populations.

The tolerability data of Biopolymer L 112 from the present weight reduction study indicate that 2-3% of the population can expect to have skin manifestation in form of rash. This can probably be due to seafood intolerance. Gastrointestinal problems are the dominating nuisance in people taking Biopolymer L 112 and first of all it is obstipation that is the major problem. The obstipation problem can probably be greatly reduced and even avoided if the users have a proper water intake of about 1.5-2 l per day.

Biopolymer L 112 represents an interesting and well tolerated treatment principle for patients with overweight and hyperlipidemia by reducing the fat from dietary intake.

Future research on the Biopolymer L 112 should concentrate on defining those patients that can profit from the intake of the product.

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Conformation of chitosan ascorbic acid salt

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Summary
A well-defined X-ray fiber pattern of chitosan ascorbic acid salt indicated that the crystalline unit cell of the ascorbic acid salt was a monoclinic (pseudo-orthorhombic) with the following dimensions: a = 1.122; b = 1.177; c (fiber axis) = 1.040 nm; and β = 90°. The similarity of the fiber axis to that of the unreacted chitosan (1.043 nm) suggested that the extended two-fold helical conformation of chitosan is retained in the backbone chitosan chain of the salt. Based on the fiber repeat an energetically favorable conformation of the salt was proposed.

Introduction

Chitosan makes salts when reacts with inorganic or organic acids since it has a regular distribution of aliphatic primary amino groups on the chain. X-ray fiber diffraction study on several inorganic acid salts of chitosan has suggested that chitosan acid salts take up two different conformations depending on kind of acid [1]. One is called Type I salt where the backbone chitosan chain retains the extended two-fold helix of unreacted chitosan [2, 3] although they were different crystals to one another. The other called Type II salt has a less-extended two-fold helical conformation composed of tetramer of glucosamine residues as an asymmetric unit [4]. Crystals of Type I salts are mostly anhydrous forms, whereas, those of Type II, hydrated [1]. Salts formed with HNO_3, HBr, and HI take up the former structure, and those with HF, HCl, and H_2SO_4, the latter. Despite differing anion sizes, all the Type II salts gave very similar fiber patterns not only to one another but also to that obtained by Cairns *et al.* with a chitosan crystal [5], i.e., they have identical unit cell dimensions suggesting that these anions are not present regularly in the crystals and that the fiber patterns are of the backbone chitosan chains [1]. A solid-state ^{13}C NMR study of these crystalline salts revealed that the two types of helical structures were easily distinguishable by their spectra [6]. Organic acid salts of chitosan have been found to be classified into two types, as well, but they showed some difference to the inorganic salts [7-9]. That is, some acid salts of chitosan showed both polymorphs (Type I and II) depending on their preparation temperature. Optically active acids showed different affinity with chitosan between their L- and D-isomers. And, spontaneous removal of monocarboxylic acids accompanying dehydration of the corresponding chitosan salts was observed during the storage of these salts resulting in the anhydrous crystal of chitosan [8-13].

Chitosan and partially *N*-acetylated chitosan are expected to be applied in medical fields such as drug carriers in DDS [14, 15]. Chitosan makes a salt when reacts with ascorbic acid, vitamin C, suggesting that chitosan may act as a carrier of ascorbic acid. Such a

function of chitosan depends undoubtedly on the three-dimensional structure (conformation) of chitosan itself and of its ascorbic acid salt. In this paper crystal structure of the salt was reported. In addition, an energetically favorable chain conformation of the chitosan ascorbic acid salt was proposed.

Materials and Methods

A chitosan specimen having a high molecular orientation and high crystallinity is required for this study. So that, tendon chitosan was prepared from the chitin of a crab tendon, *Chionecetes opirio* O. Fabricus, by a complete *N*-deacetylation described in the previous paper [16]. Since chitosan dissolves with an aqueous ascorbic acid solution leading loss of not only molecular orientation but also crystallinity, chitosan-ascorbic acid salts were prepared by immersing the tendon chitosan in a mixture of an aqueous ascorbic acid solution and isopropyl alcohol (1:3, v/v) under nitrogen atmosphere for 3 h, followed by washing with 75% aqueous isopropyl alcohol and isopropyl alcohol and dried in air. A high concentration (4M) of ascorbic acid and high temperature (70°C) were required in order to get a fiber pattern of the chitosan-ascorbic acid salt [16]. The decomposition of the ascorbic acid which may be expected to occur during the heating even though under nitrogen atmosphere could be ruled out since the similar treatments for the chitosan in a mixture of aqueous dehydroascorbic acid and isopropyl alcohol (1:3, v/v) gave the chitosan's tendon polymorph only indicating no reaction between chitosan and dehydroascorbic acid. Ascorbic acid is known to have two pK values: pK_1 4.25 and pK_2 11.79. In the present experimental condition, the dissociation of ascorbic acid is considered to occur at pK_1, that is, only the hydroxyl group at C3 is dissociated (Fig. 1). The monoanion reacts with the amino group of chitosan chain to make the salt.

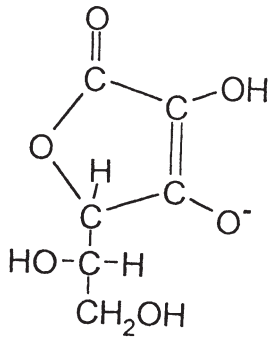

Figure 1. Ascorbic acid

The density of each chitosan ascorbate was measured by a flotation method in a carbon tetrachlorideethylene dibromide at 25°C. The X-ray fiber diffraction patterns were recorded by using a flat-film camera at 100% relative humidity in a helium atmosphere or under vacuum with a Rigaku Gerigerflex X-ray diffractometer employing Ni-filtered Cu Kα radiation generated at 40 kV and 15 mA.

Results and Discussion

Based on a fiber pattern of chitosan ascorbic acid salt (Fig. 2), the salt was considered to be crystallized in terms of a monoclinic (pseudo-orthorhombic) unit cell with the following dimensions: a = 1.122; b = 1.177; c (fiber axis) = 1.040 nm; and β = 90° [17]. The absence of (0 odd 0) reflection on the equatorial layer line suggested that the probable space group is $P2_1$ with b axis unique. The observed density (1.59 cm^{-3}) was in good agreement with the density calculated (1.63 cm^{-3}) for 4 glucosamine ascorbic acid residues per unit cell. The absence of water molecule in the cell was supported by that there was observed no change in the fiber pattern when the chitosan ascorbic acid salt was x-rayed at 100% relative humidity or under vacuum. The presence of odd meridional reflections, (0 0 1) and (0 0 3), indicated no 2_1 screw axis along the c-axis. However, the similarity of the present c-axis length (1.040 nm) with that for free chitosan (1.043 nm) [2, 3, 10-13] suggested that the backbone chitosan chain

of the salt molecule still retained the extended two-fold helical conformation. This and the presence of $P2_1$ space group with 2nd setting indicate that two chains of the chitosan ascorbate are packed in an antiparallel fashion to each other in the unit cell.

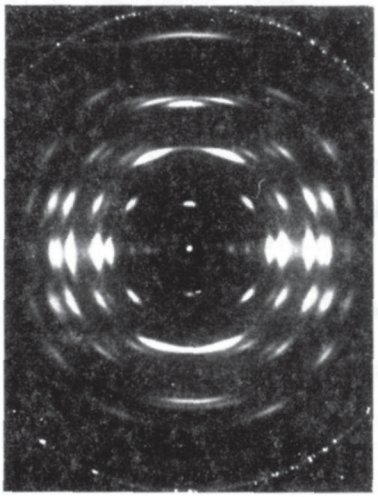

Figure 2. Fiber Diffraction Pattern of Ascorbic Acid Salt of Chitosan.
Fiber axis is vertical.

In the present ascorbic acid salt of chitosan the extended two-fold helical conformation of the unreacted chitosan chain were retained, and the crystal was anhydrous indicating that it is classified into type I salt of the chitosan acid salts [1].

Molecular modeling: An energetically preferable chain conformation of the chitosan ascorbic acid salt was searched based on the X-ray result that the backbone chitosan chains are suggested to be retained their original extended two-fold helices. So that, we used the geometry of chitosan molecule in the crystal of the anhydrous polymorph [11] as the initial atom coordinates of the backbone chitosan chain of the salt. Those of ascorbic acid and its connection to the amino group of chitosan were from the structure of L-arginine L-ascorbate [18]. Conformational energy calculation was done by MNDO-PM3 semi-empirical SCF-MO method [19] with keywords EF and SYMMETRY, as implemented in the WinMOPAC V. 2.0 program [20] by the following conditions.

(1) The geometry (coordinates) of all atoms in chitosan and ascorbic acid was fixed to the X-ray data [11, 18], respectively.

(2) The partial optimization was carried out as follows. The geometry of ascorbic acid was linked symmetrically to that of the chitosan molecule because the salt took up the extended two-fold helix revealed by the present X-ray study. The bond distance between ionic oxygen atom at C3 of ascorbic acid (Fig. 1) and nitrogen atom of chitosan and the orientation of the ascorbic acid were optimized. At the same time, protons of hydroxyl and ammonium groups were optimized.

The present calculation led an energetically favorable conformation of chitosan ascorbic acid salt shown in Fig. 3 although the exact positions of ascorbic acid will be decided by further analysis of the X-ray pattern (Fig. 2) which is underway.

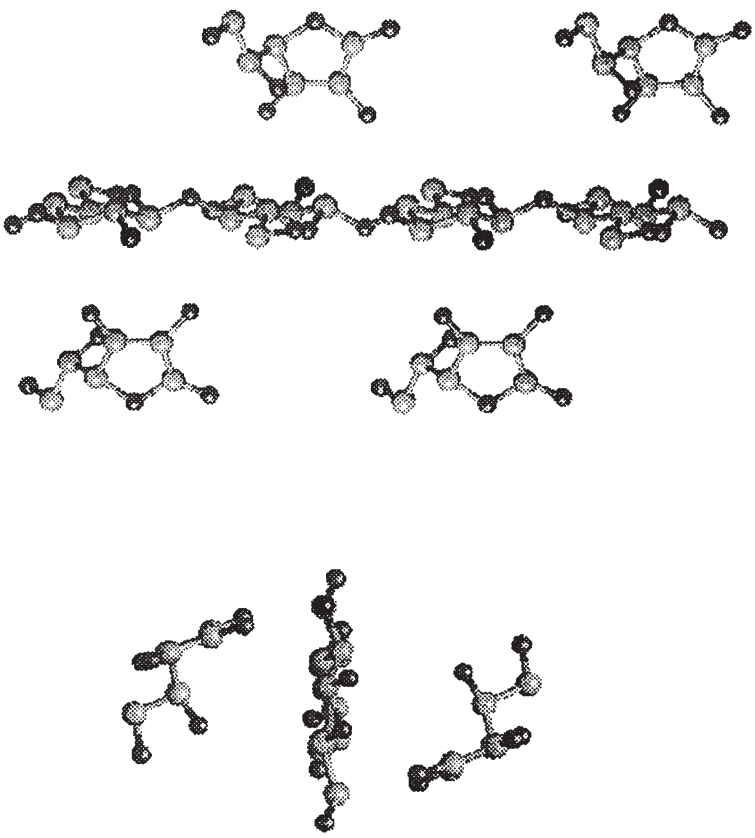

Figure 3. An energetically preferable conformation of chitosan ascorbic acid salt, projected perpendicular (upper) and parallel (bottom) to the helix axis.
Atoms drawn by large ball are carbon; small, oxygen; and black, nitrogen. Hydrogen atoms are omitted.

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Trimethylated Chitosans as Safe Absorption Enhancers for Transmucosal Delivery of Peptide Drugs

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Summary
N-trimethyl chitosan (TMC) polymers are quaternized chitosans in different degrees of substitution. To investigate the safety of these novel absorption enhancers cytotoxicity and ciliotoxicity studies have been performed. Intestinal Caco-2 cell monolayers were chosen to study possible membrane damaging effects, using CLSM visualization of nuclear staining by a membrane impermeable fluorescent probe during transport of the paracellular marker Texas red dextran (M.W. 10,000). Ciliated chicken embryo trachea tissue was used to study the effect of the polymers on the ciliary beat frequency (CBF) in vitro. No substantial cell membrane damage could be detected on the Caco-2 cells treated with 1.0% TMCs, while the effect on the CBF in vitro was found to be marginal. TMC60 and TMC40 enhance paracellular transport of Texas red dextran in Caco-2 cell monolayers, whereas TMC20 is ineffective. Thus, TMCs of high degrees of substitution may be effective and safe absorption enhancers for peptide and protein drug delivery.

Introduction
In recent years, several derivatives of chitosan have been synthesized which are water-soluble over a wider pH range. Among these is N-trimethyl chitosan chloride (TMC), which has been synthesized in different degrees of substitution [1]. These methylated chitosan derivatives have been evaluated in vitro and in vivo as permeation enhancers in intestinal and nasal epithelia [2-4]. A major conclusion from these studies was that the highly quaternized TMC with a degree of trimethylation of 60% (TMC60) was the most efficient permeation enhancer at neutral pH values when compared to TMC with lower degrees of quaternization of 20 and 40%. High charge density promotes the solubility and the permeation enhancing effect of this polymer. It is believed that TMC60 like chitosan (when dissolved in acidic environment) opens the tight junctions of the epithelia, allowing for the paracellular transport of hydrophilic high M.W. compounds, but does not interact with mucosal membrane components and hence does not induce transcellular transport.

The mechanism of the opening of the tight junctions under the influence of either acidic chitosan or the quaternized derivatives has not yet been elucidated. Chitosan like most cationic macromolecules such as protamine and polylysine, can interact with anionic components (sialic acid) of the glycoproteins on the surface of the epithelial cells. Furthermore, the interior of the tight junction (pores) is highly hydrated and contain fixed negative charges. An alteration in the relative concentration of specific ion species in the pore volume would result in substantial alterations in tight junction resistance, which might lead to losening or opening of the pore [5].

For the evaluation of novel absorption enhancers safety studies are required in order to guarantee the absence of tissue damaging effects. In the present study both Caco-2 cell cultures and ciliated chicken embryo trachea tissue in vitro are used as models of epithelial membranes to assess the safety of the cationic polymer TMC. The Caco-2 system is one of the most established in vitro model for intestinal epithelia, and this cell culture has also been used in preformulation studies of the cellular effects of various pharmaceutical excipients [6]. Studying the effects on the ciliary beat frequency of chicken embryo trachea in vitro has proven to be a valuable tool in the design of safe nasal drug excipients [7].

Materials and Methods

TMC polymers: N-trimethyl chitosan chloride (TMC) was synthesized in different degrees of substitution (D.S.), resulting in TMC20 (D.S. 18%), TMC40 (D.S. 39%) and TMC60 (D.S. 65%) as characterized by ^{1}H-NMR [1,4].

Effects on Caco-2 cell cultures: Caco-2 cell culturing was performed as described previously [2,4]. For all experiments cells were used 23-25 days after seeding. Apical applications with or without TMCs and Texas red dextran texas were prepared in HBSS-HEPES at pH = 7.2. Texas red dextran M.W. 10000 (as paracellular transport marker) was dissolved in HBSS-HEPES at a concentration of 0.06%. For co-application 1.0% TMC60, TMC40 or TMC20 was incorporated and 1ml of this solution was applied on Caco-2 cell monolayers. The cells were incubated for 4h at 37° C. Before visualization by Confocal Laser Scanning Microscopy (CLSM), YO-PRO™-1 (a membrane impermeable fluorescent probe) was added to the apical solution at a concentration of 1.2μM, and the cells were incubated for 5 min more. Then, the apical solution was removed and the supporting filter with the monolayer was sandwiched between two coverslips for CLSM [8]. The YO-PRO™-1 excitation/emission wavelengths are 491/509 (nm/nm), and it was excited with a 488nm argon-ion laser. Texas red dextran has characteristic excitation/emission wavelengths of 595/615 (nm/nm), and was excited by a 643nm HeNe laser. For dual labeling a block with a DC 580 and a LP600 filter was used for the detection of both probes [9].

Effects on ciliary beat frequency (CBF) in vitro: CBF measurements were performed with ciliated tissue of chicken embryo trachea [7]. Briefly, the chicken embryo trachea was dissected from the embryo, and sliced into small rings of approximately 1 mm thickness. The slices were placed in stainless steel supporting rings, and were allowed to recover for 30 min in Locke-Ringer (LR) solution at 33°C. Then the tissue samples were put in a well containing 1.0 ml of the TMC solution in Locke Ringer (pH = 7.0) and placed under an Olympus BH-2 light microscope. The microscope table was connected with a thermostat to maintain the temperature of 33°C. The CBF was subsequently monitored using a photoelectric registration device. The CBF was measured every 5-10 min. Control experiments were performed on tissue samples in pure LR solution at pH 7.0. Data were calculated as the relative frequency of the initial frequency measured in the LR solution at the start of the experiment, the latter being expressed as 100%.

Results and Discussion

Effects on Caco-2 cell cultures: For all TMC- treated cells the number of the stained nuclei was not different from those of the control (untreated cells), and nuclear staining was limited to less than 1% of the total cell population. As a positive control a 0.1% (w/v) SDS solution was used.

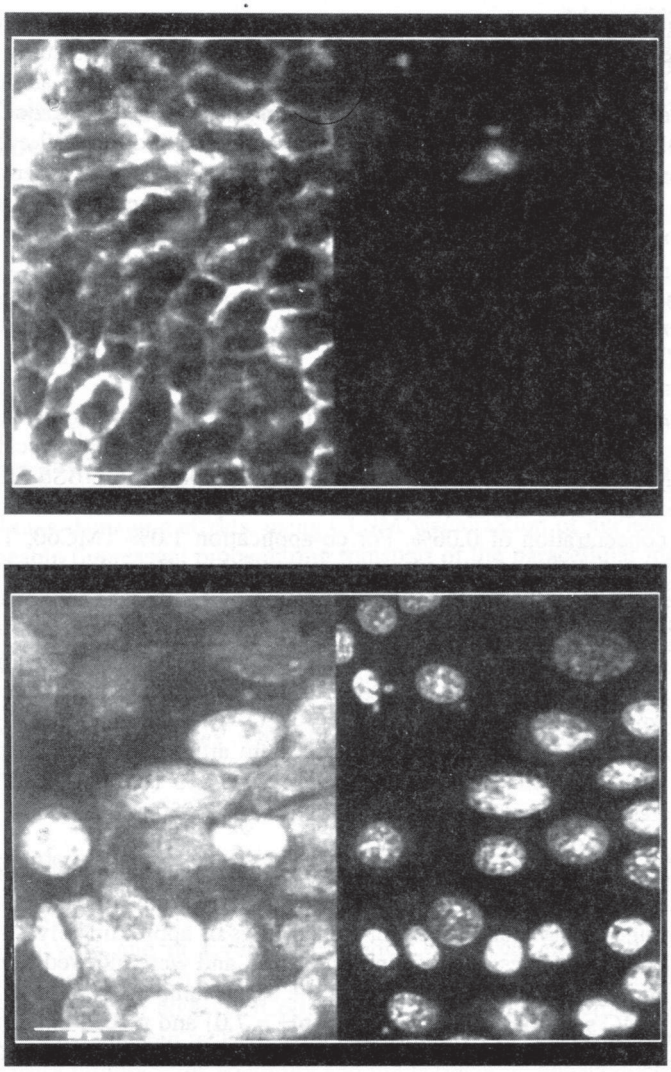

Figure 1. CLSM horizontal cross sections of Caco-2 cell monolayers. (a) Treated with Texas red dextran 10,000 for 4 hours and YO-PRO™-1(dual labelling). Left part, image visualization of the Texas red dextran channel; paracellular fluorescence was observed only at the apical level. Right part, image visualization of the YO-PRO™-1 channel; limited number of cells showed intracellular fluorescence. (b) Treated with SDS 0.1% (w/v) and Texas red dextran (M.W. 10,000) for 10 min.; nuclear staining was performed using YO-PRO™-1. Left part; the paracellular marker is diffused between and into the cells. Right part; all cells have stained nuclei.

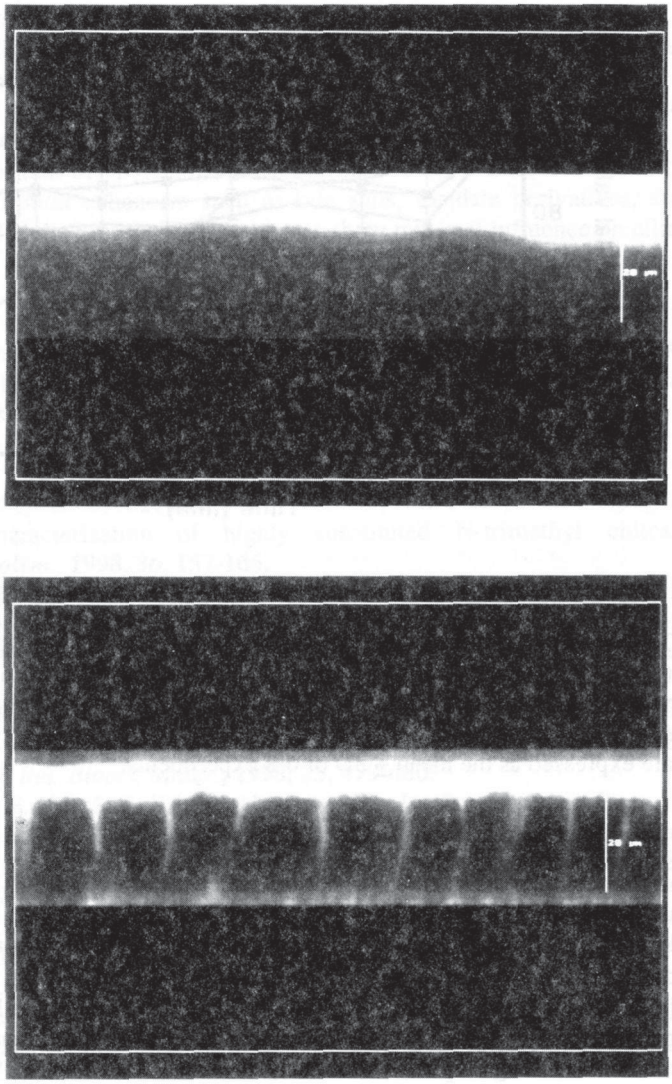

Figure 2. CLSM vertical XZ cross- sections through Caco-2 cell monolayers. Upper panel: Cells were treated only with the fluorescent markers texas red dextran for 4 hours and YO-PRO™-1. Lower panel: Cells were treated additionally with 1.0 % (w/v) TMC60 for 4 hours. Note the paracellular presence of texas red dextran 10000 and the absence of intracellular YO-PRO™-1 staining.

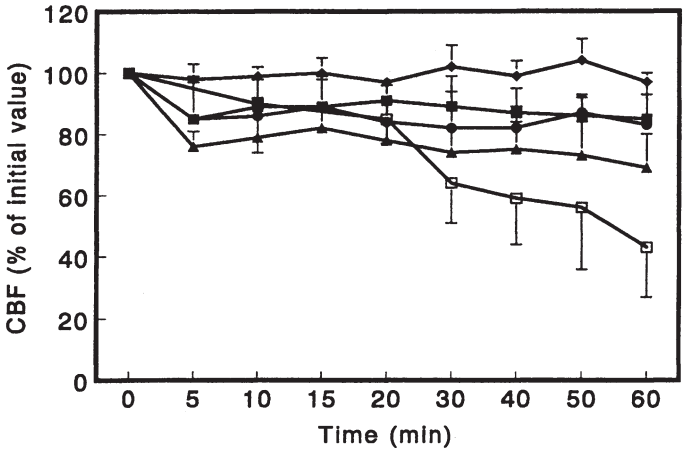

Figure 3. The effects of 1.0% (w/v) TMC polymers (pH = 7.0) and physiological saline (0.9% NaCl) on the ciliary beat frequency (CBF) of chicken embryo trachea in vitro. Control solutions: Locke-Ringer (pH= 7.0) (◆) and physiological saline (0.9% NaCl; □). TMC polymers: TMC20 (●), TMC40 (▲), TMC60 (■). Data are expressed as the mean ± SD of 6-8 experiments.

After 10 min of incubation almost all of the cells had stained nuclei. Furthermore, tight junctions and intercellular contacts, could not be detected anymore (Fig. 1b). When the cells were incubated only with Texas red dextran (control) and YO-PRO™-1, the fluorescence of Texas red dextran was limited only at the apical side between the cells and no further than about 1μm depth from the apical level (Fig. 1a).

In Fig.2 vertical cross-sections of Caco-2 monolayers are shown. In the upper image, cells were incubated only with the paracellular Texas red probe and the nucleic acid staining probe, while the lower image represents monolayers treated also with 1.0% (w/v) TMC60. It is obvious that in the latter case (treatment with TMC60) the Texas red dextran fluorescence is also present in the intercellular space and at the basolateral side of the monolayers. The fluorescence staining of the paracellular routes is evident and rather homogenous in the whole Caco-2 monolayer. No staining of transcellular passage of the fluorescent probe could be detected. Similar results were obtained with 1.0% TMC40, whereas 1.0% TMC20 was ineffective in enhancing the paracellular transport of Texas red dextran.

In all Caco-2 cell monolayers tested the effect of highly substituted TMCs on the paracellular transport of the marker dextran was found to be independent on possible cytotoxic effects of TMCs. Furthermore, a previously observed difference in efficacy between TMC20 and TMCs with higher degrees of trimethylation [2,4] was also clear in the present study for polymers applied on cell monolayers at pH = 7.2. From these CLSM visualization studies it is evident that the effect of TMCs on the paracellular permeability of the Caco-2

intestinal epithelia is not occurring by damaging effects of the polymers on the cell membranes, but because of opening of the tight junctions and thereby facilitating the paracellular transport of the marker dextran.

Effects on ciliary beat frequency in vitro: As apparent from Fig. 3, the effect of all TMC polymers tested, in concentrations of 1.0% (w/v), on the ciliary activity in vitro is very mild, and even less than that of physiological saline (0.9% NaCl). Thus compared to other well-known nasal absorption enhancers such as bile salts, fusidate derivatives, surfactants and phospholipids [7,10] the present TMC polymers show minimal influence on ciliary activity *in vitro*.

Conclusion: Because of the absence of significant cyto- and ciliotoxicity, TMC polymers (particularly with high degrees of trimethylation) are expected to be safe absorption enhancers for improved transmucosal delivery of peptide and protein drugs.

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Chitosan derivatives as intestinal penetration enhancers of the peptide drug buserelin *in vitro* and *in vivo*

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Summary
Macromolecular drugs, like peptides, are administered mainly through the parenteral route. Their molecular size and hydrophilicity have excluded them from being peroraly administered due to their limited permeation across the epithelia. Chitosan is able to increase the permeation of hydrophilic macromolecules through epithelia, acting as an absorption enhancer. Nevertheless, its poor solubility at neutral pH values led to the design of chitosan derivatives displaying better solubility at these pH values. *N*-trimethyl chitosan chloride (TMC), a quaternized chitosan derivative, was evaluated as an intestinal absorption enhancer of the peptide buserelin *in vitro* in Caco-2 cells and *in vivo* in rats. In both cases substantial permeation and/or absorption was found. These results demonstrate that TMC is a potent absorption enhancer of the peptide drug buserelin.

Introduction
A number of therapeutics is administered through the inconvenient parenteral route. Peptides like insulin, peptide analogues, and heparins are administered daily by frequent subcutaneous injections. These agents demonstrate poor bioavailability when they are administered perorally. Poor oral bioavailability can be explained by the three major barriers present in the intestine: 1) The mucus, consisting of polyssacharides and glycoproteins, forms a diffusional barrier to hydrophilic macromolecules. 2) Proteolytic enzymes secreted by the pancreas (for instance trypsin) or present in the intestine (for example the mettaloproteases carboxypeptidases) can degrade peptide drugs to inactive metabolites. 3) Since most of the peptide drugs are hydrophilic and of high M.W., they are transported via the paracellular route. The tight junctions are forming the last and most important absorption barrier for peptides and other macromolecular therapeutics. Tight junctions are present at the apical side of epithelial cells and are composed by the transmembrane protein occludin with its anchoring proteins ZO-1 and ZO-2. Their function is to regulate the transport of liquid and solutes from the luminal to the serosal side of the gut [1].

It has been found that compounds, which are able to open the tight junctions of epithelia, are also able to increase the paracellular permeation of hydrophilic therapeutics and therefore increase their systemic bioavailability after peroral administration. These compounds are generally named permeation or absorption enhancers. An ideal permeation enhancer should have the following desirable properties: 1) The absorption enhancing action should be immediate and unidirectional; the duration of the effect should be specific, predictable and transient. 2) The enhancers should show no systemic toxic side effects; they

should not irritate or damage the applied membrane surface. Most permeation enhancers studied so far do not comply with these requirements.

A novel approach for safe permeation enhancement is the use of functional, biocompatible and non-absorbable polymers, which have a specific and reversible effect on the tight junctions' integrity. One of these polymers frequently explored as permeation enhancer (by means of opening the tight junctions) is chitosan. This polymer has found a number of applications in the biomedical field because of its biocompatibility, biodegradability and absence of systemic and local toxicity. However, its poor solubility at pH values above 6.5 hinders chitosan to be used as permeation enhancer at the intestinal site of absorption.

Chitosan is not able to open the tight junctions of intestinal epithelia at neutral pH values, lacking positive charge density, due to aggregation and precipitation phenomena [2]. To overcome these drawbacks, N-trimethyl chitosan chloride (TMC) has been synthesized and evaluated at different degrees of substitution as safe and efficient permeation enhancer *in* vitro [3-5]. Recently, TMC has proven to be a potent enhancer of both nasal and rectal insulin absorption *in vivo* in rats, especially at neutral pH values where chitosan salts are ineffective [6]. TMC does not exert any cyto- or ciliotoxic effects, as tested with Caco-2 cells and ciliated chicken embryo tissue [7]. Therefore, it can be safely used as absorption enhancer for nasal and intestinal epithelia.

In the present study the effect of TMC polymers on the intestinal permeation was investigated. TMCs of two different degrees of substitution (40 and 60% degree of trimethylation; TMC40 and TMC60) were initially tested for their efficiency to increase the permeation of the peptide drug buserelin, a metabolically stable LHRH analogue, across intestinal mucosae *in vitro* using the Caco-2 cell model. These TMCs were further studied for their potential to increase the intestinal absorption of buserelin following intraduodenal administration in rats *in vivo*. In both cases the pH of application/administration was kept at 7.2, being a representative pH value for the intestinal environment.

Materials and Methods

Preparation of TMC polymers: N-trimethyl chitosan chloride (TMC) was synthesized in different degrees of substitution (D.S.) [8]. Briefly, sieved chitosan with a particle size of 200-400μm was mixed with methyliodide in an alkaline solution of N-methylpyrrolidinone at 60°C for 75 min. The product was isolated by precipitation with ethanol and subsequent centrifugation, and consisted of TMC20 (15-20% of trimethylation). This product underwent a second step of reductive methylation, to yield the final products TMC40 and TMC60 iodide, dependent on the duration of this second reaction step. The product was precipitated by addition of ethanol and isolated by centrifugation. The purification step of the final products included the exchange of the counterion iodide with chloride in a NaCl solution and extensive washing with ethanol and diethylether. The products were dried in vacuo and measured for their degrees of quaternization by ^{1}H-NMR using a 600 MHz spectrometer (Bruker, Switzerland). In all experiments TMCs of two different D.S. were studied: TMC40 (D.S.39%) and TMC60 (D.S. 63%).

In vitro transport studies in Caco-2 cell cultures: Caco-2 cell cultures of passage number 78 were used for the experiments. The cells were seeded on tissue culture polycarbonate membrane filters (pore size 0.4μm, area 4.7 cm^2) in Costar Transwell 6- well plates at a seeding density of 10^4 cells /cm^2. The medium was changed every second day. The cell cultures were kept at a temperature of 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. For all experiments cells were used 23-25 days after seeding. Two hours before the

experiments the medium was changed to DMEM (Dulbecco's Modified Eagle Medium) buffered to pH 7.4 with 40mM n-(2-hydroxyethyl) piperazine-N-(2-ethanosulfonic acid) (HEPES).

TMC40 or TMC60 and buserelin acetate were dissolved in DMEM-HEPES at concentrations of 1% (w/v) and 200µg/ml, respectively. The pH of application was adjusted at 7.2. Apical applications of 2.5ml, containing the polymers and the peptide, were applied on the cells and transport of the peptide was monitored by serosal sampling over 4 hours. Samples of 200µl were added to 800µl solution of phosphoric acid (pH=2) and analysed by HPLC-UV_{220nm} for their content of buserelin.

In vivo studies in rats: The protocol for the animal studies was approved by the Ethical Committee of Leiden University. The experimental procedure was slightly different from previously reported studies [9].

In brief, male Wistar rats SPF (average body weight 250g) were obtained from Harlan (Zeist, The Netherlands). The animals were fasted for 18h prior the experiment, with free access to water. The animals were anaesthesized with Hypnorm® (1.5ml/kg body weight) and Dormicum® (500µg midazolam/ kg body weight). Body temperature was monitored rectally and kept at 36.5-37°C. After buserelin administration (intraduodenally or intravenously), blood sampling was performed through a cannula previously inserted into the right carotic artery. TMC40 and TMC60 polymer solutions and chitosan hydrochloride dispersions were prepared at concentrations of 1% (w/v) in 50mM MES/KOH buffer, (pH = 7.2) containing 250mM mannitol. An amount of 250µg buserelin acetate was dissolved per ml of the control (MES/KOH buffer) and the different polymer preparations. In order to administer the buserelin formulations intraduodenally, a teflon tube connected to a syringe was inserted by a small incision into the corpus of the stomach and guided through the pylorus about 5 to 10mm into the duodenum. Then 2 ml of the control or polymer containing formulations were administered slowly. Afterwards the tube was removed and the incision in the stomach was closed. The stomach was subsequently washed with physiological saline at the site of incisionand the abdomen was closed. In order to determine the pharmacokinetic parameters of buserelin a group of 6 animals received buserelin intravenously (i.v.). The analysis of serum samples on buserelin concentrations was performed by radioimmunoassay.

Results and Discussion

In vitro transport studies: The transport profiles of buserelin across the Caco-2 cell monolayers are depicted in Fig. 1. Both TMCs managed to significantly increase the transport of buserelin compared to the control. However, the effect of TMC60 was more prominent, reaching 6.0% of the applied dose transported after 4h of incubation. These results are in agreement with previously reported results using Caco-2 cells and ^{14}C-mannitol as a hydrophilic marker for paracellular transport. The P_{app} and the enhancement ratios R (R defined as $P_{app\ polymer} / P_{app\ control}$) are presented in Table 1. TMC40 and TMC60 induced a 17- and 46-fold enhancement of the transport of buserelin. Buserelin transport across polymer-untreated cell monolayers was found to be very minor, indicating that buserelin per se poorly permeates the Caco-2 monolayers.

In vivo absorption studies: The mean serum concentrations of buserelin after i.v. bolus injection were analyzed in terms of a two-compartment model and were found to be similar as previously reported [9]. Fig.2 shows the serum buserelin levels after intraduodenal application

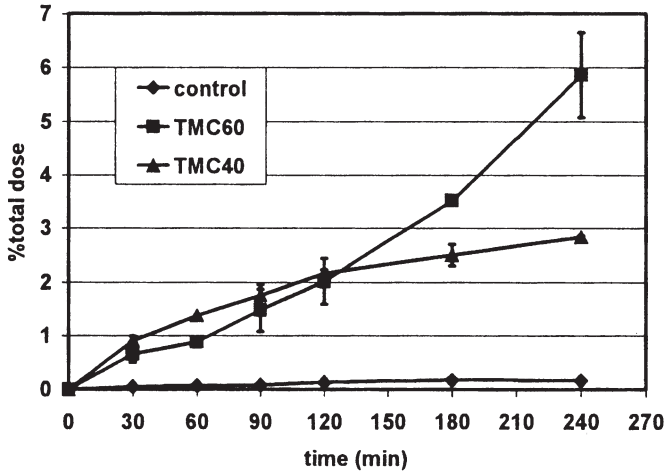

Figure 1. Transport of buserelin acetate across Caco-2 cell monolayers, polymer-untreated (control) and treated with 1.0% TMC40 and TMC60 (pH= 7.2; n=3; mean ± SE).

Table 1. Apparent permeability coefficient (P_{app}) and enhancement ratio (R) values for buserelin acetate across Caco-2 cell monolayers, polymer-untreated (control) or treated with 1.0% TMC40 and TMC60 (pH = 7.2; n=3; mean ± SE).

Application	$P_{app} * 10^{-6}$ (cm/sec)	R
Control	0.04 ± 0.01	1
TMC40	0.85 ± 0.02	17.2 ± 1.3
TMC60	2.40 ± 0.27	46.4 ± 5.7

of buserelin in buffer solution (control) and in the presence of 1.0% TMC40 or 1.0% TMC60. A remarkable increase in buserelin serum concentrations was observed after co-administration of the peptide with both polymers, whereas buserelin alone was poorly absorbed. In agreement with the present *in vitro* results, TMC60 managed to increase the absorption of buserelin at higher levels than TMC40. Fig. 2 also depicts the buserelin serum levels after co-administration of the peptide drug with 1% chitosan HCl. This chitosan HCl dispersion did show a slight increase in buserelin absorption compared to the control, but it did not manage to increase the buserelin concentrations at the levels achieved with both TMCs. The pharmacokinetic parameters of intraduodenally administered buserelin with or without the polymers are presented in Table 3. TMC60 reached the highest C_{max} and the highest peptide bioavailability. The absolute bioavailabilities of buserelin after co-administration with TMC40 and TMC60 were 6.3 and 13.0% respectively, indicating 8- and 16-fold increases in comparison with intraduodenal administration of buserelin alone.

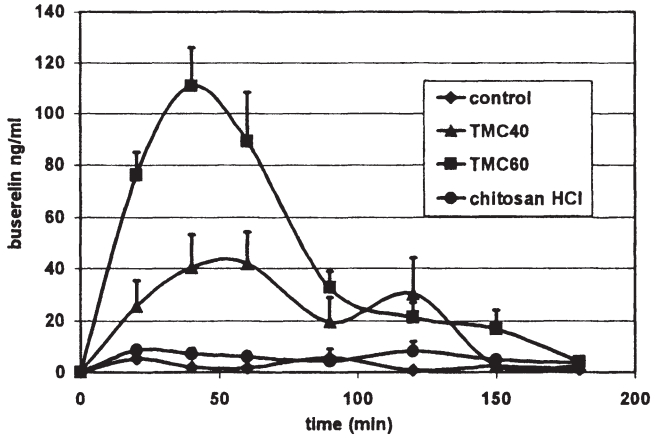

Figure 2. Buserelin serum levels after intraduodenal administration of buserelin acetate (500µg/rat) alone (control) and after co-administration of buserelin with 1.0% TMC40, 1.0% TMC60 or 1.0% chitosan HCl (pH of administered formulations 7.2; n=6; mean ± SE).

TMCs represent a novel group of polymeric mucosal permeation enhancers. The positive charge density of the chitosan polymers has been found to play an important role in the effect on the tight junction regulation [4]. In the present study the D.S.-dependent effect of TMC on facilitating the paracellular permeation *in vitro* in Caco-2 cells could also be demonstrated *in vivo* in rats. TMC60 in concentration of 1.0% shows increased intestinal absorption of buserelin when compared with TMC40 as well.

TMCs are a novel class of polymeric permeation enhancers, which are able to open the paracellular pathway in a reversible way. Being themselves hydrophilic macromolecules, they do not interact with membrane compounds and are not absorbed.

Table 2. Pharmacokinetic parameters after intraduodenal administration of buserelin (500µg/rat). Data are presented as mean ± SE of 6 animals. (Abbreviations: T_{max}, time to reach serum peak concentration; C_{max}, serum peak concentration; F, absolute bioavailability). Statistical evaluation: [a], significantly different from the control ($p<0.005$); [b], significantly different from TMC40 ($p<0.005$).

Polymer	T_{max} (min)	C_{max} (ng/ml)	AUC (ng/ml*min)	F(%)
Control	90	5.8 ± 3.3	510 ± 152	0.8 ± 0.2
Chitosan HCl	20, 120	8.2 ± 3.7	1070 ± 235	1.7 ± 0.4
TMC40	40-60	41.9 ± 12.3 [a]	4018 ± 1037 [a]	6.3 ± 1.6 [a]
TMC60	40	110.9 ± 15.3 [a,b]	8199 ± 392 [a,b]	13.0 ± 0.6 [a,b]

From the present study, it is evident that both TMC40 and TMC60 are able to increase the permeation of the peptide drug buserelin across intestinal epithelia *in vitro* (Caco-2 cell monolayers) and *in vivo* (rats). The type of interactions of the TMCs with the components of the tight junctions is presently under investigation.

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Chitosan microparticles for oral vaccination: optimisation and characterisation

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Summary
Although oral vaccination has numerous advantages over parenteral injection, both degradation of the vaccine in the gut and low uptake in the lymphoid tissue of the gastrointestinal tract still complicate the development of oral vaccines. These problems can sometimes be avoided by associating the vaccines with microparticles.

In this study chitosan microparticles were prepared according to the method of Berthold *et al.* [1] and optimised for oral vaccination with respect to size, zeta potential and ovalbumin loading and release. The properties of these microparticles were observed to be very promising for oral vaccine delivery.

Introduction
The introduction of an efficient oral vaccine would diminish costs, the need for qualified personnel to administer the vaccine and increase patient compliance. Despite these advantages, oral vaccines still have to be administered parenterally due to several factors. Oral vaccines first have to pass the stomach with its low pH. Then the vaccines have to be to taken up by the M-cells in the intestinal epithelium. These M-cells are specialised in the transport of antigens across the epithelial barrier. After uptake, these antigens are transported to the Peyer's Patches. Peyer's Patches are part of the Gut Associated Lymphoid Tissue (GALT). In these Peyer's Patches systemic immune reactions can be provoked against antigens taken up from the food. The M-cell uptake of vaccines and delivery to the Peyer's Patches is the first essential step in oral vaccination. Several studies have shown that by associating the vaccine to a microparticulate drug carrier system, the uptake by M-cells can be enhanced and the degradation in the gastrointestinal tract can be prevented [2-6].

Chitosan is a good excipient for oral drug delivery systems, because of its biocompatibility, biodegradability, low costs and ability to open intercellular tight junctions [7]. Numerous studies of chitosan as a drug absorption enhancer have recently been published and advances in microparticulate drug delivery research have opened up the way to apply these techniques for oral vaccination.

In this study chitosan microparticles were developed and optimised for oral vaccination. Because of their good antigen binding properties, chitosan polymers are expected to be good candidates for oral vaccination [8]. Chitosan microparticles were made according to the method of Berthold *et al.* [1] and adapted for oral delivery of the model antigen ovalbumin.

Materials and Methods

Solutions of 0.15-0.25% Chitosan (13 mpas, generous gift from Primex, Avaldsnes, Norway) in 2% acetic acid and 1% Tween 80 were made. Thereafter, 1-2 ml of 10% sodium sulphate was added dropwise under stirring and sonication. After 20 min microparticles were centrifuged for 25 min (2750 rpm). All reagents were of analytical grade. The pellet was resuspended in MilliQ water to wash the microparticles and centrifuged again. This washing procedure was repeated twice before freeze-drying.

The size was analysed using an Accusizer 770 (PSS, Santa Barbara, CA, USA) and the zeta potential was determined using a Malvern 2000 zetasizer (Herrenberg, Germany).

Morphology was first studied by means of light microscopy (Zeiss Axioskop, Weesp The Netherlands). Then Scanning Electron Microscopy (SEM) was used to visualise the microparticles in to more detail. For SEM the freeze-dried chitosan microparticles were gold-sputtered for 3 min and analysed using a Philips SEM 525 electron microscope (Eindhoven, The Netherlands).

Loading of microparticles was done by incubating 1% of microparticles in 0.5-2% ovalbumin under shaking in Phosphate Buffered Saline (PBS) at 25°C. After incubation the suspension was centrifuged to remove the unloaded ovalbumin. Loading was determined by quantifying the non-bound ovalbumin in the supernatant by means of the Lowry protein assay. Both loading capacity (LC) and loading efficacy (LE) were determined:

LC=(total amount ovalbumin)-(free ovalbumin)/weight microparticles

LE=(total amount ovalbumin)-(free ovalbumin)/total amount ovalbumin

Ovalbumin release from the microparticles was determined in PBS (pH 7.3). An amount of 3.5 ml of a 1% (w/v) chitosan microparticle suspension containing 0.5% (w/v) ovalbumin was incubated for 3 hours to load the microparticles. After centrifugation (1400 rpm for 30 min) the loaded microparticles were resuspended in PBS to make a 1% (w/v) microparticle suspension. The samples were incubated under mild shaking. After 15, 30, 45, 60, 90, 120, 180 and 240 minutes, the tube was given a spin off and samples of 250µl of the supernatant were taken. Release was determined by measuring the non-bound ovalbumin in PBS after centrifugation.

Results and Discussion

By adding 2ml of 10% (w/v) sodium sulphate to 200 ml 0.25% (w/v) chitosan solution, the most suitable chitosan microparticles were obtained. Light microscopic studies showed that these microparticles did not from a gel when resuspended in water, were not forming large aggregates and were not accompanied by fibres. Furthermore, they all showed reasonable uniformity (Fig. 1a). SEM revealed that the surface of the chitosan microparticles was very

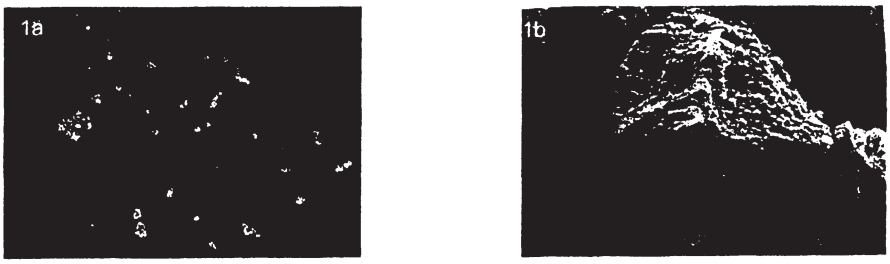

Figure 1. (a) Light microscopic fotograph of chitosan microparticles (1000x). (b) SEM micrograph of chitosan microparticle (5700x).

rough (Fig. 1b).

The particle size was found to be 4.23 ± 0.67μm (±SD, n=10). Since uptake of microparticles by M-cells appears only when they are smaller than 10μm [6], the present chitosan microparticles are in that respect suitable for M-cell uptake. The zeta potential was 19.9 ± 3.5 mV (±SD, n=3). Since cell membranes are negatively charged, positively charged microparticles are more likely to be attached to and subsequently taken up by the M-cells than negatively charged microparticles.

Dependent on the total amount ovalbumin added in the incubation solution, both loading capacity and loading efficacy were determined. The optimized chitosan microparticles showed a high loading capacity (about 40%; Fig. 2a). The amount of ovalbumin added to the microparticles did not change the loading capacity. In further experiments 0.5% ovalbumin was chosen, because in that case most of the ovalbumin is associated to the microparticles (85%) and not lost during the loading process.

With these loaded microparticles the ovalbumin release in PBS was determined, as presented in Fig. 2b. The release profile showed that about 85% of the ovalbumin remain inside the chitosan microparticles. Since the microparticles are designed to be taken up by the M-cells, this protein content may be released after biodegradation of the microparticles within these cells.

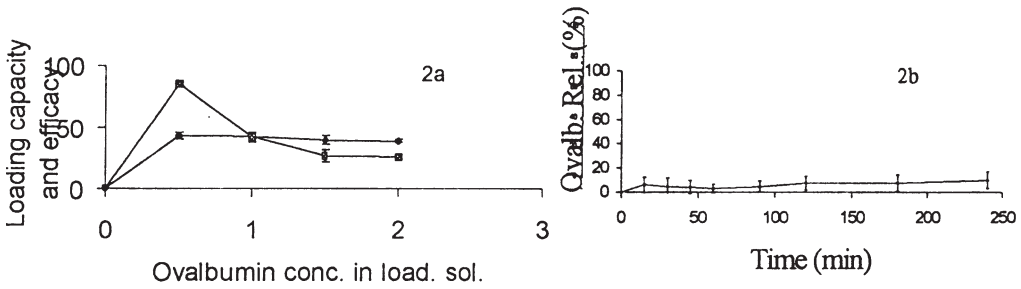

Figure 2. (a) Loading Capacity (LC; ♦) and Loading Efficacy (LE; □) dependent on different amounts of ovalbumin (0.5-2.0 % (w/v)) added during the loading (n=10). (b) Release of ovalbumin from loaded chitosan microparticles (n=10).

Chitosan microparticles, presented in this study, have suitable size and zeta potential to be taken up by the M-cells of Peyer's Patches. These microparticles show a high loading capacity and loading efficacy for the model antigen ovalbumin. About 85% of the ovalbumin remained in the microparticles after release studies for 4 hours in PBS, indicating that high amounts of ovalbumin can be transported into the Peyer's Patches. Further research is now being performed to load the present microparticles with real vaccines, and *in vivo* studies will be carried out to examine the uptake of vaccine- loaded particles by the M-cells of the Peyer's Patches.

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Effect of chitosan in enhancing drug delivery across buccal mucosa

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Summary
In this study, the effect of chitosan in facilitating the penetration of two potential therapeutic compounds, hydrocortisone, and transforming growth factor beta (TGF-β), was investigated. Chitosan gel was prepared at 2% concentration in dilute acetic acid and the drugs were incorporated into the gel. The effect of chitosan as a permeabilizer was determined by measuring the flux of compounds across porcine oral mucosa in an *in vitro* system. The localization of compounds within the oral mucosa was determined by horizontal sectioning and counting. Chitosan was found to exert a marked permeabilizing effect on buccal mucosa for the candidate compounds tested.

Introduction

A major challenge in formulating topical agents for the oral cavity is the need for adhesion to the moist surface of the mucosa and to resist the flushing action of saliva. The delivery system in which any therapeutic agent is incorporated must therefore be formulated to prolong retention of the drug in the moist environment of the oral cavity. The use of bioadhesive gels reduces the frequency of application and the amount of drug administered and thus can improve patient compliance and acceptance. Optimising the retention time of the drug is also important in improving the clinical effectiveness. Finally, for the mucositis patient, the occlusion and lubrication of a bioadhesive gel reduces the discomfort of this inflammatory and ulcerative condition.

The first materials to fulfill some of these requirements were based on mixtures of polyethylene in mineral oil containing hydrophilic polymers such as pectin and gelatin. One such compound, marketed as Orabase [1] has been widely used as an occlusive dressing to protect ulcerated areas of oral mucosa. However, such products suffer from a variety of problems, particularly the difficulty of application to sensitive surfaces and the relative ease with which they are dislodged and dissolved. Consequently, a great variety of hydrophilic, bioadhesive, polymers such as polyacrylic acid, an anionic polymer with a high concentration of carboxyl groups, and polyacrylic acid-hydroxypropyl methylcellulose systems, have been developed. These compounds are flexible and sufficiently adhesive to be retained for periods of six hours or longer [2,3]. However, they are often complex systems that are expensive to manufacture, difficult to apply, and few have been marketed on a large scale. Some of those that

are available have undesirable properties which can cause great discomfort at an ulcerated surface.

Chitosan is a biologically safe polycationic biopolymer, that has been proposed as a bioadhesive polymer for oral mucosal drug delivery [4,5] and initial studies showed that it has an extended retention time on the oral mucosa [6]. With its bioadhesive and antimicrobial properties and its biocompatibility, chitosan is an excellent candidate for treatment of oral mucositis, offering not only the palliative effects of an occlusive dressing but the potential for delivering therapeutic compounds where a sustained and long lasting pharmacokinetic and pharmacodynamic profile is desired. Chitosan has also been shown to have potential as an enhancer of mucosal drug absorption [7-9].

The aim of the present study was to investigate the effectiveness of chitosan gel for delivering the antimicrobial and anti-inflammatory compounds necessary for palliative therapy of mucositis and of the bioactive peptides that may be able to provide protection or promote healing of the oral mucosa. Two candidate drugs were used in this study, hydrocortisone, a topical anti-inflammatory agent, and transforming growth factor beta (TGF-β), which is a bioactive peptide.

Materials and Methods

Preparation of formulations: Chitosan-H (Lot 337) (degree of deacetylation: 80%) (Dainishiseika Colour and Chem. MGF Co. Ltd, Japan) was used to prepare a 2% gel in dilute lactic acid solution. Phosphate buffered saline (PBS) was used as the control solution.

1% hydrocortisone (HC) was incorporated into the gel and into the control solution. Both formulations were labelled with tritiated HC (NEN Life Science Products) to achieve a concentration of 2μCi per application.

Transforming growth factor beta (TGF-β), a peptide of molecular weight approximately 25Kda (supplied by Oncogene Sciences, NY) was incorporated into gel or control solution. Both formulations were labelled with I^{125} to achieve a concentration of 2μCi per application.

Flux studies: Porcine buccal mucosa, which has a similar structure and permeability as human tissue [10] was dermatomed to a thickness of approximately 700 μm and mounted in through-flow mucosal perfusion cells designed by Squier *et al.* [11], and maintained at 37°C. Chitosan gel or PBS containing the drug was applied to the epithelial surface of 7 replicates for 1h and then removed. The tissue surface was rinsed with PBS, and a solution of artificial saliva was applied. Perfusate was collected from the connective tissue side for intervals up to 12 hours and counted in a scintillation counter to determine a flux (dpm/cm^2/min). Labelled water was used as benchmark control since it is inert and there is a large amount of data available on its flux across a variety of tissues.

Distribution of drug within the mucosa: At the time of the formulation removal and at 1, 2 and 7 h afterwards, 3 specimens were harvested from each group and snap frozen onto corks in liquid nitrogen so that sections could be cut parallel to the epithelial surface in a cryostat. These sections were solubilized in TS2 tissue solubilizer and counted to determine the quantity of drug (mean±sem) in four tissue strata: outer epithelium (~100μm thick), middle epithelium (~100μm thick), inner epithelium (~100μm thick) and the

remaining underlying connective tissue. The value for the amount of drug in the tissue was then used to calculate the percentage of the total drug applied that was present in each stratum for each time point.

Microscopic examination of histological sections of porcine buccal mucosa exposed to chitosan gel for upto 8h did not show any deleterious effects as compared to controls treated with PBS.

Results and Discussion

There was a significant ($p<0.05$) increase in maximum flux for HC in gel compared to PBS. The flux reached a value of 131 ± 49 dpm/cm^2/min after 1 hour, which was significantly higher ($p<0.05$) than the value for PBS of 21 ± 3 dpm/cm^2/min. The flux for the gel remained elevated, reaching a peak value of 276 ± 140 dpm/cm^2/min after 7 h; that of HC in PBS declined to a level of 7.6 ± 3 dpm/cm^2/min in the same time period.

Similarly, for TGF-β, flux was significantly ($p<0.05$) increased with chitosan gel. TGF-β is a large peptide which resulted in a greater lag time than for the other compounds. The flux obtained after 1.5h was 241.75 ± 95.98 dpm/cm^2/min with gel, whereas with PBS it was only 39.59 ± 18.13 dpm/cm^2/min.

Tissue sectioning revealed that, in general, chitosan increased the quantity of compounds in the superficial layers of the epithelium as compared to PBS. However the hydrophilicity of the compound also had an effect; for HC, there was relatively less penetration into the deeper tissue layers whereas more of the more water soluble TGF-β reached the deeper layer (Figs.1 and 2).

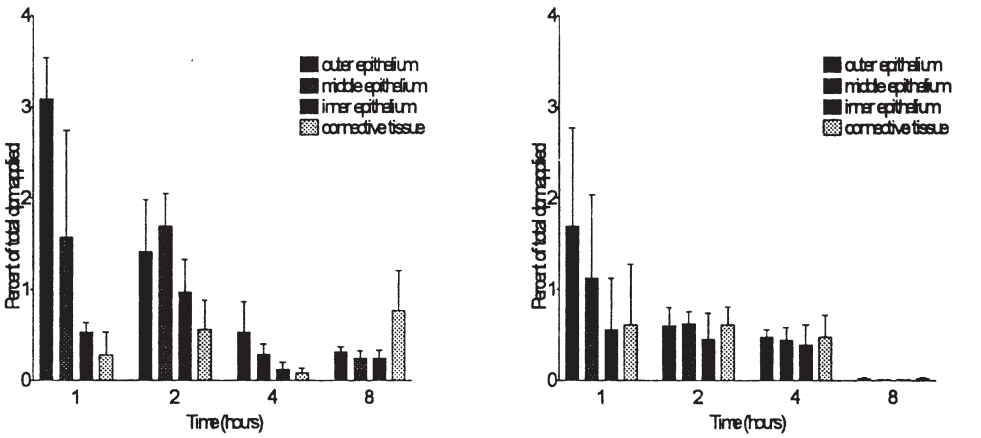

Figure 1.1 Distribution of HC in different tissue strata applied for 1 h: (a) in chitosan gel, and (b) in PBS

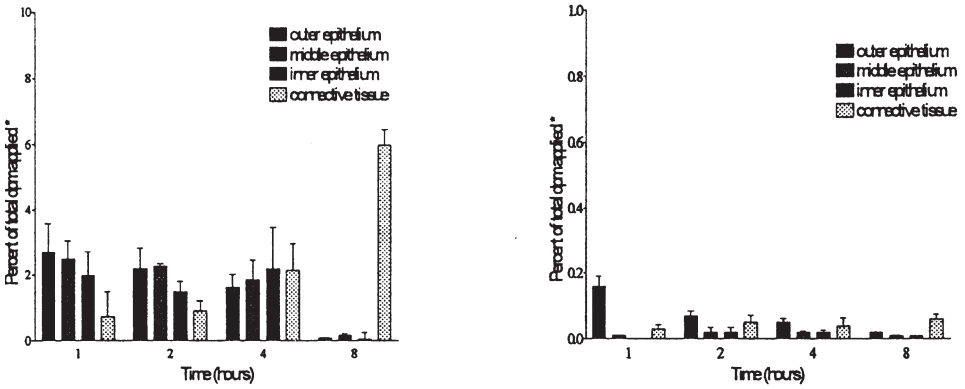

Figure 2. Distribution of TGF-ß in different tissue strata applied for 1 h: (a) in chitosan gel, and (b) in PBS (*Note: The graphs are plotted on different scales)

The results show a six to seven fold enhancement of permeability by chitosan for the compounds to which the oral mucosa is normally relatively impermeable. One explanation for this effect may be the bioadhesive nature of chitosan, which increases the retention of the drug at its application site. Another important factor may be binding to the epithelial surface, mediated through the positive charge on the gel, which could contribute to its continued effect on epithelial permeability after physical removal from the surface.

The results suggest that chitosan may be of value for enhancing oral mucosal drug delivery, particularly to large compounds or relatively hydrophobic molecules. This can be particularly valuable where a sustained and long lasting pharmacokinetic and pharmacodynamic profile is desired.

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Influence of chitosans on permeability of human intestinal epithelial (Caco-2) cells: The effect of molecular weight, degree of deacetylation and exposure time

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Summary

The effect of chitosan chloride on transepithelial membrane permeability has been studied *in vitro*. Confluent monolayers of Caco-2 cells were used as a model. The transepithelial electrical resistance (TEER) was measured before and after exposure to chitosan. It was found that chitosan with a high degree of deacetylation (DA%) and a degree of polymerization, $DP_n > 50$ induced the greatest effect on opening tight junctions, although the difference between low and high DA (DA = 60-100%) was small. Oligomers of glucosamine, $(GlcN)_1$ and $(GlcN)_6$, had no effect on TEER, demonstrating that the positive charge itself is not enough to open the tight junctions. Factors which contributed to the reversibility of chitosan-induced opening of tight junctions were treatment with chitosan chloride having a low degree of deacetylation, high molecular weight chitosan chloride, and short exposure times ($\leq$ 1 hour).

Introduction

Drug delivery by nonparenteral routes has gained much attention in recent years. Polar drugs as peptides and proteins are not well absorbed across the mucosal epithelia, due to the tight junctions between the epithelial cells which form a strong absorption barrier. Many attempts to overcome these absorption barriers have been undertaken, using both enzyme inhibitors and absorption enhancers [1, 2]. Chitosan has been shown to increase the transport of polar drugs across epithelial surfaces due to its bioadhesive properties and to a transient opening of the tight junctions between epithelial cells [3]. It has been shown by Schipper *et al.* [4] that the absorption enhancing and toxic effects of chitosans on epithelial cells are dependent on their chemical composition and molecular weight. Aspden *et al.* [5] studied the effect of five types of chitosan with varying molecular weights and degree of deacetylation on the nasal epithelium using a frog palate model, and found no correlation between the degree of deacetylation or the molecular weight and the absorption enhancer efficacy. Lower molecular weight chitosans appeared to cause slightly more cell damage and protein release than higher molecular weight chitosans.

In this study we have used the Caco-2 cell mololayer as a model for evaluating the effect of various chitosan chlorides on the integrity of tight junctions. Not only the ability of chitosan to open tight junctions but also the re-establishment of the permeability barrier will be studied. Previous studies have not investigated the effect of a range of chitosan molecules, from oligomers to high molecular weight chitosan, on tight junction. Here we demonstrate the

correlation between the molecular weight and the effects on tight junctions, and the correlation between the degree of deacetylation and the effect on tight junctions. The effects of chitosan on the re-establishment of tight junctions after different exposure times are also studied.

Materials and Methods

Materials: Chitosan chlorides (PROTASAN™ chloride) having varying degrees of deacetylation and molecular weights were manufactured by Pronova Biomedical a.s (Olso, Norway). Different chitosans with DA = 60%, DA = 73%, DA = 84% and DA = 95% and an apparent viscosity of ~70 mPa.s (corresponding to M_w~170,000 g/mol) were compared. Furthermore, for study of the effect of different molecular weights, a chitosan chloride with DA = 84% and M_w = 240,000 g/mol was degraded to lower molecular weights, 170,000 g/mol (thermal degradation) and 34,000 g/mol (nitrous acid degradation). The degradation to oligomers (DP_n ~ 13, DP_n ~ 50, DP_n ~ 100) was performed by Nobipol (NTNU, Trondheim, Norway) by acid degradation to prevent the formation of new reducing ends. A high molecular weight chitosan with DA ≥ 99.8 % was also provided by Nobipol. The degree of deacetylation was determined by UV spectra [6] or proton NMR spectroscopy [7]. The weight average of the molecular weight was determined by size-exclusion chromatography with multiple angle laser light scattering (SEC-MALLS) by Nobipol, and the DP_n of the acid hydrolysed chitosan was determined by NMR analysis and intrinsic viscosity analysis.

α–D-Glucosamine x HCl (2-amino-2-deoxy-α-D-glucopyranose x HCl) was obtained from GLYCON Biomedicals GmbH, Marburg, FRG. The hexamer of glucosamine x HCl was obtained from Bio-Test A/S, Oslo, Norway.

Cells: Caco-2 cells (ATCC HTB 37), grown *in vitro* as monolayer cultures, were seeded on Transwell Clear membranes (0.4 μm pore size) from Costar (The Netherlands). Eagles minimum essential medium , MEM (Sigma), supplemented with 10 % heat-inactivated FCS (Foetal Calf Serum) was used as the culture medium. The cells were kept at 37°C in an atmosphere of 95% air and 5% CO_2, and the medium was changed every second day. The cells were used for experiments 21-24 days after seeding.

Preparation of chitosan solution: Chitosan or chitosan oligomer solutions (1%, w/v) were prepared in tissue culture quality water (Milli-Q). The chitosan weight was adjusted according to the dry matter content and the salt content of the sample. The chitosan solution was filtered through a 0.45 μm sterile Millipore filter. Double strength (2x) medium, adjusted to pH 6.3, was added proportionally, 1:1, to the chitosan solution to give a final chitosan concentration of 0.5% (w/v). The control medium consisted of MEM + 10% FCS adjusted to pH 6.3.

Exposure of cells to chitosan: The Caco-2 cells cultured on Transwell Clear membranes were placed on a heating block maintained at 37°C whenever removed from the incubator. The transepithelial electrical resistance (TEER) across the Caco-2 monolayer was determined by using a Millicell ERS meter (Millipore). Treatment of cells with control or chitosan-containing medium was initiated by replacing medium in the apical chamber. New medium not containing chitosan was also added to the basolateral chamber. TEER values were recorded immediately after adding the chitosan solution. Chitosan was removed after a defined exposure time and the cells were washed three times with Hanks' balanced salt solution (pH 6.3) and one time with Hanks'(pH 7.4), and replaced by the medium (MEM + 10% FCS). TEER was measured at one to three days after removing the chitosan to study the reversibility effects. Between every measurement, the cells were kept at 37°C in an atmosphere of 95% air and 5% CO_2. Experiments were performed in triplicate.

Results and Discussion

The effect of chitosan chloride (Mw~170,000) with different degrees of deacetylation on the TEER of Caco-2 cell monolayers is shown in Fig. 1.

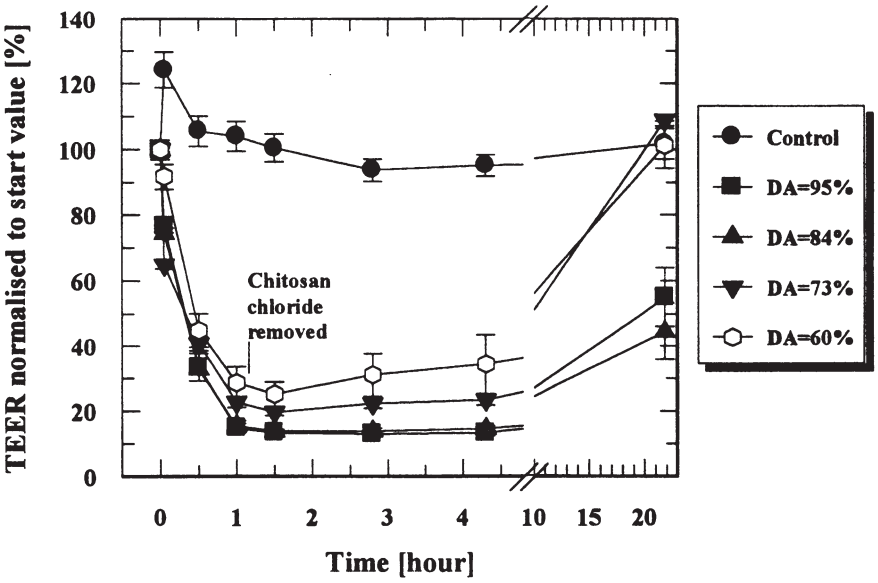

Figure 1. Effect of chitosan chloride (Mw~170,000, concentration: 0.5% (w/v)) with different degrees of deacetylation on TEER of Caco-2 cell monolayers. Each data point represents the mean of 3 replicate samples. Error bars indicate the standard error of the mean.

Addition of chitosan to the apical side of the Caco-2 cell monolayers resulted in an immediate and pronounced lowering in TEER values as compared to the control group. Only a slight additional decrease in TEER resulted when the exposure time to chitosan chloride was extended from 1 to 1.5 hours. The decrease in TEER values was in the order DA = 95%, DA = 84% > DA = 73% > DA = 60%. Our results demonstrate that all of the chitosans tested were able to decrease TEER values of the Caco-2 monolayers. Furthermore, this effect is about 15% greater for a chitosan with DA=95% than for a chitosan with DA = 60% and the same molecular weight. These results differ from the results obtained by Schipper *et al.* (1996) [4], who found that the action of increased epithelial permeability is much quicker for a chitosan with DA = 65% than for a chitosan with DA = 99%.

The reversibility of the effects of these chitosans on the TEER of the cell monolayers can also be seen from Figure 1. By replacing the chitosan solution with fresh MEM + 10% FCS after 1.5 h, the tight junctions slowly began to be re-established. A slight increase in TEER values towards the initial values was found. TEER values had returned back to initial values 24 hours post-exposure with chitosans of low degree of deacetylation (DA = 73%, DA = 60%), while TEER values following treatment with chitosans of high degree of deacetylation (DA = 95%, DA = 84%) had only reached approximately 50 % of the initial TEER values 24 hours after exposure. Because of the bioadhesive character of chitosan, it is difficult to remove all of the chitosan from the cell surface without damaging the cells. The

better reversibility of TEER observed for chitosans with low degree of deacetylation may be due to lower bioadhesive properties than for chitosans with higher degree of deacetylation. In a previous study [4] it was found that chitosans with a high degree of deacetylation (DA = 85-99%) showed a clear dose dependent toxicity by using an intracellular dehydrogenase activity to assess cytotoxicity.

The effect of chitosan chloride (DA = 84%) with different molecular weights (Mw > 34,000 g/mol) on the TEER of Caco-2 cell monolayers is shown in Fig. 2.

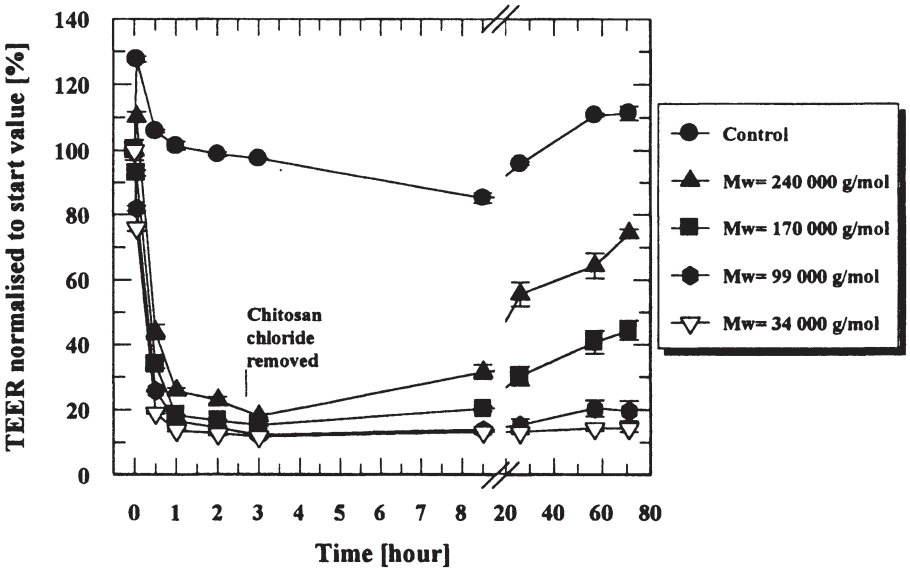

Figure 2. Effect of chitosan chloride (DA = 84%, concentration: 0.5% (w/v)) with different molecular weights (Mw > 34,000 g/mol) on TEER of Caco-2 cell monolayers. The data represent the mean of 3 replicate samples. Error bars indicate the standard error of the mean.

A pronounced and rapid lowering in TEER values resulted during the first hour after chitosan was applied to the Caco-2 cell monolayers. Furthermore, after a 3 hour exposure, TEER values were reduced to approximately 20% of the initial TEER values. The data in Figure 2 also demonstrate the reversibility of the effect of the different molecular weight chitosans on TEER values. As compared to initial values, an increase in TEER was only found for the two chitosans having the highest molecular weight (170,000 g/mol, 240,000 g/mol). TEER values did not return to a control level even after 3 days post-exposure. Because of the lack in reversibility observed in these data, we also investigated the relationship between exposure time to chitosan and reversibility of TEER.

In Fig. 3 the effect of chitosan chloride (DA = 84%) with high and low molecular weight on TEER of Caco-2 cell monolayers after different exposure times is shown.

The data in Fig. 3 demonstrate that the exposure time of the cell monolayers to chitosan is an important parameter for the reversibility of TEER. The TEER of Caco-2 cells exposed to chitosan chloride with high and low molecular weight for 1 hour, returned to initial values within a 24 hour post-exposure period. For cells exposed to chitosan with high

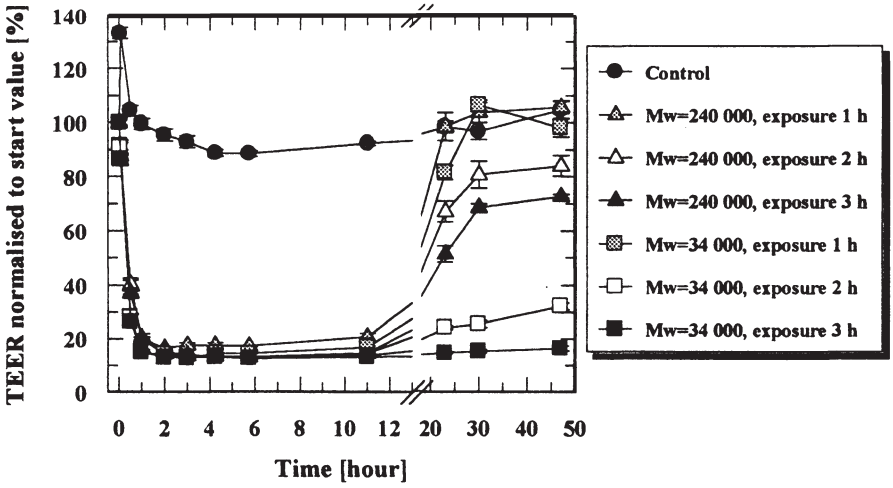

Figure 3. Effect of chitosan chloride (DA = 84%, concentration: 0.5% (w/v)) with high and low molecular weights on TEER of Caco-2 cell monolayers after different exposure times. The data represent the mean of 3 replicate samples. Error bars indicate the standard error of the mean.

molecular weight for 2 and 3 hours, a complete reversibility was not observed following a 2 day post-exposure period. TEER was re-established to only about 80 % of control values. Further-more, for cells exposed to low molecular weight chitosan for 2 or 3 hours only a slight recovery of TEER values was observed. Results shown here demonstrate, therefore, that the reversibility of TEER decreases when the molecular weight of chitosan is reduced and the exposure time increased. After completion of these experiments, the cells were also analysed by transmission electron microscopy (TEM). For cells exposed to chitosan with Mw = 34,000 g/mol for 3 hours, discontinuities and a reduced number of microvillies were found. This is in agreement with the results reported by Aspden *et al.* [5]. They found that lower molecular weight chitosans cause some cell damage and protein release. Additional experiments must be performed to further investigate the effect of low M_w chitosan on cells.

Fig. 4 shows that glucosamine monomer and hexamer had no effect on TEER. These results indicate that the positive charge itself is not enough to open the tight junctions between epithelial cells. It is apparently important that the chitosan polymer also is of a sufficient molecular weight.

To investigate the effect of chitosan chloride with Mw < 34,000 g/mol and DPn > 6, a chitosan was degraded by acid to prevent new reducing ends. The effects of the acid hydrolysed chitosan of low molecular weights on TEER of Caco-2 cell monolayers are illustrated in Fig. 5.

Fig. 5 shows that a pronounced lowering in TEER values to approximately 20% of initial values was induced following treatment of cells with chitosan having a $DP_n \geq 50$. The oligomer of $DP_n \sim 13$ induced only a slight 30% reduction in TEER. Acid hydrolysis caused some additional deacetylation. The oligomer of $DP_n \sim 13$ had a DA > 99.9 %. Therefore, a high molecular weight chitosan with DA > 99.8% was also tested, as shown in Figure 5. This high MW, highly deacetylated chitosan induced a large effect on TEER. This result indicates that lack of effect on TEER was due to low molecular weight of the oligomer. The slight

effect seen with $DP_n \sim 13$ may be due to the presence of larger molecular weight fractions within the molecular weight distribution in the sample, and is is probably this fraction of the sample that contributes with the reduction.

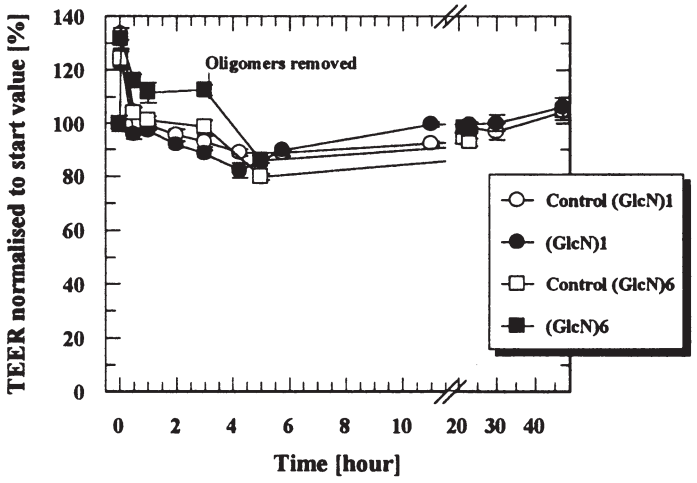

Figure 4. Effect of glucosamine $(GlcN)_1$ (concentration: 0.5% (w/v)) and glucosamine hexamer $(GlcN)_6$ (concentration: 0.5% (w/v)) on TEER of Caco-2 cell monolayers. The data represent the mean of 3 replicate samples. Error bars indicate the standard error of the mean.

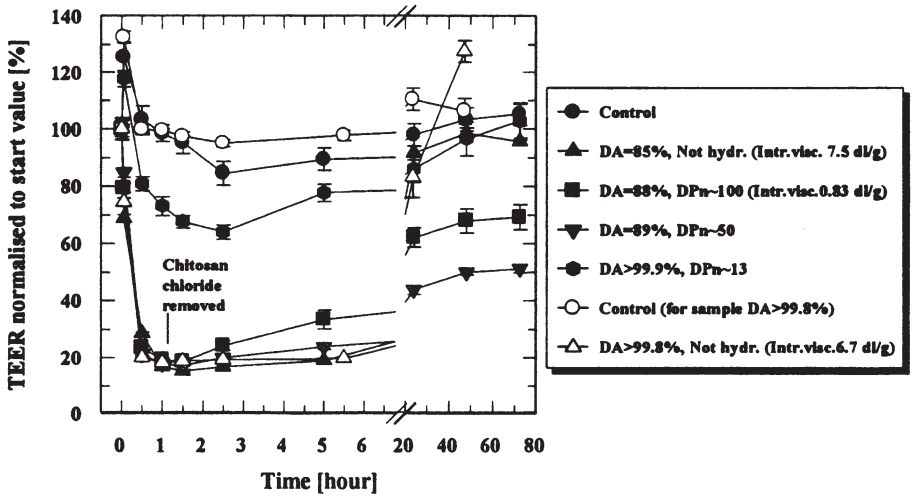

Figure 5. Effect of acid hydrolysed chitosan chloride (concentration: 0.5% (w/v)) with different chain lengths on TEER of Caco-2 cell monolayers. The data represent the mean of 3 replicate samples. Error bars indicate the standard error of the mean.

In conclusion, chitosan with DA = 60-100% and $DP_n > 50$ has been shown to increase the permeability of human intestinal epithelial cells. The effect on tight junctions was reversible if cells were exposed for a short duration (≤ 1 hour), and if the chitosan used was of a high molecular weight ($M_w > 200,000$ g/mol).

Acknowledgements: The authors are grateful to Nobipol for their assistance in preparing acid hydrolysed chitosan and in carrying out the NMR and SEC-MALLS analysis.

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Oral polymeric *N*-acetyl-D-glucosamine as potential treatment for patients with osteoarthritis

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Summary

We have evaluated the use of the orally ingested polymer of N-acetyl-D-glucosamine (POLY-Nag®) for sustained release of glucosamine in the treatment of osteoarthritis. Subjects received either the polymer or a placebo and were evaluated for pain relief and impact on quality of life. In addition, serum samples were analyzed for glucosamine and N-acetylglucosamine by high performance liquid chromatography. Results showed that oral ingestion of 1.5 gram per day of POLY-Nag® increased the serum concentration of glucosamine and improved the clinical assessment. Washout studies suggest that oral POLY-Nag® sustains a longer serum half-life than monomeric glucosamine. These data suggest that POLY-Nag® may be useful in the treatment of osteoarthritis.

Introduction

Osteoarthritis (OA) is the most common form of arthritis and a major cause of morbidity and disability of the elderly. It is estimated to affect approximately 12% of the U.S. population, increases with age and it is found in most persons over the age of 65 [1]. Pain is the primary symptom of OA. In the past, pain management has utilized acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs). The problem with this approach is that it does not address the underlying degenerative disorder, and some of these medications have serious adverse side effects.

The potential use of glucosamine, and its derivatives such as glucosamine sulfate, and N-acetyl-D-glucosamine (NAG) for the treatment of dysfunctions of connective tissue has been recognized for several years, first in veterinarian medicine and later in the treatment of OA [2]. Glucosamine is a substrate for glycosaminoglycan (GAG) synthesis, but also stimulates its synthesis and inhibits its degradation [3,4]. A variety of clinical trials have verified the safety and efficacy of glucosamine and its derivatives in the treatment of OA. For example, Qui, et al. [5] studied 178 patients suffering OA of the knee in a four-week, double-blind protocol comparing daily treatment of either glucosamine sulfate (1.5 g) or ibuprofen (1.2 g). Glucosamine sulfate was more effective and significantly better tolerated than ibuprofen. Reginster [6] conducted results of a randomized, placebo-controlled, double-blind, three-year trial with glucosamine sulfate. This study of 212 patients with knee OA showed

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Abbreviations: POLY-Nag®: Poly [N-acetyl glucosamine]; Registered trademark of Lescarden, Inc., N.Y., N.Y.; OA: Osteoarthritis; GAG: Glycosaminoglycan; NAG: *N*-acetyl glucosamine

25% improvement in the subjects receiving glucosamine, while placebo controls suffered a worsening in symptoms.

Although the use of glucosamine and its derivatives for the treatment of OA has a mechanistic basis, and clinical trials continue to support its use, questions remain concerning bioavailability, optimal dosage and delivery. The pharmakokinetics of glucosamine have been measured after oral, intravascular and intramuscular administration. Unfortunately, the half-life of glucosamine in the blood is relatively short [7]. Thus, a sustained release form of glucosamine would have many of attractive features.

We have examined the use of a polymeric form of NAG (chitin, or poly [N-acetylglucosamine] [POLY-Nag®, Lescarden, Inc., New York, N.Y.]). These studies were designed to assess (a) the bioavailability of NAG and glucosamine from oral administration of POLY-Nag® and (b) the effects of POLY-Nag® when administered to subjects with OA. A portion of the studies has been published previously [8].

Materials and Methods

POLY-Nag® was supplied by Lescarden, Inc.

Bioavailability study: This was a randomized, blinded, crossover study of 10 healthy subjects (5 men and 5 women; age range 36-50 years) who were randomly divided into two groups. On day one, a fasting blood sample was collected. Subjects in one group then orally ingested 1 gram of NAG and the subjects in the other group ingested 1 gram of POLY-Nag®. On day two, the subjects again ingested 1 gram of the appropriate test substance and on day three a fasting blood sample was obtained at T=0. Subjects then consumed the final 1 gram of the appropriate substance and blood samples were obtained at 1, 2, 4, 8, 24, and 48 hours thereafter. After a five-day washout period the two groups were switched and the experiment was repeated with the previous NAG ingestors taking POLY-Nag® and *vice versa*. The statistical evaluation of the data are presented elsewhere [8].

Clinical study: In a randomized, double-blinded study, five subjects received POLY-Nag® (1.5 gram per day) and five others a placebo. After randomization, all subjects were removed from any medications or dietary supplements containing glucosamine. After two weeks, a blood sample was obtained and baseline values established. The groups then initiated daily oral ingestion of either POLY-Nag® or the placebo. After an additional six weeks, they were reexamined and a blood sample analyzed. Clinical assessments included patient self-reported pain and physician assessment of pain, and an arthritis impact scale of quality of life. Routine laboratory analyses at each visit included complete blood counts, serum chemistries, and urinalysis. In addition, sera were monitored for levels of glucosamine and NAG HPLC analyses.

Results and Discussion

Bioavailability: Fig. 1 shows a profile of serum NAG levels during a 48-hour washout period after having ingested either NAG or POLY-Nag for three days. Serum levels of NAG (and glucosamine: data not shown) increased over the next few hours. After four hours, serum NAG began to decrease in subjects who had ingested NAG. In contrast, serum NAG levels in the POLY-Nag®-ingestors remained elevated.

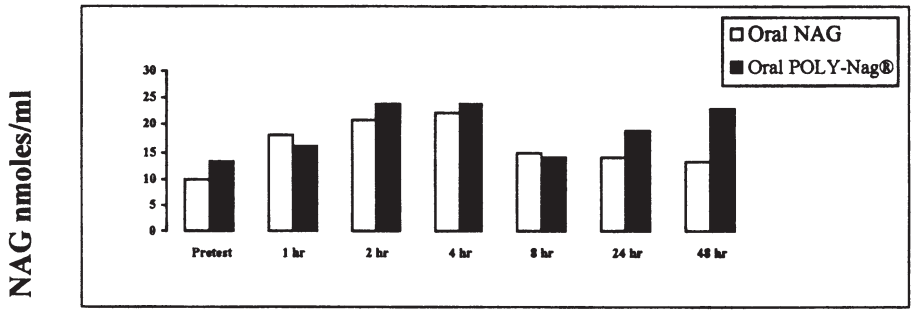

Figure 1. Changes in serum N-acetyl-D-glucosamine (NAG) following ingestion of either NAG (open bars) or POLY-Nag® (shaded bars). Subjects received the material orally for three days prior to this test as described in the text. Blood sera was analyzed at the indicated times during this washout period.

These data demonstrated that orally administered POLY-Nag® is hydrolyzed *in vivo* to NAG and glucosamine, and both are absorbed and distributed via the serum. It also appeared that oral ingestion of POLY-Nag® was providing a sustained delivery of NAG. There were no adverse reactions to ingestion of these levels of either NAG or POLY-Nag®.

These short-term pilot studies were encouraging and thus a clinical pilot study was undertaken using a different protocol. All subjects had existing OA, and the length of time for the oral administration of POLY-Nag® was increased. After initial screening and randomization, in a double-blinded study, five subjects received orally POLY-Nag® (1.5 gram per day) while the other five received a placebo.

Table 1 shows that after a six-week period the POLY-Nag®-ingestors showed an increase in both serum NAG and glucosamine. This is in contrast to the placebo group who showed a decline in levels of glucosamine.

Table 1. Changes in serum glucosamine and N-acetyl-D-glucosamine after ingestion of either POLY-Nag® or placebo.

Changes in Serum Glucosamine and N-Acetylglucosamine[a)]

Ingestion of:	Glucosamine	Nag
POLY-Nag®	+ 310.4	+175.1
Placebo	-333.2	- 76.6

[(a)] = Change in concentration (nmoles/ml) from analyses at T=0 (after 2 week washout) and 6 weeks later after ingestion of either POLY-Nag® or placebo.

Bioavailability: From the initial bioavailibility studies, it was clear that oral administration of POLY-Nag® resulted in hydrolysis of the polymer and elevation of levels of glucosamine and N-Acetylglucosamine in the serum. The data also suggested that levels of glucosamine and NAG reached approximately the same levels whether the polymer or the monomeric NAG was orally administered. Moreover, these short-term studies suggested that following cessation of administration of the compounds, serum levels of glucosamine and NAG were sustained at elevated levels longer than when the monomer was supplied.

Clinical Results: This brief pilot study done in a randomized, placebo-controlled fashion demonstrated that POLY-Nag® yielded symptomatic improvement in all the subjects who received it. There was a clear difference in the visual analog scale pain scores, patient global assessment and physician global assessments between the two groups. The impressive clinical outcomes in this pilot study will be confirmed in longer and larger studies.

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Clinicoimmunological efficiency of the chitin-containing drug Mycoton in complex treatment of a chronic hepatitis

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Summary
Studies of immunomodulating properties of Mycoton in the complex treatment of patients with chronic hepatitis were conducted in the clinic. The results of the research held have shown that Mycoton has an immediate influence on the immunocompetent cells normalizing of the production of interleukin-1 and level of immunoglobulins, increasing proliferatic activity of lymphocytes and phagocytic activity of neutrophils, normalising amount of CD4+ and CD8+ cells. The detoxication influence on an organism is also connected with the decrease of a circulating immune complex contents and increase an albumin transport function is also. Thus, the absence of the expressed by-effects with normalization of a combination of biochemical and immunological parameters allows positively estimating the results of preparation Mycoton application in a complex treatment of a chronic hepatitis.

Introduction

Chronic hepatitis takes one of the major places in the diseases of digestive organs. It is attributed to a high disease rate of acute hepatitis, alcoholic addiction, wide use of fertilizers and toxic chemicals as well as misuse of medicinal preparations [1, 2]. One of the major syndromes in the clinic of chronic hepatitis is the syndrome of endogenic intoxication whose acuity conditions the course and result of the disease [1, 3]. As a result, the organism accumulates intermediate and final products of normal metabolism with decompensation of regulatory systems and formation of their effector components in toxic concentrations. It results in the depression of both humoral and cellular immune response, thus fostering development of the secondary immune deficit [3, 4].

In this relation it is really urgent and advisable to search for optimal methods of detoxification as well as the ways to correct different forms of the immune system misbalance. One of the recognized and rather efficient detoxification methods is the enterosorption – the method based on fixation and removal of endogenic and exogenic substances, submolecular structures and cells from the alimentary tract with medicinal and preventive aim. Hemicellulose, pectin, lignin, carbons, silica gels organomineral sorbents are used as sorbents most frequently [4]. In the last several years a number of complex natural preparations have been created. One of them is a new chitin-containing drug Mycoton [7].

The objective of our work was to evaluate clinic-immunological efficiency of using Mycoton application in complex treatment of patients with chronic hepatitis.

Materials and Methods

Mycoton is a complex of natural biopolymers obtained from cell walls of Higher Basidiomycetes [5, 6]. It contains chitin in microfibrous form – 70%, β-1.3 and β-1.6 glucans in the amorphous form – 20% and melanin pigments – 10%. Mycoton possesses sorption, antioxidant and immune-modulation properties simultaneously. It is not toxic and licensed by the State Sanitary-Hygienic Expertise of the Health Care Ministry to be used as biological additive in meals.

Research is done at the Laboratory of Clinic Immunology and Allergology of the National Medical University in Kiev. Two groups of patients have been tested. Group 1 is experimental. These are 30 patients with chronic hepatitis aging from 28 to 62 years. They were treated with Mycoton along with traditional medicinal preparations used for chronic hepatitis treatment. Mycoton was prescribed for a patient *per os* by 0.5 g three times a day 1.5 hour before meal during 10 days. Group 2 is a control one. These are 10 patients with chronic hepatitis treated traditionally.

Before treatment and after it the patients were tested to identify the following indices:

1 – biochemical: concentration of common protein and its fractions, indirect and direct bilirubin, a level of aminotranferase activity (alaninaminotransferase – ALT and aspartateaminotrasferase – AST) and alkaline phosphotase, thymol test, fixing ability of albumin (tested in Congo Red marker);

2 – immunological: the quantity of T- and B-lymphocytes by the method of indirect immunofluorescence with monoclonal antibodies CD3+, CD4+, CD8+, CD19+, concentration of IgG, IgM, IgA by the method of radial immunodiffusion after Mancini, concentration of circulating immune complexes by the method of polyethylene glycol precipitation, interleukin-1 concentration (IL-1) using immono-enzyme method, quantity and functional activity of phagocytes by their ability to absorb latex particles.

Results and Discussion

Mycoton tolerance was good in all cases with no side effects. The research results are presented in Tables. Table 1 presents biochemical indices for patients with chronic hepatitis of two groups before and after treatment. The following changes have been observed in patients of group 1 which were treated with Mycoton along with traditional treatment: a 3-4 times decrease in content of conjugated and direct bilirubin in blood serum, a 3-fold decrease of aminotransferase activity, a 1.5-fold decrease of the level of alkaline phosphotase and thymol test, an increase of the total content of albumin by 53% and equalization of globulin fraction correlation, enhancing of transport functions of albumin, i.e. its fixing ability to the marker which confirms a detoxification effect of the drug.

Table 2 presents immunological indices of patients with chronic hepatitis in two groups before and after treatment. The following changes have been observed in patients of group 1 treated with Mycoton: an increase of the number of T-lymphocytes (particularly T-helpers) by 35%, a decrease of the level of CIC complexes 3-3.5 times, normalization in the content of IgG, IgM, IgA almost to the normal level, increase of the absorbing ability of phagocytes under inconsiderable decrease of their number. Changes in patients from group 2 (control) treated traditionally were observed later and were not so pronounced.

Table 1. Biochemical indices for patients with chronic hepatitis of two groups before and after treatment. p = 0.05

Indices	Normal level	Treatment with Mycoton		Traditional treatment	
		Before treatment	After treatment	Before treatment	After treatment
Common protein, g/l	65-85	58.2±2.6	76.2±2.1	59.1±2.8	65.1±2.8
Globulin, g/l	20-30	40-45	30-35	40-45	35-40
α_1 ,%	3-4	5.6±1.8	2.4±1.4	5.7±1.6	4.2±1.1
α_2 ,%	5-6.5	10.2±2.2	5.8±1.1	10.3±2.1	8.8±1.8
β, %	7-12	16.2±2.0	9.2±1.2	16.6±2.0	14.1±1.6
γ , %	10-16	31.6±2.3	18.1±1.1	31.8±2.1	23.7±2.4
Albumin, g/l	40-50	30-35	40-45	30-35	38-40
Common bilirubin, mmol/l	8-20	92±12	28±10	93±11	38±8
Direct bilirubin,mmol/l	5-12	68±8	20±6	69±7	28±5
Indirect bilirubin, mmol/l	2-8	24±4	8±4	24±4	10±3
Thymol test, Unit	0-4	7.3±1.2	3.2±1.3	7.1±1.1	4.8±0.8
AST activity, mmol/g*l	0.1-0.68	2.48±0.42	0.82±0.11	2.20±0.41	1.1±0.26
AST activity, mmol/g*l	0.1-0.45	1.96±0.30	0.55±0.10	1.92±0.32	0.98±0.21
Alkaline phosphotase mmol/g*l	1-3	7.5±1.,5	3.0±1.1	7.4±1.6	4.8±1.6
Fixing ability of albumin, mkg Congo Red/mg protein	0.054-0.170	0.02±0.01	0.11±0.03	0.021±0.011	0.038±0.012

Table 2. Immunological indices for patients with chronic hepatitis of two groups before and after treatment. p = 0.05

Indices	Normal level	Treatment with Mycoton		Traditional treatment	
		Before treatment	After treatment	Before treatment	After treatment
IgG, g/l	11.5±3.05	26.7±4.8	14.8±2.1	26.9±4.2	20.6±2.3
IgM, g/l	0.99±0.27	2.88±0.3	1.48±0.21	2.84±0.2	1.98±0.19
IgA, g/l	1.3±0.2	1.98±0.2	1.41±0.22	1.97±0.2	1.58±0.21
CIC, g/l	0.04-0.09	0.48±0.06	0.13±0.02	0.47±0.08	0.28±0.03
CD3+	40-60	33.8±5.2	56.6±6.08	33.6±4.8	42.1±3.2
CD20+	20-26	31.2±4.15	27.4±4.25	31.4±4.05	29.2±3.12
CD4+	33-41	26.4±1.2	38.3±1.4	22.8±1.1	28.1±1.2
CD8+	10-19	21.6±1.4	16.1±1.8	21.8±1.2	19.2±1.1
Phagocytic cells, %	40-90	81.2±3.75	61.1±2.3	81.7±3.25	75.1±2.4
Phagocytic absorption activity, unit	2-8	6.21±0.37	10.2±1.1	6.28±0.31	8.1±1.1

Table 4 presents the results of research of the Mycoton effect on the level of IL-1 content in blood of patients with chronic hepatitis. The control group consisted of practically healthy people (table 3). Spontaneous and induced production of IL-1 was investigated. As is seen from the table 4 a decrease of IL-1 production down to the index norm was observed in all tested patients.

Table 3. Influence of Mycoton on IL-1 secretion in vitro (control group). p=0.05

Blood-donors	Spontaneous production of IL-1, pg/ml	Induced production of IL-1 (with Mycoton), pg/ml
1	190.77	220.92
2	183.32	209.07
3	144.74	192.42
4	130.92	170.14
5	155.38	165.71
6	177.86	213.25
7	146.11	169.43
8	130.16	158.52
9	170.56	196.33
10	151.25	186.66
M ± m	158.10±6.42	188.24±6.55

Table 4. Mycoton effect on the level of IL-1 content in blood of patients with chronic hepatitis. p=0.05

Patients	Spontaneous production of IL-1, pg/ml		Treatment with Mycoton, pg/ml	
	before	after	before	after
1	212.9	102.03	210.3	109.9
2	196.82	144.38	166.3	140.56
3	264.25	129.9	190.5	129.7
4	200.6	132.2	170.43	144.31
5	274.1	151.8	269.0	162.6
6	267.9	158.8	256.7	165.4
7	243.2	179.8	220.9	196.2
8	244.15	160.2	205.4	166.4
9	258.9	182.34	252.5	180.9
M ± m	240±9.3	149±7.9	215 ±11.7	155±8.3

The results of the research in vivo have shown that Mycoton has an immuno-modulative effect. Mycoton efficiency is determined by a complex of medical-biological properties of macromolecules in its composition – chitin, glucans and melanins which are well known in the medicine. ***Chitin*** adds to Mycoton unique high sorption properties in relation to heavy metal ions (Pb, Hg, Bi, Sr) and radionuclides (U, Pu, Am, Cs, Sr and others) which are delivered to an organism together with food. At the same time chitin is indifferent to basic biogenic microelements (Na, K, Ca and others) [7, 8] which means that chitin does not disturb salt metabolism in the organism. Mycoton sorbs bacteria, viruses and many endotoxins accumulated as a result of liver, kidney and alimentary tract disturbances. Besides, it facilitates regeneration of liver cells and liver better functioning. Research of the action of polysaccharides on immune reaction has shown that there are no components in the immune system that react to immunomodulators of polysaccharide nature. The action of fungal ***glucans*** is related to macrophages and lymphocytes as basic components of the organism protection system. However, the nature of the immunomodulative action of glucans has not

yet been fully investigated. ***Melanins*** are powerful bioprotectors due to the presence of lots of paramagnetic centers able to neutralize free radicals which appear in the organism under the action of radiation, chemical and bacterial toxins [8, 9]. Besides, fungal melanins are good sorbents for heavy metals [10, 11].

According to the results obtained, Mycoton is a good radioprotector [12] while chitin-glucan complex exposes strong bacteriostatic properties [13]. Mycoton is a good haemostatic and a sedative remedy. It considerably accelerates healing of different wounds.

Thus, clinic-immunological efficiency of Mycoton is determined by its immune-modulative effect which is attributed both to the deintoxication action of the drug proved by the increase of the transport function of albumin and decrease of the level of circulating immune complexes and to direct immunomodulating action on immune-competent cells. Both data from the literature and our experimental data testify to this fact.

The above makes it advisable to recommend Mycoton for complex treatment of chronic hepatites and testifies to the necessity to further research mechanisms of Mycoton action which is promising.

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Interactions of chitin, chitosan, N-lauryl chitosan and N-dimethylaminopropyl chitosan with olive oil

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Introduction

Chitosan is being marketed for human consumption in various countries. The claimed efficacy of chitosan in reducing hypercholesterolemia, hypertension and body weight, stimulated industrial production of chitosan tablets in spite of inadequate scientific knowledge about the reactivity of chitosan towards lipids. Certain promotional campaigns presented chitosan as a fat-binder active toward all kinds of lipids with no supporting scientific evidence, thus rising the protest of consumer associations.

Scope of the present study was the verification of the effect of chitosan, chitin, N-lauryl chitosan and the new N-dimethylaminopropyl chitosan on olive oil, i.e. their respective capacity to retain preferentially some component when contacted with the oil.

Experimental

Olive oil (Olio Sasso, Italy) was percolated through a bed of polysaccharide powder. Both the effluent and the imbibition fraction were retained for analysis. The analytical procedure is presented in Scheme 1. Squalane, $C_{30}H_{62}$, internal standard (10 µl of 10 % benzene solution) and nonadecanoic acid C19:0, internal standard (200 µl of 1 % benzene solution) were added to the olive oil (1 g). Then the saponification procedure was applied. The fatty acids were methylated with diazomethane in ether, and analysed with a Carlo Erba Mega 5160 gaschromatograph equipped with a Mega 2 integrator. The analytical conditions are in Table 1.

Preparation of N-DMA chitosan

3-Dimethylaminoacrolein, $(CH_3)_2N\text{-}CH{=}CH\text{-}CHO$, offers a pre-formed dimethylamino function, and high reactivity as an unsaturated aldehyde. It would easily lend itself to Schiff reaction: preparations have been carried out under homogeneous and heterogeneous conditions, the latter being devised to obtain a powder of the same grain size as the chitin and chitosan.

Table 1. *Gaschromatographic working conditions.*

	Fatty acids	Diacylglycerols	Unsaponifiable*
Stationary phase	SP 2330	TAP	TAP
Internal diameter (mm)	0,25	0,32	0,32
Length (m)	60	25	25
Film thickness (μm)	0,25	0,1	0,1
Injection technique	split system	split system	Split system
Carrier gas (kPa)	1,3	2	1
Split ratio	1/60	1/80	1/80
Carrier gas	He	He	He
Oven temperature (°C)	150 →230	200 →355	200 →300
Temperature rate	Isoth. 1'; 3 °C/min	3°C/min	Isoth. 1'; 3°C/min
Detector temperature (°C)	240	360	340
Injector temperature (°C)	240	360	340
Detector	F.I.D.	F.I.D.	F.I.D.

* Analytical determination made on the whole unsaponifiable.
F.I.D. = flame ionization detector.
TAP = Chrompack.

Preparation of N-lauryl chitosan

Chitosan (10 g) was suspended in a water-methanol 1:1 mixture (134 ml), lauryl aldehyde (15 g) was added and stirring protracted for 30 min. Reduction was carried out as indicated above, and left overnight. After filtration and washing with water-methanol and methanol, the powder was dried at 50°C. Yield 25 g.

When olive oil was percolated through a bed of chitin or modified chitin of comparable mesh size, the amounts of oil retained in the dry powder varied depending on the chemical functions carried by the polysaccharide. In practice, when 15 g of oil were percolated at 25°C, 10.8 g were retained in the chitin, 5.5 g in the DMAP-chitosan and 4.0 g in chitosan or in N-lauryl chitosan, as shown in Table 2 for 2 g of each powder.

The fatty acid composition of the oil was not appreciably altered, as a consequence of the contact with the polysaccharides: Table 3 shows that the concentrations of twelve fatty acids remained substantially the same in the percolated and in the retained fractions for all systems tested, in spite of the different amine nitrogen content, moisture content and hydrophobicity of the powders, and regardless of the degree of insaturation of the fatty acids (compare for instance C18:0, C18:1 and C18:2 in Table 3.

Table 2. ***Quantity of oil percolated and retained by 2 g of chitin derivatives***

Polysaccharide	g	Oil, g	Percolated, g	Retained, g
Chitosan	2	15	11	4
Chitin	2	15	4.2	10.8
Lauryl chitosan	2	15	11	4
DMA chitosan	2	15	9.5	5.5

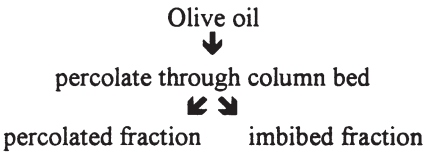
Table 3. ***Composition (%)* of fatty acids in the percolated and retained fractions. Determined as methyl esters.***

Sample	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$
Oil	<0.1	12.9	1.2	<0.1	0.1	2.6	72.7	9.2	0.6	0.4	0.2	0.1
Chitosan												
Percolated	<0.1	12.9	1.2	<0.1	0.1	2.6	72.6	9.2	0.6	0.4	0.3	0.1
Retained	<0.1	12.9	1.2	<0.1	0.1	2.6	72.5	9.2	0.6	0.4	0.3	0.1
Chitin												
Percolated	<0.1	12.9	1.2	<0.1	0.1	2.6	72.8	9.3	0.6	0.4	0.3	0.1
Retained	<0.1	12.9	1.2	<0.1	0.1	2.6	72.7	9.2	0.6	0.4	0.3	0.1
Laurylchit.												
Percolated	<0.1	11.9	1.2	<0.1	0.1	2.1	74.0	9.4	0.6	0.3	0.3	0.1
Retained	<0.1	13.1	1.2	<0.1	0.1	2.5	72.4	9.2	0.6	0.4	0.3	0.1
DMAChitos.												
Percolated	<0.1	12.5	1.1	<0.1	0.1	2.6	72.9	9.3	0.6	0.4	0.3	0.1
Retained	<0.1	12.8	1.2	<0.1	0.1	2.6	72.6	9.2	0.6	0.4	0.3	0.1

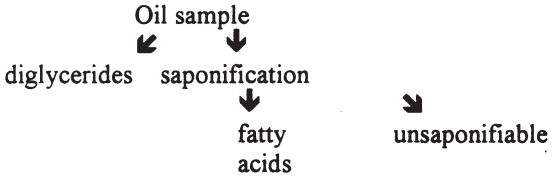
* Percent calculated on the total fatty acids, based on the relevant HRGC areas.
$C_{n:m}$ (n = number of carbon atoms, m = number of double bonds).
$C_{14:0}$ = mirystic; $C_{16:0}$ = palmitic; $C_{16:1}$ = palmitoleic; $C_{17:0}$ = margaric; $C_{17:1}$ = heptadecenoic; $C_{18:0}$ = stearic; $C_{18:1}$ = oleic (ω-9); $C_{18:2}$ = linoleic (ω-6); $C_{18:3}$ = linolenic (ω-3); $C_{20:0}$ = arachitidonic; $C_{20:1}$ = gadoleic; $C_{22:0}$ = beenic.

On the other hand, the diacylglycerol content of the percolated oil was deeply altered. The percolated oil was depleted of diacylglycerols (lowered to 42 % of original concentration) when contacted with chitosan and N-lauryl chitosan, while the oil fraction percolated through chitin was 30 % enriched with diacylglycerols; DMAP chitosan was the most effective in retaining them. It might be possible that the alcohol function present in the diacylglycerols is important for establishing an interaction with the polysaccharides.

Scheme 1. ***Analytical procedure adopted for olive oil contacted with chitin derivatives.***



On each oil sample (control, percolated fraction and imbibed fraction), gas-chromatographic data were obtained on diglycerides and, after saponification, on fatty acids and unsaponifiable.



The analysis of the unsaponifiable fraction revealed that the concentrations of the steroids present in the olive oil were altered as a consequence of the contact with the polysaccharides. Remarkable cases were those of campesterol, stigmasterol and avenasterol, which were enriched in the fraction retained on chitin and on N-lauryl chitosan, while the β-sitosterol concentration increased slightly in the fraction retained on chitosan and N-lauryl chitosan. α-Tocopherol did not appear to be retained by chitin and chitosan. As for the triterpene alcohols 24-methylenecycloartanol and citrostadienol, their concentrations were high in the fraction retained by chitin. The steroid profile of the unsaponifiable fraction was remarkably altered as a consequence of the passage through the polysaccharide powders.

Conclusions

The present preliminary study provides for the first time analytical data on the interactions between chitin and dietary oil, and indicates that certain olive oil components may be preferentially retained in the oil fraction the imbibes the chitin or the chitin derivative. This is verified in particular for plant steroids. Chitin is more effective in holding olive oil, and enriching the retained oil fraction with steroids.

Table 4. Composition (%)* of the unsaponifiable fractions

Sample	Squalene	α-Toco-pherol	Campe-sterol	Stigma-sterol	β-Sito-sterol	Δ^5-Avena-sterol	24-Methylene cycloartanol	Citrosta-dienol	o.p.
Oil	57.8	0.2	<0.1	0.9	24.0	4.1	9.0	4.0	<0.1
Chitosan									
Percolated	57.5	0.2	1.0	0.9	24.5	2.0	9.0	3.5	1.4
Retained	64.3	<0.1	<0.1	<0.1	29.6	1.8	1.5	0.8	2.0
Chitin									
Percolated	67.7	0.4	1.0	0.9	25.0	<0.1	4.1	0.5	0.4
Retained	58.1	<0.1	1.0	0.9	25.9	1.7	8.6	3.2	0.6
Laurylchit.									
Percolated	60.0	0.2	<0.1	0.4	25.7	<0.1	2.7	0.3	10.7
Retained	56.4	0.2	0.9	1.0	33.9	1.9	0.9	3.9	<0.1
DMAchit.									
Percolated	60.0	0.1	1.0	0.9	25.4	1.7	8.0	2.9	<0.1
Retained	73.6	0.1	0.8	0.4	19.9	<0.1	4.1	1.1	<0.1

* Percent calculated on the whole of HRGC areas in terms of TMS

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The chitin-containing preparation Mycoton in a pediatric gastroenterology case

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Summary
A new strategy of the children chronic gastroduodenites treatment is proposed. The key points of the strategy are its active influence on infection factor and organism detoxication using chitin-containing preparation Mycoton having simultaneously powerful sorption, anti-infectious and immunomodulation activities. Mycoton showed its high effectiveness during treatment of children. Its use rapidly diminished organism infection, blood plasma immunotoxicity, normalized immune response parameters, decrease autoallergy and level of autoimmune reactions.

Introduction

Chronic gastroduodenites (CGD) make up 50-60% of all digestive organ diseases and are widely spread among children. During CGD infection, the intoxication syndrome connected with proper infection, immunologic disorders and vegetative disfunctions play an important role. Traditional medical tactics is not effective. Despite the treatment undertaken, disease takes a course with frequent relapses and sharp complications threatening health and life of children. These circumstances substantiate the necessity to improve the CGD therapy, to search new, effective and pathogenically appropriate methods of the treatment.

The key points of new strategy are its active influence on infection factor and organism detoxication using chitin-containing preparation Mycoton, which simultaneously possesses anti-infective, detoxicative and immunomodulative activity.

Patients

In the Kiev Consultative Gastroenterologic Centre complex clinic-laboratory examination of 192 children at the age of 4-15 years with chronic gastroduodenites was carried out. Among patients there are: 53 patients with *Candida albicans* infection – C group; 49 - with *Herpes vulgaris* – H group; 47 - with *Staphylococcus aureus* and *St. epidermidis* – S group,: in monoculture – 50.9%, in associations with: conditional-pathogenic microflora – 28.6%, and-fungal *Candida albicans* infection – 12.8% of cases. Intestinal disbacteriosis was revealed in 92.1%, modification of oral cavity biocenosis was observed in 78.4% of cases.

Sick children were divided into two groups: patients who received treatment with preparation Mycoton and patients who received traditional treatment.

Control group was 107 children practically healthy pupils of the same age.

Materials and Methods

To estimate colonization resistance we studied the sIgA level in the saliva and indices of cytological examination of oral cavity mucous tunic in a test of radial precipitation.

Adhesive lymphocytes peculiarities were studied *in vitro* on the model of:

- rosette formation with sheep erythrocytes,
- in complimentary rosette formation (with sheep erythrocytes, processed by complement C3-fraction and antibodies Fc-fragment, B-lymphocytes),
- in rosette formation with own erythrocytes, T-autosensitized lymphocytes.

Besides, specific antigens, red blood cells express HLA-antigens as well: HLA-A2, B5, B40, B17 [1]. The natural resistance system activity was estimated by the capacity of peripheral blood neutrophils and monocytes to restore nitroblue tetrazolium. The Mycoton action was studied *in vivo* on 32 children with CGD. Mycoton was used *per os* 0,5 g thrice a day during 30 days. Blood samples were made before and after the course of treatment.

Results and Discussion

During CGD the stimulating agent is both by constantly acting infectious antigens and by those of autologic tissues in combination with products of microbe metabolism, medicinal remedies and tissue detritus. Chronic infection in child organism (regardless of the microbe type) is associated with NBT-test parameters, which are considered to be indicators of the phagocyte function and at the same time of the antimicrobic activity of the neutrophils and monocytes. High level of immunogram indices is another proof of the intensive stimulus presence in inner media. Often CGD is combined with allergic diseases and with reactions of medicamental and alimentary allergy. Histamine (classic allergy mediator) added to test-systems of children with CGD, is able to stimulate considerably initially elevated T-lymphocytes adhesion with autoerythrocytes. Authors claim that it may be an evidence of allergic component presence at CGD. So, antiallergic treatment is needed.

The other peculiarity of CGD that was revealed in children – the capacity of own blood plasma to increase the T-lymphocytes adhesive activity with own erythrocytes. This fact is usually associated with immunointoxication properties calls for sorption therapy. It is necessary to define adaptation reaction towards such adverse factors as: the presence of intensive stimulus (infection and own tissue antigens, toxins, etc); allergic reactions involvement in the immunopathogenesis; autologic plasma immunotoxicity.

Manifestation and type of this reaction depend on individual characteristics of the organism and on stimulus intensity: weak stimuli – training reactions; average intensity stimuli– activation reactions (calm and higher); strong stimuli – stress reactions. It was determined that each of the reactions is characterized by the complex of complicated and mutually conditioned changes in central nervous and endocrine systems and which affects morphological composition of white blood – leukogram.

To define the predominant type of adaptive reaction in children with CGD, leukograms were studied. A tendency to increase total pool of circulating lymphocytes especially during chronic infection caused by *Herpes vulgaris* virus is inherent. The most considerable growth is shown by lymphocytes, mature neutrophils and eosinophils, which on the whole are natural for stress reaction. Under such conditions even ordinary therapeutic doses of pharmacological

compounds act as independent additional "stressors". Therefore the strategy is that which is oriented to reduce doses and number of medicines to be used only.

It is necessary to use complex polyvalent remedies with the following properties: to decrease the level of catabolism products and free radicals (which is the base of endointoxication) and to restore activity of the hematological and immune system responsible for the realization of systemic general inflammatory reaction.

We propose Mycoton - ***chitin-containing preparation,*** as an active agent for CGD treatment. Mycoton is received from cell walls of fungi (*Higher Basidomycetes*). This preparation has the following components: chitin - 70%, β-1,3- and β-1,6-glucans – 20%, and melanin pigments - 10% [2]. Preparation Mycoton is not toxic and has a permission of Ministry of Health of Ukraine and to be used as an biological active nutritional supplement.

Chitin and its derivatives are known to have the following properties:

1) sorption activity towards: serum albumin, alpha-globulin and fibrinogen [3]; bacterial endotoxins [4]; bacteria – *Escherichia coli, Staphylococcus aureus, St. epidermidis, Salmonella typhi, Pseudomonas aeruginosa, Bacillus subtilis, Micrococcus luteus, Streptococcus mutants, Vibrio cholerae, V. parahaemolyticus Proteus vulgaris*; *Enterobacter, Klebsiella* [5, 6, 7];

2) antiviral activity towards: hepatitis viruses [8]; phages [9]; pathogenic viruses [10];

3) fungicidal activity: *Candida albicans* [5, 11].

4) immunomodulating activity: increase the production of some cytokines, activate macrophages and natural killer cells [3, 12-14]; activate of specific immune response and normalise of autoimmunity [15].

Due to high specific surface of chitin microfibrils (more than 1000 m^2/g) Mycoton is effective in small doses. The extreme sorption activity of this preparation relative to heavy metals (Pb, Hg, Bi, Cr) and radionuclides (U, Pu, Am, Cs, Sr and other and chitin is indifferent to basic biogenic elements (Na, K, Ca, etc.) [16].

Fungal glucans are effective as immunomodulators. Their activity is associated with macrophages and lymphocytes, which are the main components of organism protection system. Melanins are powerful bioprotectors, due to the presence of large amounts of paramagnetic centers in molecules, able to neutralize free radicals which emerge in the organism under the influence of penetrating irradiation, chemical and bacterial toxins [4, 17].

Pronounced positive clinical dynamics when Mycoton correlated with qualitative leukogram changes (Table 1). As a result of Mycoton treatment of children with CGD already after the very first course there was observed a tendency to diminishing of all types of cells, especially eosinophils, stab and mature neutrophils and, that is leukogram changed towards the normal level. The data received are an integral index diminishing the intensity of stimulus influence on inner media of child body with CGD.

Table 1. Mycoton course action on leukogram.

Leucogram index, cells quantity x10^9/l	**Before treatment**	**After treatment**	**% indices deviation**
Leukocytes	5826 ± 493	5150 ± 374	- 12.0
Lymphocytes	2774 ± 216	2508 ± 168	- 10.0
Neutrophils: mature	2495 ± 390	2163 ±255	- 13.4
stab	69 ± 18	50 ± 7	- 25.4
Monocytes	218 ±32	216 ± 19	- 1.0
Eosinophils	270 ± 39	185 ± 13	-31.5

Similar trends were registered during research of Mycoton influence on functional indices of the natural resistance system responsible for anti-infectious protection (Table 2).

Table 2. NBT-test indices before and after the Mycoton therapy course.

Index, cells quantity x10^9/l		Before treatment	After tratment	% indices deviation
Neutrophils:	spontaneous	1720±195	1489±161	-13.5
	induced	1813±201	1597±175	
Monocytes:	spontaneous	168±18	144±16	-15.3
	induced	169±19	165±19	

As a result of Mycoton treatment of the disease with expressed infection process a tendency was observed to a decrease of the excessively expressed phagocytating cells activity.

According to authors point of view the above data confirm a decrease in infectious agent influence on the colonized organism. Mycoton inclusion into therapy led to changes in *Lactobacteria* quantity and increased colonizing resistance of oral cavity tunic (Table 3).

Table 3. Colonization resistance of oral cavity tunic during the treatment process.

Group of children	Before treatment	After treatment
With Mycoton therapy	4.3±0.1 lg	5.9±0.1 lg
With traditional therapy	4.6±0.2 lg	4.7±0.1 lg
Healthy children	6.9±0.1 lg	-

We also revealed the growth of sIgA. SIgA is known to be responsible for the state of local immunity humoral mechanisms against viruses, bacteria, fungi (Table 4).

Table 4. The state of local immunity of oral cavity in children with CGD

Index	Mycoton-therapy		Traditional therapy		Healthy children
	before	after	before	after	
sIgA, g/l in saliva	0.72+0.01*	1.16+0.02*	0.69+0.02**	0.92+0.01**	1.20+0.02
Mature neutrophils	13.84+1.13*	2.99+0.27**	14.06+1.09**	9.05+0.9*	2.02+0.19

*$p<0.05$ concerning data before treatment and healthy children,
**$p<0.05$ concerning data after treatment and healthy children,
***$p<0.05$ concerning data after treatment and healthy children.

Main mechanisms of anti-infection effect of the proposed treatment:

1) direct absorption of infective agent (for example, in digestive tract),
2) indirect stimulation of specific immune function by polysaccharide complexes:
   - induction of direct bactericidal activity of induced polymorphonuclear cells and antimicrobic cytokines such as tumor necrotic factor and interleukin - 1;
   - activation of haloid myeloperoxide antibacterial system of micro- and macrophages;

- normalization of free-radicals processes in tissues by natural antioxidants.

Taking into consideration aseptic activity mechanisms of the proposed treatment we consider that this treatment does not cause complications such as antibiotics do. The data demonstrate a decrease in T and B-chain indices of specific immune response. Especially it concerns the part responsible for interaction with organs and tissue antigens represented on the own erythrocytes. It shows high probability of autoimmune mechanisms involvement into general inflammatory reaction (Table 5).

Table 5. Immunological indices of children before and after the Mycoton therapy.

Index, cells quantity x10^9/l	**Before treatment**	**After treatment**	**% of indices deviation**
T-lymphocytes	2100±224	1485±156*	-29.3
B-lymphocytes	1849±196	1338±141*	-27.6
T-autosensitized lymphocytes	375±39	118±15*	-68.5

*$p<0.05$ between data before and after therapy

The normalizing action of the Mycoton therapy is also revealed during dynamic estimation of immunotoxic properties of autologic plasma in children with CGD (Table 6).

Table 6. Autoplasma influence on adhesive capacities of T-cells in children.

Index, cells quantity x10^9/l	Before treatment	After treatment
T-autosensitized lymphocytes	375±39	118±15
T-autosensitized lymphocytes +plasma	435±42*	116±18

*$p<0.1$

The data obtained show that the use of sorption potentialities of Mycoton rapidly lowers the content of T-lymphocytes in blood circulation which interact with antigens of own tissues, and also of immunotoxic properties of native autoplasma (Table 7).

Table 7. Histamine action on the adhesive capacity of T-lymphocytes in children.

Index, cell number x 10^9/l	Before treatment	After treatment
T-autosensitized lymphocytes	375+39	118+15
T-autosensitized lymphocytes +histamine	615+71*	130+15

*$p<0.1$

These data evidence for a rapid decrease of lymphocytes quantity in the process of Mycoton therapy and also for a reduction of autoallergy reactions.

Conclusions

1. CGD is a complex disease. It is characterized by the: presence of infection, which induces intoxication syndrome, with expressed autoallergy and immunity activation.

2. New strategy to treat CGD was proposed. This strategy lies in an active affect on infection factor and absorption of toxic substances of different genesis by a chitin preparation.

3. Chitin-containing preparation Mycoton was tested as a remedy having simultaneously powerful sorption, anti-infectious and immunomodulation activities.

4. Mycoton showed its high effectiveness during treatment of children with CGD. Its use rapidly diminished organism infection, blood plasma immunotoxicity, normalized immune response parameters, decrease autoallergy and level of autoimmune reactions. The results received testify that the CGD therapy with application of Mycoton is on the average 1.6 times as effective as a traditional one.

5. Chitin preparations are promising treatment of diseases with complicated genesis, which are followed by infection, intoxication, allergy and immunodeficit conditions.

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Antifungal activity and release behaviour of cross-linked chitosan films incorporated with chlorhexidine gluconate

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Summary
Free and cross-linked chitosan films were prepared by solvent casting method. Chlorhexidine gluconate (Chx) which is an effective therapeutic agent for oral candidiasis was chosen as the candidate drug. The films were characterized by determination of water absorption capacity, drug release and antifungal activity. Water absorption capacity and the release of drug from the films decreased with increasing cross-linking agent, tripolyphosphate concentration. The release of Chx from chitosan films was maintained for 4h reaching a plateau after 1.5h without a lag-time. The antifungal activity of Chx incorporated into chitosan film was found to be more pronounced when compared to that of Chx itself.

Introduction

A major difficulty for the successful eradication of fungal infections appears to be the dilution and rapid elimination of the drugs from the oral cavity due to the flushing action of saliva. The delivery system is therefore an important consideration in prolonging retention of drug in the oral cavity. Bioadhesion has been reported to improve the oral therapy for periodontal diseases and mucosal lesions by prolonging the retention time. A controlled release system could increase patient compliance and reduce side-effects compared to conventional dosage forms because less drug is required to achieve the desired therapeutic effect as a result of the continuous presence of the drug at the site of action.

Using natural polymers as drug carriers has received considerable attention in recent years. Chitosan is a natural cationic polymer, which has been used as a potential vehicle for application of drug to the oral cavity due to its bioadhesive and antifungal properties [1,2].

Chlorhexidine was chosen as the candidate drug. It is a broad-spectrum antimicrobial that is effective in both the prevention and treatment of oral candidiasis [3]. Incorporating Chx into chitosan gel was found to increase its antifungal activity indicating that chitosan gel formulation would enable application of Chx at lower concentrations [2]. This might be important because of the staining effect of Chx on teeth [4].

In this study chitosan in film form, either free or cross-linked was investigated as a suitable vehicle for local delivery of Chx into oral cavity. Antifungal activity of chitosan films either with or without Chx incorporated was studied.

Materials and Methods

Chitosan-H (Lot 337) (Dainishiseika Colour and Chem. MGF Co., Ltd. Japan), chlorhexidine digluconate (Lot 65HO427) (Sigma Chem. Co., St. Louis, MO, USA), tripolyphosphate pentasodium salt (TPP) (Sigma Chem. Co.) and lactic acid (E. Merck Germany) were used in this study. All other chemicals were of analytical grade.

Preparation of chitosan films: The chitosan films were prepared by solvent casting method. 1% chitosan gel was prepared in dilute lactic acid solution (1% w/v). Glycerine was added at 10% (w/w) concentration as plasticiser. Chitosan gel either containing Chx or not was poured into glass petri dishes (0.5 g per 1 cm^2) and oven dried at 40°C. Cross-linking was done by soaking the films into aqueous solution of TPP for 5 minutes. TPP was used at two different concentrations (0.1% or 0.5% (w/v)). The films were than placed on a glass plate and oven dried at 40°C. Thicknesses of the films were between 300 to 400 μm which being thicker by cross-linking.

Water absorption capacity: The water uptake was determined gravimetrically. Weighed films were placed in the bottles containing 50 mL distilled water and shaken at 37°C. At 5, 15, 30, 45, 60 and 90 minutes the weight of the films were determined (n=3). The water uptake of the films were calculated according to the following equation (Eq. 1):

$$\text{Water uptake (g/g film)} = (Wt - Wo)/Wo \quad \text{(Eq. 1)}$$

where Wo is the initial weight, and Wt weight at t time.

Drug Content: Chitosan films were cut into 1cm^2 pieces and weighed. Then the films were dissolved in dilute lactic acid solution. After filtration Chx concentration was assayed at 254 nm using UV 160A Shimadzu Spectrophotometer.

In vitro release of Chx: The release of Chx from films was studied by using Franz diffusion cells with a 2.52 cm^2 diffusion area. Distilled water was used as the receptor phase (20 mL) at 37°C, uniform mixing of the receptor medium was provided by magnetic stirring. Samples of 1 mL were taken from the receptor side at certain time intervals for 4h, replaced with the same amount of distilled water. The samples were filtered and assayed for Chx at 254 nm.

Antifungal activity: A strain of *Candida albicans* was isolated from a patient suffering from candidiasis and identified using a Candifast Diagnostic Kit (International Microbio, France). *C. albicans* was grown in Yeast Nitrogen Base Medium (YNB: Difco Laboratories Detroit MI, USA) overnight at 37°C. After incubation, standardization of the inoculum was done by counting the yeast in Malessez cell (International Microbio, France) and the concentration of the inoculum was adjusted to 2.5-3.5x10^3 cfu. The final concentration was confirmed by colony forming unit (cfu) method. The inoculum was dispersed into petri dishes and dried. Chitosan films were cut into 11 mm diameter discs and weighed before placing onto YNB agar dishes. 100 μL of Chx solution (20% w/v) were pipetted into uniformly spaced 11 mm diameter wells as control. Inhibition zone diameters were measured after 48 h incubation at 37°C.

Results and Discussion

Water absorption capacity: The uncross-linked (free) chitosan films absorbed large quantity of water and gelation was observed in 15 minutes. When cross-linked with 0.5% (w/v) TPP, swelling of the films was 6 fold of their original size and remained intact for 4h in water. Hydration of films plays an important role in bioadhesion however, excessive hydration can lead to a weakening of the bioadhesive bond probably as a result of a dilution of functional

groups available for adhesive interactions at the interface between bioadhesive and substrate [5].

Drug loading and release: Drug loading efficiency was between 58-66% for film formulations. The release of Chx from chitosan films was maintained for 4h reaching to a plateau after 1.5 h without a lag-time. The released amount of Chx decreased with cross-linking. In 1h, 53.5±3.3 percent was released from free chitosan film containing 0.2% Chx whereas from films cross-linked with 0.1% and 0.5% TPP, the released percentage was 32.8±11.6 and 20.4±5.1, respectively (Fig.1). Chx release was not affected by the increased TPP concentration from the films containing 0.1% Chx.

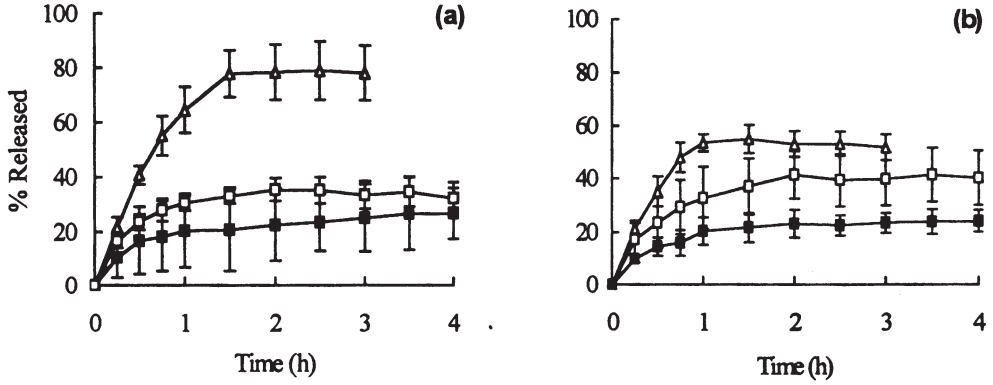

Figure 1. Chx release from chitosan films containing 0.1% Chx (a) and 0.2% Chx (b) Free (Δ); crosslinked with 0.1% TPP (□) and 0.5% TPP (■)

Cross-linking is found to prolong the release of drug from the films. One explanation for this can be the slow hydration of cross-linked films when compared to that of free films.

Antifungal activity: Drug content of the films and inhibition zone diameters are given in Table 1. The typical inhibition zones are shown in Figs. 2a and b. Cross-linking did not have any effect on antifungal activity. No inhibition zone was observed with drug free chitosan film. When the concentration of Chx applied on the diffusion medium is taken into consideration, it was observed that the inhibition zone with Chx solution was significantly lower when compared to that of Chx incorporated films.

Table 1. Inhibition zone diameter for chitosan films (n=3).

Formulation	Inhibition zone* (mm)±SD	Drug content (mg)
0.1% Chx-Uncross linked	14.3±0.6	0.334
0.1% Chx-0.1% TPP	14.0±0.0	0.297
0.1% Chx-0.5% TPP	14.3±0.6	0.498
0.2% Chx-Uncross linked	12.0±0.0	0.790
0.2% Chx-0.1% TPP	17.0±0.0	1.030
0.2% Chx-0.5% TPP	14.7±0.6	0.848
Chx solution (20% w/v)	21.0±0.0	10.00

*application diameter: 11 mm

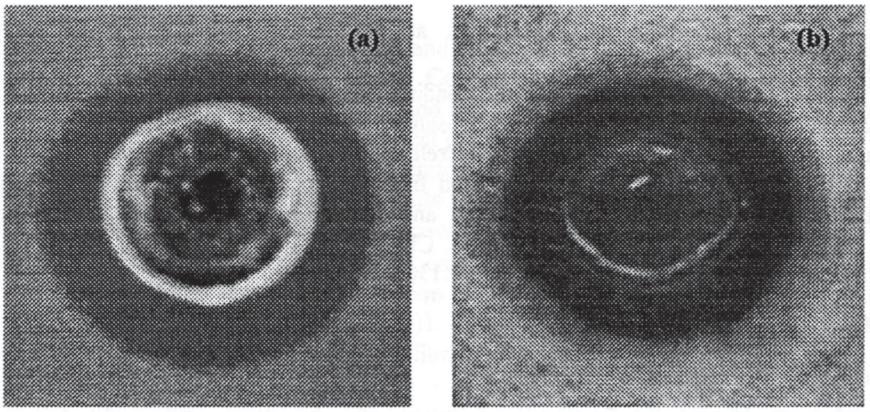

Figure 2. Inhibition zones obtained with Chx solution (a) and 0.2% Chx incorporated into chitosan film (b)

The antifungal activity of Chx is enhanced when incorporated into chitosan film, which allows administration of Chx at lower concentrations thus reducing the undesirable effects. It can be concluded that chitosan with its antifungal activity and bioadhesive properties is a suitable delivery system in film form in achieving controlled release of drugs into the oral cavity. Hydration and the release properties of Chx from the film can be controlled by the concentration of the cross-linking agent.

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Release of *N*-acetyl-D-glucosamine from chitosan in saliva

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Summary
The aim of this in vitro study was to examine human saliva influence on chitosan degradation and N-acetyl-D-glucosamine formation. Chitosan ascorbate used for the tests was the same as the one used in earlier clinical studies i.e. 67- 70% deacetylation grade and the ratio of chitosan to ascorbate equal to 1:1. This in vitro research has confirmed the process of chitosan degradation in saliva. Significant increase of the released N-acetyl-D-glucosamine was observed after 8, 20 and 24 hrs of incubation. In the case when an experimental mixture contained saliva only, practically no release of N-acetyl-D-glucosamine was found.

Introduction
We have recently studied clinical application of chitosan ascorbarte as a multifunctional dressing that could be used in the oral cavity. The chitosan preparation was assessed taking into consideration its haemostatic, hygroscopic and film – forming properties as well as the degree of adhesion to soft tissues [5, 6]. Upon control examination of the patients we were able to notice that chitosan dressing had remained functionally intact in the oral cavity for the time of several to a dozen hours or so, and in some cases even for two days. As we observed the preparation had remained longest within interdental spaces and the crevice among other sites of oral cavity [5, 6]. It is obvious that during this time the chitosan dressing must have been in close contact not only with the soft tissue covered by it but with the surrounding saliva as well.

Saliva being the essential environment of the oral cavity is a very complex mixture. It contains many enzymes both exogenous i.e. of bacterial origin and endogenous. It is possible that some of them like lysozyme (which concentration in saliva varies individually from 2 to 1000 μg/ml or more) may take part in the chitosan biodegradation [3, 7]. This is of crucial significance that hydrolytic degradation of chitosan goes along with the formation of N-acetyl-D-glucosamine moieties, which play an important role in wound – healing process [1, 2, 8, 9, 10, 11, 12, 14, 15].

The purpose of this in vitro study was to examine whether N-acetyl-D-glucosamine moieties can be possibly released from chitosan suspended in saliva.

Materials and Methods
In our study we have used sterile chitosan ascorbate in the form of powder. The preparation was obtained from Sea Fisheries Institute of Gdynia. Chitosan was produced from the shell of *Antarctic krill.* The ratio of ascorbate to chitosan in the preparation was equal to 1 and the

chitosan deacetylation grade equalled 67 – 70%. It is worth mentioning that the same kind of chitosan preparation has been recently used in our clinical studies [5, 6].

We took 36 saliva samples from healthy volunteers, men and women aged from 25 to 62 years. Sample collection was performed in the morning (9.30 – 10.00) two hours after breakfast. There were 7 ml of resting mixed saliva obtained from each volunteer Then using the collected saliva samples we prepared 36 separate experimental kits. Each of them was made up of 3 different incubation mixtures, marked A, B and C:

- Mixture A consisted of: 20 mg chitosan ascorbate + 1 ml of buffer solution (0.6 M sodium citrate, 1.2 M Na_2HPO_4, pH 5.1) + 3 ml of fresh, non-centrifuged saliva;
- Mixture B consisted of: 20 mg chitosan ascorbate + 1 ml of buffer solution (0.6 M sodium citrate, 1.2 M Na_2HPO_4, pH 5.1) + 3 ml of bidistilled water;
- Mixture C consisted of: 3 ml of fresh, non-centrifuged saliva +1 ml of buffer solution (0.6 M sodium citrate, 1.2 M Na_2HPO_4, pH 5.1).

The incubation of all mixtures of a kit (A, B and C) was carried out simultaneously at 37^0 C in a water bath under constant shaking for 26 hrs. We took samples of 300 µl from each mixture before (initial sample) and then after 1, 8, 20, 24 and 26 hrs of incubation. Immediately after collection each sample was analysed to detect N-acetyl-D-glucosamine according to the modified method of Reissig et al. [4, 13] for N-acetyl-aminosugars determination. Absorbance was measured at 544 nm by Beckmann DU-64 spectrophotometer. The study was performed at the Department of Biochemistry, Medical University of Gdańsk.

Results and Discussion

On the basis of the results obtained from the in vitro study, we confirmed that in the presence of human saliva chitosan ascorbate underwent biodegradation releasing N-acetyl-D-glucosamine (Table 1). The concentration of N-acetyl-D-glucosamine was increasing in the following samples collected in the course of incubation reaching its maximum after 24 hours. Then it did not seem to change and after 26 hrs was the same as in the samples taken after 24 hrs of incubation (Figure 1).

Chitosan biodegradation rate was much higher in the presence of saliva (mixture A) than buffer solution (mixture B) where the significant increase in N-acetyl-D-glucosamine concentration was not observed before 20 hrs of incubation. Anyway even after 24 or 26 hrs the concentration in samples from mixture A was about three times higher than that measured in samples from mixture B. In the case of samples containing solely saliva with no chitosan (mixture C) we could hardly detect formation of N-acetyl-D-glucosamine (Figure 1).

The results of our experiments imply that under in vitro conditions human saliva stimulated chitosan degradation and thus N-acetyl-D-glucosamine release. It is possible that a similar process goes on also in vivo in the oral cavity for a dozen hours or so (or even longer) after having applied a chitosan dressing by a dentist. As mentioned above chitosan covering the tissue is in close contact with saliva being a natural environment of the oral cavity. However, it is still to be examined whether the products of chitosan in vivo biodegradation have a significant impact on wound – healing and tissue regeneration process. Also the mechanism of chitosan biodegradation in saliva is to be elucidated.

Table 1. Mean concentration (x ± SD) of N-acetyl-D-glucosamine determined in samples taken before (initial sample) and in the course of 26-hour incubation

	incubation mixture	number of samples	incubation time: initial sample (0 hrs)	1 hr	8 hrs	20 hrs	24 hrs	26 hrs
			µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
A	chitosan ascorbate + buffer + saliva	36	6,0 ± 3,3	6,3 ± 2,7 ***	9,5 ± 3,4	17,3 ± 7,8 ***	23,7 ± 5,9 ***	23,2 ± 7,4
B	chitosan ascorbate + buffer + water	36	3,0 ± 2,4	3,2 ± 1,9	3,4 ± 2,1	6,2 ± 3,1 ***	7,0 ± 4,3	7,7 ± 3,5
C	saliva + buffer	36	5,4 ± 2,4	5,9 ± 2,0	5,6 ± 2,3	6,1 ± 3,1	5,9 ± 3,1	6,3 ± 2,4

*** $p < 0,001$ (difference significance was calulated applying the paired Student test).

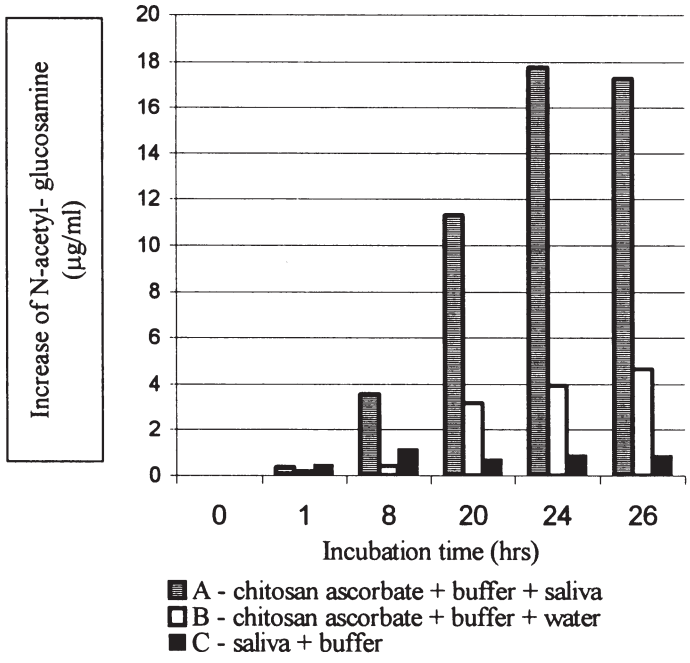

Figure 1. Mean increase of *N*-acetyl-D-glucosamine concentration in samples taken in the course of incubation as compared to the initial sample

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Recent Approach Of Metal Binding By Chitosan And Derivatives

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Summary

Chitosan has been known for a long time for its capability to strongly bind metals especially through a chelation process. This kind of interaction can occur with chitosan either in solution or in the solid state. In the first case, pH and polymer concentration play a major role. Thus, in the case of multivalent metal ions, below C*, the critical concentration of polymer chain entanglement, only a simple coordination process corresponding to "pending" structures should occur although at concentrations over C*, a gel formation can be observed. When chitosan is used in the solid form, dispersed in water, in addition to pH and concentration, the crystallinity and the accessibility of the amorphous parts to water also play a role on both the kinetics of metal uptake and the maximum capacity of chelation by chitosan chains. The chemistry of the metal species in a given condition is a last parameter which has to be accounted for the complete interpretation of the structure of the formed complexes.

Introduction

If we consider the chemical structure of chitosan, it corresponds to the series of the copolymers of glucosamine and N-acetyl glucosamine and then is a highly functionalized structure. It bears a great variety of functions such as alcools, amine, amide and ethers. Among these functions some of them can be ionized in different pH ranges, some others bear atoms such as oxygen an nitrogen which possess doublets of free electrons. As a consequence, chitosan should interact with metals by means of different types of interactions, especially ionic interations and complexation.

Ionic interactions

In the ammonium form corresponding to the presence of $-NH_3^+$ groups, chitosan interact with various anionic forms of metals. This kind of interaction is conditioned by the value of DA, pH and the nature and concentration of the metal species studied. It is necessarily favoured in acidic media. Hydroxyl groups of chitosan can also be ionized in the form of alcoolates. The relatively high pK_a values of these functions need a high pH (over 10) to be formed in significative amounts. Then, some cationic species present for some metals on

these pH's can allow an ionic interaction with the alcoolate forms of chitosan. In numerous circumstances, wether at low or high pH's, the metal specie present on these pH's can be of the same sign as the chitosan chain and then, an increase or a decerease of pH is often the origin of the destruction of true complexes.

Complexation

The possible complexation between chitosan and some metal species is due to two favorable conditions. The one is related to the chemical structure of chitosan which contains possible ligands due to the presence of doublets of free electrons on atoms such as nitrogen of the free amine groups or oxygen of hydroxyl group. The second is related to the electronic structure of numerous metals which contains free quantic boxes, especially of level d and f. This situation is that encountered with all the transition metals. The consequence is that chitosan can bind all the metals except those of the alkaline family. Nevertheless, this complexation is subjected to the role of various important parameters.

General rules

The complexation between chitosan and metal species is governed by the problem of the interaction metal-electron donors and is related to the orbital frontiers: the HOMO concerning the doublet of free electrons and the LUMO for the metal. If the different orbitals are of equivalent energy, a transfer of electrons is possible and a dative bond close to a covalent bond can be formed. When the difference of energy is important, only electrostatic interactions can be observed.

Role some important parameters

Role of DA

DA regulates the yeld of metal uptake since only the free amino groups are necessary to this property. Indeed, the interaction between metal and fully acetylated structures has never been demonstrated.

In the case of electrostatic interactions involving the ammonium form of the glucosamine residues and anionic structures of metals, the optimal pH is then located near 3-4 and the interaction decreases necessarily on increasing DA.

Although it has not been clearly demonstrated, we can consider a possible interaction by means of alcoolate forms of hydroxyl groups. If this situation occurs, we must take into account the high values of the pK_a of alcool functions generally located within 10-12. Then, the pH of the media must be over 10 and there also, the interaction should increase on decreasing DA.

In the case of the formation of true complexes, the role of this parameter has been rarely studied. The difficulty for a quantitative evaluation is to be in conditions of polymer saturation by metal species. Nevertheless, this role has been recently demonstrated in the case of the interaction between chitosans of different DA's and uranyl ions [1]. The study was

performed at pH 6.5 from dispersions of chitosan particles in water. In this case, tacking into account the number of free amino groups in the amorphous domains of chitosan particles it was clearly shown that for DA's below 30%, the apparent capacity of fixation at equilibrium is directly dependant on the number of amino groups and that the presence of acetylated residues plays no role on the mechanism. In the case of chitin, the results obtained in the same conditions show that for DA of 70%, after correction as above, the capacity of fixation is below that for DA's below 30%. The role of DA in this case can be related to the fact that over 50%, the intrinsic pK of the amino groups increases to become close to that of the monomer and then, for a same pH, the concentration of ammonium groups increases with DA thus disfavouring the complexation. It is also important to remind that for a 100% acetylated structure, the total absence of metal uptake was demonstrated in the case of CuII and HgII [2]. As a conclusion, N-acetylated residues do not take part directly in the complexation and then, DA has no influence on the capacity of sorption related to $-NH_2$ groups in the solvated parts as much as pK_0 remains constant.

Role of pH

pH induces the ionisation state of chitosan, the nature of the metal species present in solution and then the nature of the interaction and the structure of the complex formed. In the case of true complexes, the interaction is governed by the sum of two reactions as follows;

$$-NH_3^+ \Leftrightarrow -NH_2 + H^+ \qquad (K_a)$$

$$M^{n+} + y \Leftrightarrow -NH_2\ [M\ (-NH_2)_y]^{n+} \qquad (K_c)$$

$$\Sigma \qquad y\ -NH_3 + M^{n+} \Leftrightarrow [M\ (-NH_2)_y]^{n+} + y\ H^+ \qquad (K)$$

The first depends on DA and the intrinsic pK_a, the second on the complex formed. y often equals 1. Then, the overall constant $K = K_a.K_c$. Assuming $pK = pK_a + pK_c$, we can consider that complexation becomes not negligible for $pK < -5$. If pK_0 is close to 6.5, we find $pK_c < -11.5$-12 which signifies that the complexation constants must be necessarily high to observe the interaction with chitosan. It is also obvious that this reaction depends on DA and pH. We can conclude that for a given metal specie, complexation increases on decreasing DA and increasing pH. This explains why it is generally difficult to observe high complexation at pH below 4. Due to the presence of incompatible metal structures, it is also difficult in most cases to form complexes over pH 10.

Only few examples of the literature can be used to illustrate the role of pH. Thus, in the case of chitosan in solution [3], in the presence of CuII, circular dichroism experiments can be used to demonstrate that the same complex is formed whatever the concentration of copper and the pH in a range located within pH 5-6.5. It is interesting to notice that no complexation is observed at low pH's where the concentration of free amine groups is negligible. Moreover the study allows to demonstrate the effective participation of pH since the complex concentration is multiplied by 8.6 when pH increases from 5 to 5.5. The study of the variation of pH when a solution of copper perchlorate is added to a solution of chitosan shows clearly a consumption of $-OH^-$ during the addition of metal ions, in relation with a complex involving the hydroxylated forms of the metal. In the case of the interaction between low DA chitosan and uranyl ions [4], we also demonstrated that no significative complexation was observed for pH below 5.1 and the presence of only one kind of complex which formation is stronghly related to pH. These results confirm quite well the general mechanism of complexation described above. In the case of chitin which corresponds to highly acetylated

structures, as shown in the case of manganese ions [5], depending on the metal concentration, the complexation does not take place before pH 7.5. As already mentioned, this behaviour has to be related to the fact that for high DA's, the value of the pK_0 of the amine groups should tend towards that of the monomer which is close to 7.4. In this case the role of the concentration of metal on the metal uptake is related to the equilibrium law of the interaction but possibly also to the influence of this parameter on the repartition of the metal species present for a given condition.

The possible selectivity of metal uptake by chitosan, according to the pH value, has been quite well demonstrated, especially on two series of divalent and trivalent metals in the range of pH's located within 2-6.5 [6]. Unfortunately, the nature of the metal species present in solution or in interaction were not described. For a given pH, in the case of trivalent metal ions, the selectivity is as follows: Fe>>In>Ga>Al. In the case of divalent species, the sequence is: Cu>>Ni>Cd>Pb>Zn>Co. Nevertheless, the interaction below pH 6 remains particularly weak in relation with a low concentration of free amino groups. It is also important to consider that in this case it is necessary to avoid the dissolution of the polymer chains, essentially by crosslinking, a modification which also contributes to decrease the number of interactive sites. This selectivity should be related to the distribution of the metal species which at low pH can certainly vary from one metal to one another and also to the participation of ionic interactions with anionic species especially of metal oxydes.

The role of the nature of the metal species present at a given pH certainly plays an important role on the metal uptake by chitosan, especially in the ranges of pH where the species differ from one metal to one another. This is particularly the case at pH's below 6 or over 8. As a consequence, it seems important to know the distribution of these species for a given condition (pH, concentrations, ionic strentgh...). This information is rarely given in the literature on chitosan and only recent papers mention this aspect of the problem. Although the knowledge of the majoritary species is not sufficient to determine the structure of the complexes, it is necessary for a rigorous interpretation of the mechanisms of interaction. Diagrams of distribution can be easilly obtained by means of the constants of the equilibrium of formation of the various possible derivatives of metals. The literature on classical metals contains a great number of constant values and the use of some softwares such as the TOT software allows an easy drawing of such diagrams. We can mention the case of the distribution of uranyl ions [1] which was calculated in the presence of various other species such as carbonate ions which in some circumstances must be considered as competitors of chitosan. From such a diagram we can clearly see that the specie $(UO_2)_3(OH)_5^+$ is largely majoritary on pH's located within 6.5-7.5 . If we consider the values of the decontamination factors (DF) obtained in the presence of chitosan for a solution containing uranyl, carbonate and nitrate ions [1], they corroborate quite well the fact that the specie $(UO_2)_3(OH)_5^+$ plays the major role in the interaction between chitosan and uranyl ions (1 signifie no metal uptake).

pH	**6**	**7**	**7.5**	**8**	**9**	**11**	**12.5**
DF	**9**	**9**	**8**	**2.5**	**2**	**1**	**1**

Then, the maximum complexation is observed for pH's within 6.5-7.5, with a decomplexation which progressively appears over pH 7.5, certainly in relation with the predominance of some species such us $UO_2(OH)_3^-$ which do not interact with chitosan.

The case of molybdenium is particularly interesting. In acidic media, this metal exists under a great variety of oxyde forms which most of them are negatively charged. If we

consider the diagrams of distribution of these oxydes at pH's below 6 [7], they reveal the very important role played by the metal concentration and pH on this distribution. Among the possible species, the most negatively charged are predominant for pH's ranging within 3-5. Then, working with partially crosslinked chitosan to avoid dissolution, it is interesting to observe that the maximum metal uptake occurs for pH's within 3-4 corresponding to the maximum anionicity. This interaction depends on the initial metal concentration and desappears suddenly at pH over 5. This behaviour must be related to a pure mechanism of ionic interaction between ammonium sites of chitosan and negatively charged metal species.

Competitiveness of some non metalic species

This problem is particularly important when chitosan is used for the decontamination of effluents which are generally complex systems. Nitrates are relatively frequent in waste water. If we consider the case of uranyl ions in presence of nitrates, the most important specie, $UO_2(NO_3)^+$ desappears at pH over 6. Therefore, over this pH, nitrates have no influence on the decontamination factor even at concentrations 10^6 times that of uranyl ions. We only notice a slight decrease of the kinetics of sorption on increasing their concentration. Carbonate ions play an important role in the case the treatment of waste water of nuclear industry. Indeed, they are good complexing agents of numerous metal species and as already mentioned, two derivatives, UO_2CO_3 and $UO_2(CO_3)_2^{2-}$ are important competitors of chitosan at pH 6 and 9 respectively. The case of sulfate ions is particular. They disfavour the complexation of metal ions by chitosan due to their high dehydrating power (scale of Hofmeister) and also because (although not clearly demonstrated), they should complex themselves with amino sites of chitosan thus reinforcing the insolubility of the polymer and contributing to the subtraction of numerous binding sites. If we consider the study of the kinetics of sorption of uranyl ions the presence of sodium sulfate at a concentration no more than 0.1M contributes to divide the metal uptake at equilibrium by a factor close to 4 compared to the absence of this salt [1].

Parameters related to the solid state

Solid state of chitosan is the most important physical state for the metal uptake. Indeed, the insolubility of this polymer observed at pH over 6 (for low DA's) constitutes an interesting property for an easy collection and concentration of metals. It also favours an easy regeneration of the polymer and/or the metal. In addition pH's over 6 correspond to the range where the complexation is maximum with chitosan. In this state various parameters must be considered. The crystallinity plays an important role since chitosan is a semi-crystalline polymer and that only the amorphous parts are accessible to the metalic species. As a consequence, the accessibility to the amorphous domains is essential. We can mention the case of the study of the role of chitosan crystallinity and morphology on uranyl ion sorption [4]. It is shown that chitosan hydrochloride in the lyophilized form constitutes the most interesting system with the lowest crystallinity and the highest accessibility to the amorphous parts. In addition, this material is easily dispersed in water to give particles of average size largely below 50μm. Transmission electron microscopy gives interesting information on the morphology of the different chitosan materials. Thus, raw chitosan which has never been dissolved contains true closed pores. It is highly crystalline and the general morphology is similar to that of the cuticle from which it is originated [8]. In this case, the

amorphous parts correspond to the surface of the crystallites, flaws and dislocations. Therefore, the particle size plays a very important role since it is related to the accessibility to amorphous parts. In the case of regenerated chitosan in the free amino form, the crystallinity due to the step of dissolution is lower and the accessibility to amorphous parts becomes better. In the case of lyophilisates of chitosan hydrochloride, the crystallinity is minimal, the crystallite size is very law and we can assume that most of them are destroyed during the dispersion in water where the dispersion is very high (particle size <50μm). The material corresponds to a highly expanded three-dimensional network with a very high porosity. In this case, the particle size has no great importance since we observe a pure diffusion process. If we consider the maximum capacity of sorption of uranyl ions by chitosan (saturation of the polymer) [4], we notice a linear variation with the degree of crystallinity with a negative sloop and a point of zero sorption when the crystallinity is extrapolated at 100%.

Whatever the physiscal form, the kinetics of metal uptake are tightly related to the kinetics of hydration of the particle dispersed in water. In fact, the complete hydration of the particles in the case of raw chitosan needs at least 10 hours and less than 2 hours with lyophilisates of chitosan hydrochloride. Thus [8], when the polymer has been hydrated at least 12 hours, the kinetics of uranyl uptake is relatively high and more than 85% of the value at equilibrium is achieved after only 10 minutes although, for the same material, only hydrated 30', the value at 10' is less than 25% that at equilibrium. In the case of lyophilisates of chitosan hydrochloride, the hydration is completely achieved within 2 hours.

Role of chemical modifications

Numerous papers concern the chemical modification of chitosan in relation with the metal uptake. Unfortunately the comparisons between unmodified and modified chitosan are rarely reported. These modifications are made for various reasons: to avoid dissolution in acidic media or to improve the chelating properties in the solid state. They are generally performed on raw chitosan. We can mention the results reported by K. Kurita et al. [9]. It is particuliarly interesting to compare the results of copper sorption corresponding to N-acetylation and N-nanoylation of chitosan. In both cases, the chemical modification contributes to decrease the cristallinity of the materials and then to favour the complexation. Nevertheless, in both cases, this improvement is limited to low degrees of substitution since over a DS of 0.2%, the cristallinity shows a new increase, less important in the case of reacetylation and, complexing sites are consumed by the modification.

Complexed species, geometry

There are only few papers on this important aspect of the metal uptake by chitosan. Thus, we have to know the distribution of the metal species corresponding to a given condition *i.e.* for a given pH, concentration, ionic strength and temperature. It allows us to define the majoritary specie for this condition. Nevertheless, this specie is not necessarily that having the highest complexation constant and the structure of the complex can also be different from that of the specie which actually reacts with the polymer. In solution it has been shown that for numerous metals, at pH's located within 5-6, only one complex was formed (see above in the case of Cu an U). In the solid state we can mention the following

result [4]. If we consider the IR spectra of chitosan samples isolated after interaction with various metals (Al, Ag, Cu, U), in a range of pH within 5-7, the spectra show a broad band near 1625 cm^{-1}. The second derivative applied in this region reveals the presence of 3 bands. One at 1660 cm^{-1} corresponding to the residual amide-1 band, a second at 1595 cm^{-1} related to the residual uncomplexed free amine groups and a third at 1635 cm^{-1} associated to a band at 1525 cm^{-1}. These later bands are due to the formation of complexes involving free doublets of electrons of the N atoms. They are relatively similar to amide bands. Thus, whatever the metal mentioned above, it seems that the complexes correspond to the same involvement of Nitrogen atoms. and as others, we propose to consider that the 'pending structure' could be formed in all cases with the structure $-NH_2-M^n(OH)_n$, except for solutions at a concentration over C* where a metal can be coordinated with several amine groups to form a gel.

Conclusion

Recent works demonstrated the role of various important parameters such as: -pH on the nature of the interaction and on the nature of the metal species, - the concentration of metal on the metal species present at a given pH, - the presence of some anionic species. In the solid state the role of the crystallinity and the morphology of the amorphous domains as well as the role of the kinetics of hydration were described. The relation between chemical modifications and metal upake in relation with crystallinity changes is now clearly shown. The presence of the same kind of complexes whatever the metal (for numerous species) in the range of pH located within 5-8 is also clearly demonstrated. Nevertheless, it remains much to do: - to clearly define the exact structure of the complexes and to calculate the thermodynamic and kinetic parameters of the complexation.

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As(V) sorption on molybdate-impregnated chitosan gel beads (MICB)

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Summary
Because raw chitosan is a poor sorbent for arsenic, it was modified by impregnation with molybdate. The impregnation procedure consists of a preliminary sorption of molybdenum using chitosan gel beads. The optimum pH for arsenic uptake is around pH 3. Arsenic sorption is followed by the release of molybdenum, which can be decreased by a pre-treatment with phosphoric acid to remove the labile part of the molybdenum. Sorption capacity, over molybdenum loading, reaches almost 200 mgAs g-1 Mo. Exhausted sorbent can be recycled by desorption using phosphoric acid solution and the sorbent was successfully used over 3 sorption/desorption cycles with only a small reduction in sorption performances.

Introduction
Arsenic is widely distributed in nature due to geological/geochemical or anthropogenic sources in natural waters and wastewaters. It is currently used in microelectronics for the manufacturing of GaAs supports, as well as in pesticides and wood preservatives. Increased activity in the mining industry (Mo, Pb and Cu ores) results in arsenic contamination of streams and groundwaters. Common treatments for arsenic removal are flotation [1], co-precipitation with ferric chloride, sulfide precipitation [2] or lime softening, involving the production of highly toxic sludges, which must be further treated before being environmentally safe for disposal. Sorption processes have been investigated using several sorbents such as activated carbon, fly ash [3-5]. Though chitosan is very efficient at removing several metals such as copper, PGM, uranium, molybdenum [6-10], in the case of arsenic sorption level does not exceed 0.1 mmol g^{-1}. Elson et al. cited very low sorption capacity for arsenic sorption on chitin/chitosan materials [11]. Such low sorption levels have led to the development of new sorbents using a procedure described for activated carbon and alginate [12-13]. It consists in doping the sorbent with other metal ions such as Cu(II) or Fe(III), the impregnant being able to precipitate arsenate ions inside the sorbent particle (porous network in activated carbon, gel matrix in alginate beads). Molybdate ions react in solution with arsenate to form a complex used for spectrophotometric determination of As(V). This chemical reaction is more specific than the precipitation mechanism cited with copper and iron. Since chitosan is able to sorb molybdenum with sorption capacities as high as 7-8 mmolMo g^{-1} [10], this impregnation procedure was used to enhance arsenic uptake on chitosan, chitosan gel beads being prefered to flakes to improve mass transfer inside the porous network of the sorbent.

Material and Methods
Chitosan was provided by ABER-Technologies (Brest-France). Its characteristics were: molecular weight = 1.3 10^5, and deacetylation percentage = 87%. Chitosan gel beads were

manufactured using the alkaline casting procedure of an acetic acid solution of chitosan [10,14]. The water content of the beads was about 95 %. Their diameter was 1.5 mm. Reagents were purchased from Merck: $(NH_4)_6Mo_7O_{24}$ x 4 H_2O and Na_2HAsO_4 x 7 H_2O.

Known amounts of wet chitosan beads were put in contact with known amounts (volume and concentration) of ammonium heptamolybdate at pH 3. Dry mass was obtained by weight loss at 105 °C. The molybdate (and arsenic) concentration in the sorbent was obtained by mass balance between the liquid and solid phases. ICP-AES was used for molybdate, phosphate and arsenic analysis in solution. The MoO_3 content was also obtained by weight loss at 480 °C, until weight remained constant, and the molybdate content in molybdate imlpregnated chitosan beads (MICB) was deduced from the difference with dry mass at 105 °C. Total Mo and P concentrations in the gel phase were determined (when necessary) by disrupting the gel phase with hydrogen peroxide. MICB sorbents treated with orthophosphoric acid (MICB-PO4) were prepared by mixing MICB in a 0.1 molar solution of orthophosphoric acid for 24 hours followed by extensive rinsing with demineralized water.

The pH was adjusted with sulfuric acid and was not controlled during metal ion sorption; only the final pH was measured. Sorption isotherms were obtained through contact of a varying number of beads with a known volume (50 mL) of arsenate solution (C_o ranging between 5 and 20 mg L^{-1}) at room temperature, for at least 120 hours. Arsenic concentration was measured by ICP-AES, after filtration (1.2 µm pore size filter). For kinetic studies, 200 beads were mixed in 1 L of As (V) solution. At pre-determined times, samples were withdrawn, filtered and analysed.

Several elutants were investigated, such as tartaric acid, citric acid and phosphate, by putting a fixed number of chitosan beads (ca. 20 beads) of known arsenate concentration in contact with a fixed volume of elutant (ca. 10 mL) at a controlled pH. Arsenic content was determined by mineralization and ICP-AES analysis.

Results and Discussion

Metal ion sorption is usually strongly influenced by the pH of the solution due to protonation of the sorbent, to the speciation of metal ions and their ability to form hydroxo-complexes and polynuclear species [15]. The influence of this parameter was studied regarding both arsenic sorption and molybdenum release (Figure 1).

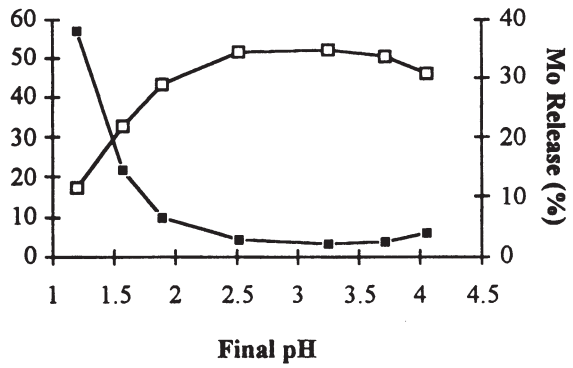


Figure 1. pH influence on As(V) sorption and Mo(VI) release (C_o: 20.5 mg L^{-1}, 50 beads: 48 mg - dry sorbent (28 mg - chitosan) in 100 mL, Mo loading : 730 mg Mo g^{-1} dry chitosan)

This figure shows that the maximum As(V) sorption occurs around pH 3 and that Mo(VI) release tends to 2-3 % around this optimum pH. A further increase in pH results in a new sharp decrease in sorption efficiency/capacity as well as considerable Mo(VI) leaching, which is complete at pH 9 (not shown). The pH may also influence sorption isotherms, which representent the distribution of the solute (i.e. As(V)) between liquid and solid phases when the residual concentration in the solution increases. Figure 2 shows that the maximum sorption capacity is almost identical at pH 2 and pH 3, but the affinity of the sorbent for As(V) decreases with increasing the pH as indicated by the initial slope of the isotherm. More interesting is the curve representing the Mo(VI) release in function of the residual As(V) concentration. While at pH 2, molybdenum release seems to be proportional to arsenic concentration with a low slope, at pH 3, molybdenum release strongly increases with an exponential trend. It appears that molybdenum relase is increased by the presence of arsenate ions. It could be suggested that this molybdenum release is related to the displacement of the complexation equilibrium between As(V) in solution and Mo(VI) sorbed on chitosan gel beads: a high arsenic concentration involves a strong complexation to Mo(VI) which is then desorbed from the sorbent and released into the solution. This release could be decreased by (a) contacting a large amount of sorbent with arsenic solution in order to strongly decrease arsenate concentration and then decrease the desorbing effect on molybdate ions bound to chitosan beads, (b) using the sorbent for the treatment of low As(V) concentration solutions.

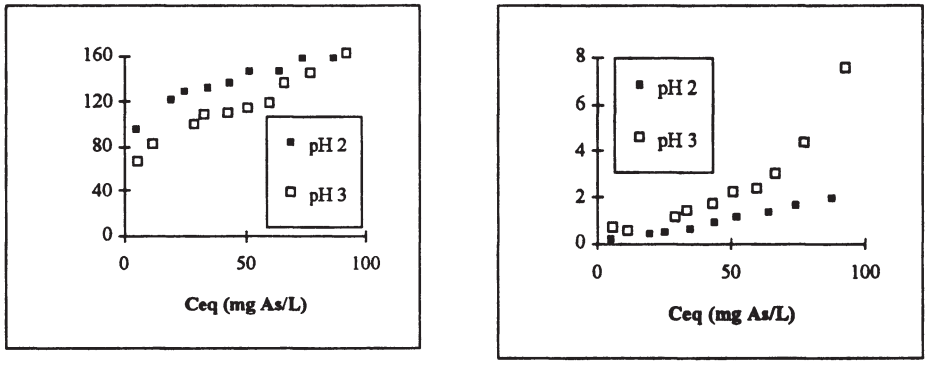
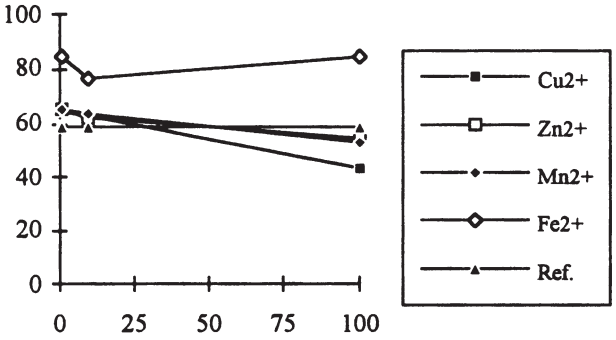


Figure 2. Influence of pH on As(V) sorption isotherms and Mo(VI) release on MICB (Mo loading 1033 mg Mo g^{-1} chitosan d.w.)

Sorption performances may be also controlled by the presence of competitor ions such as those encountered in industrial effluents and Acid Mine Drainage. The influence of anions and metal cations has been investigated and results appear in Figure 3. It appears that for the most part of these competitor ions, the presence of cations or anions has a very limited effect on As(V) sorption when concentration does not exceed 0.1 M. Ferrous ions seem to increase arsenate sorption, this enhancement in sorption performances may be explained by the precipitation effect after a preliminary easy oxydation or Fe^{2+} in Fe^{3+}. With both ferrous and ferric ions a precipitate was observed on the filter at the pH used in this study. Chloride and nitrate ions hardly influence arsenate sorption, while the presence of phosphate strongly reduces arsenate removal. This observation was expectable and may be explained by the competitor reaction between molybdate and phosphate ions to form phospho-molybdic species which, in turn, reduces the number of sorption sites available for arsenate species.



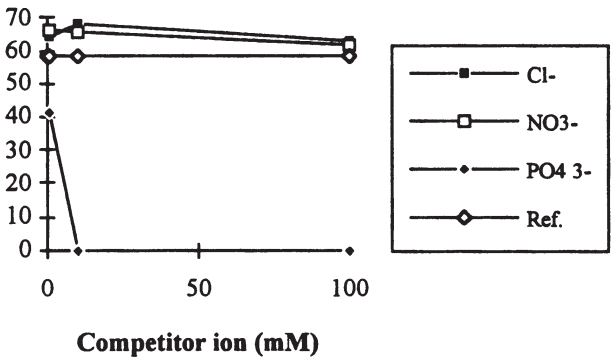


Figure 3. Influence of competitor ions on As(V) sorption at pH 3 (Co: 20 mg As(V) L^{-1}; Sorbent Dosage: 360 mg L^{-1} (d.w.); Ref.: sorption in absence of competitor anions).

This reaction of phosphate with the sorbent is confirmed by Figure 4 showing the sorption isotherm for phosphate at pH 3. This isotherm tends to the usual sigmoidal trend of the BET equation: at high residual phosphate concentration, strong sorption occurs due to condensation/precipitation mechanism. This reaction was also used for the desorption of arsenate ions and the pre-treatment of the sorbent in order to remove the excess of molybdate from the impregnated bead, i.e. the labile part of the impregnant. It is suggested that molybdate is sorbed in the chitosan beads in 2 different forms: a part which is strongly adsorbed to chitosan chains while a second fraction representing around 20 % of the total molybdenum present on the bead is absorbed in the gel phase of the sorbent. This fraction being less strongly attached to the polymer can be easily desorbed. Figure 5 shows the comparison of Mo(VI) release during As(V) sorption for 2 kinds of MICB: raw beads and phosphoric acid pre-treated beads. The pre-treatment lessened the loss of Mo(VI) during the subsequent sorption step: the labile part of Mo(VI) was removed by the phosphoric acid.

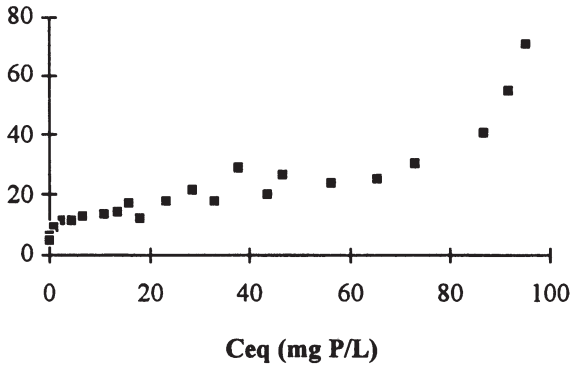


Figure 4. Phosphate sorption isotherm on MICB at pH 3.

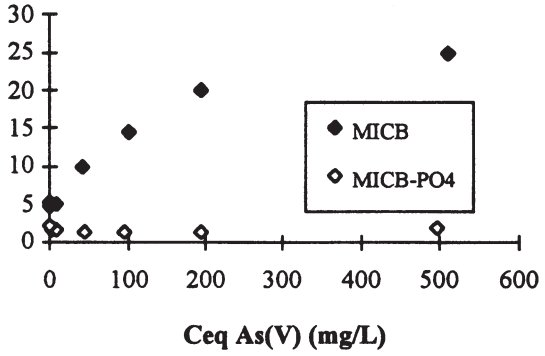


Figure 5. Influence of the pre-treatment with H_3PO_4 on Mo(VI) release from MICB during As(V) sorption (20 beads/ 50 mL; Mo-loading 1033 and 972 mgMo g^{-1} (d.w.) respectively).

Figure 6 shows As(V) sorption kinetics with MICB at 3 different initial concentrations. The change in the relative concentration in the first hours of contact is hardly influenced by the concentration or arsenate ions, while for the second section of the curves this parameter has a more marked effect. The initial section of the curve is usually controlled by extrenal diffusion while the second part is limited by intraparticle mass transfer resistance. At high initial concentration, the equilibrium is reached faster than for dilute solutions: the gradient between the sorbent and the external solution is less favourable to mass transfer and involves kinetic restrictions.

Several chemical reagents were ivestigated for arsenate desorption from exhausted sorbents in order to concentrate arsenic and recycle the sorbent. Tartaric acid, citric acid are common agents used for their ability to complex metal ions. Table 1 shows that high concentrations of tartaric acid are required to achieve 80 % of the total arsenate content. Citric acid is much more efficient at recovering arsenic from exhausted sorbent: the complete

desorption of arsenate can be achieved. However, both reagents involve a strong desorption of molybdate from the sorbent which cannot then be reused.

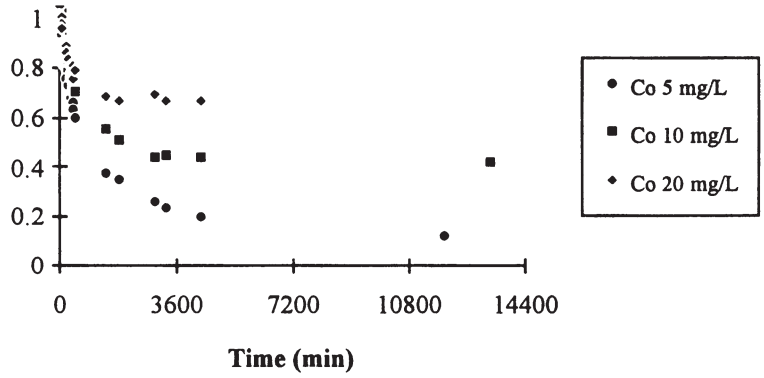


Figure 6. Sorption kinetics at different initial As(V) concentrations using MICB at pH 3 (Sorbent Dosage 360 mg L^{-1} (d.w.), Mo-Loading: 972 mgMo g^{-1} (d.w.)).

Table 1. Arsenic desorption and molybdenum release from MICB (20 beads in 10 mL - contact time: 48 h, Mo-loading 972 mgMo g^{-1} (d.w.)).

	Tartaric Acid (0.1 M)	**Tartaric Acid (0.01 M)**	**Tartaric Acid (0.001 M)**	**Citric Acid (0.1 M)**	**Citric Acid (0.01 M)**
pH	2.06	2.5	3.05	2.12	2.55
As desorption (%)	80	64	8.5	100	100
Mo release (%)	60	58	10	86	63

Alternatively, phosphoric acid solutions are potential agents and they are effective at removing arsenic from loaded MICB (Figure 7). With 0.05 M solutions of phosphoric acid, more than 95 % of the total arsenic present on the sorbent is removed while molybdenum release does not exceed 10 %. Figure 8 shows arsenate sorption and desorption for 3 successive sorption/desorption cycles. It appears that sorption capacity is weakly decreased along the 3 sorption cycles, the cumulative desorption yield reaches 86 %. Total molybdenum release in this case does not exceed 3 %.

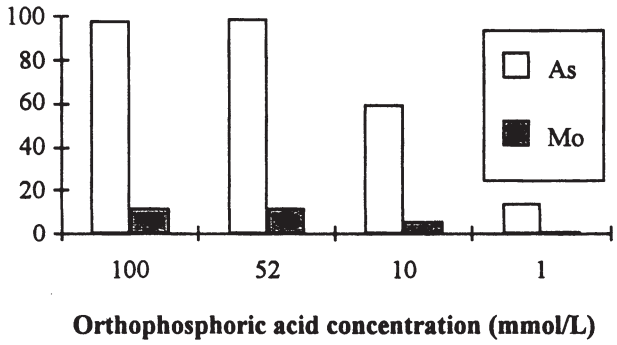


Figure 7. Influence of phosphoric acid concentration on As(V) removal and Mo(VI) desorption (20 beads in 10 mL; Mo-Loading: 972 mgMo g^{-1} (d.w.)).

Molybdate impregnated chitosan beads (MICB) are effective at removing arsenate ions from dilute effluents. Sorption capacity as high as 160 mg As(V) g^{-1} Mo(VI) (and about 200 mg As(V) g^{-1} Mo(VI) for MIBC-PO4) are obtained. Molybdate release can be decreased when the sorbent is pre-treated with phosphoric acid. Arsenate sorption is reduced by the presence of phosphate ions and the weak effect of other competitor anions such as chloride and nitrate confirms that sorption occurs through chelation mechanism rather than ion-exchange process. The desorption of exhausted sorbents can be performed using phosphoric acid at a concentration of 0.05 M allowing (a) the almost complete desorption of arsenate, (b) a low release of molybdate from the sorbent and (c) the recycling of the sorbent for further cycles of sorption/desorption.

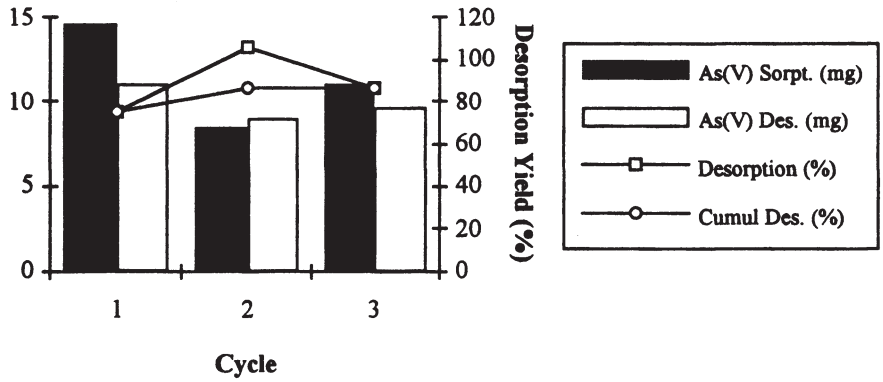


Figure 8. Sorption and desorption efficiencies along 3 sorption/desorption cycles using H_3PO_4 solutions at 0.05 M and MICB-PO4 sorbent in column system (200 beads of MICB-PO4 - desorption with 20 mL of H_3PO_4 0.1 M).

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Influence of medium pH on the biosorption of heavy metals by chitin-containing sorbents Mycoton

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Summary
Mycoton is a chitin complex produced from cell walls of the Higher fungi. The researches of sorption properties of Mycoton in relation to *Ag, Zn, Cd* and *Hg* depending on medium pH from 1 till 11 were carried out. Influence of a different Zn concentration on the character of Mycoton biosorption at different pH also was investigated. The highest level of adsorption for *Ag, Zn* and *Cd* was observed in a range of pH 5-9. The observable sorption of monovalent *Ag* was much lower than that bivalent *Zn* and *Cd*. The adsorption of *Hg* had an abnormal character. To determine the reducing properties of Mycoton have studied its redox- potential.

Introduction

Absorption of heavy metals by cell walls of fungi is named "biosorption" [1, 2]. Biosorption is usually associated with the barrier function of a cell wall, which prevents the living protoplast from the toxic action of heavy metals. The cell wall of fungi contains considerable amounts of chitin possessing the sorption properties. Fungal chitin is of microfibrillar structure. A chitin microfibrilla consists of the linear bundle of macromolecules. Its thickness is 15-20 nm and length - up to 1-2 mkm. Microfibrillas are crumbly interwoven and form the space net which fulfils the skeleton function in the cell wall. The space between microfibrillas is filled with amorphous glucan matrix, which prevent them from sticking together. The specific surface of microfibrillas in certain fungi may reach 1000 m^2/g and more [3]. Such a structure allows metal ions easy access to sorption centers of the cell wall. Short distances between microfibrillas (5-10 nm) promote high possibility of heavy metal ion catch [3, 4].

In order to obtain sorbents based on the chitin-glucan and chitin-glucan-melanine complexes have developed the method of mechanical and chemical treatment of fungal biomass [5]. The method permits the structure of the natural fungal fibre (hypha) to be preserved. As a result it was possible to produce the fibrous chitin-containing material with the thickness of fibres 3-5 mkm and the length up to several millimeters. Fibres are of hollow structure, the wall thickness being from 0.2 to 1 mkm. These materials were named "Mycoton". Mycoton demonstrates unique sorption properties in a number of heavy metals and radionuclides (*U, Pu, Am, Cm)* [4]. Its fibrous structure and good porosity may explain high sorption indices of Mycoton [6]. Depending on requirements it is possible to change the chemical composition of fibres within the wide ranges by treating the raw material: the content of chitin may be specified within 60-95%, glucans - 5-35 % and melanins - 0-10 % [7]. Chitin may be converted into chitosan by the known methods [8].

There are many works on studies of the sorption properties of fungal mycelium and chitin-containing biocomplexes [2, 3, 7, 9, 10]. In these publications special attention is focused on pH which has a significant influence on the sorption process. What ligands will take part in sorption depends to a considerable extent on the value of pH. This is especially urgent for such complex structures as a fungal mycelium and chitin-containing biocomplexes which are composed of many potential ligands [11, 12]. Also, the state of metal ions and the level of their competition with hydroxony ions for the sorption sites depend on pH [2, 10].

The main goal of this work was to study the sorption properties of Mycoton relative to *Zn, Cd, Ag* and *Hg* within a wide range of pH (1-11) and the influence of various concentrations of *Zn* (5-40 mg/l) within pH 3-9. To determine the reducing properties of Mycoton have studied its redox-potential.

Materials and methods

The chitin-glucan-melanine material Mycoton was used as an adsorbent [4, 7]. The obtained material was ground up to fine-disperse state using the "Tsiklon" mill. The test of Mycoton was performed on model systems. For their preparation pure metals (99.9%) such as *Ag, Cd, Hg* and *Zn* dissolved in the concentrated nitric acid were employed. The concentrated solution was diluted by redistillate water (pH 6.7) up to 1 mg/ml, so that pH be no less than 1.5-2 in order to avoid the formation of insoluble metal compounds. pH of the solution from 1 to 11 was obtained by applying ammonium hydroxide and nitric acid solutions of 1 and 0.1 N. The pH value of the solution was controlled before and after introduction of the sorption material using pH-meter at the room temperature.

0.5 g of air-dried adsorbent was introduced into the 50-ml solution containing the definite metal concentration. The solution was continuously mixed during 3 h for establishment of the dynamic equilibrium. After this, the liquid phase was separated from the sorption material, then the liquid phase was allowed to stay for 14 h and it was analyzed.

The concentration of metals (*Ag, Cd* and *Zn*) in solutions was determined by the method of atom-adsorption spectroscopy using the AAS-30 (*Karl-Zeiss, Jena*) device (flame: propane-air). The mercury concentration was determined by the method of "cold vapor". Mercury is fixed in the acid extract using atom-adsorption device "Yuliya-2". The error in determination of the metal concentration in the solution did not exceed 5-10 % depending on the metal concentration. The experiments were repeated thrice.

The distribution coefficient K_d was used as a sorption index:

$$K_d = [(A_1 - A_2) / A_2], V / m, \text{ where}$$

A_1 is the metal concentration in the solution before sorption, *mg/l;*
A_2 is the metal equilibrium concentration in the solution, *mg/l;*
V is the solution volume, *ml;*
m is the sorbent mass, *g.*

Results and Discussion

The distribution coefficients of silver, zinc, cadmium and mercury depending on pH, which were obtained on model systems with Mycoton, are presented in Figs 1 - 4. The results obtained have shown that the distribution coefficients in the region of low values of pH grow in a series $Ag < Zn < Cd < Hg$ and in the region of high values of pH - $Ag < Hg < Zn < Cd$. The pattern of curves for the univalent Ag^+ and bivalent Cd^{2+} and Zn^{2+} testifies that the lowest

distribution coefficients corresponds to the region of low values of pH 2-3 and with the increase of pH > 5 the sorption indices increase sharply. Under the sorption in the neutral and alkali range the curves of these metals go into the plateau at pH 5-9 and on transition to pH 11 a sorption degree decreases slightly (Figs. 1, 2).

The absorption of Hg^{2+} depends insignificantly on the acid-alkali balance within the values of pH 3-11 (Fig.3). At pH 3-7 the distribution coefficient in Hg^{2+} was practically unchanged and only on transition to pH 9 the distribution coefficient was twice as high and reached its maximal values (K_d = 14500). The minimal level of absorption was fixed at pH 1 (K_d = 830). Even at such a low distribution coefficient the level of Hg^{2+} extraction from the solution was about 90%. This indicates the high efficiency of Mycoton in extraction of mercury. Under analogous conditions at pH 2 sorption of bivalent Zn^{2+} and Ca^{2+} was considerably lower (K_d = 77 and 13, respectively).

The complex study of Zn^{2+} sorption was carried out at the concentration of 5-40 mg/l and pH 3-9 (Fig. 4). The sorption at the concentration of Zn^{2+} 5-20 mg/l increased swiftly with the transition of pH from 3 to 5 and then practically was unchanged up to pH 7. Then an insignificant decrease of the distribution coefficient was observed. At the same time the pattern of sorption curve for *Zn* at 40 mg/l differed significantly. Its sorption increased gradually with pH of the solution and reached its maximum at pH 9 (K_d=1400) (Fig.4).

During investigation of mercury sorption on Mycoton we have revealed that its relatively small part (less than 0.1% of residual mercury at C = 10 mg/l and to 20% at C = 10 mkg/l) is after sedimentation in water at the metal (element) slate. A conclusion about the presence of recovery properties in Mycoton has been drawn. It has been shown that in the regions of high values of pH Mycoton possesses rather strong recovery properties to change the valency of a number of metals.

The redox-potential of the Mycoton-water system relative to the *Ag-Cl* electrode was determined to study the recovery properties of Mycoton. Investigations were carried out within the range of pH 1.5-10.3 (Fig. 5). Titration of Mycoton with the 0.5 % solution of J_2 in O.5 N solution of *KJ* at pH 8.7 was made to estimate the concentration of the reducing centers (Fig. 6). If we suppose that one molecule of J_2 falls on one reducing center, then the ratio - one reducing center per 25-30 monomer chitin links - corresponds to it.

To explain bisorption of metal ions on Mycoton we have supposed that this process is determined by several mechanisms. One of the main mechanisms, which absorb the ions of heavy metals on chitin containing materials, is chelation [2, 9]. The ion exchange and weak interactions (Van der Waals forces) may play a lesser role in biosorption. Biosorption on fungal materials has definite differences from the sorption on chitin preparation of crustacea. The following specific mechanisms are established for chitin-containing preparations: sedimentation of insoluble compounds of metals and slow process of metal crystallization on the surface of chitin fibres [1, 9]. The presence of recovery properties in Mycoton makes it possible to suppose that the preparation may reduce mercury and silver up to metal state in the region of pH>7, which then sediment as particles on the surface of chitin microfibrillas. The formation of metal precipitates for zinc and cadmium in the range of 7<pH<12 is not possible.

Conclusions

Basing on the data available in literature on absorption of heavy metals by chitin and chitosan and on the results of our studies it is possible to conclude that chitin-containing materials Mycoton from *Higher Basidiomycetes* possess higher sorption indices than materials from

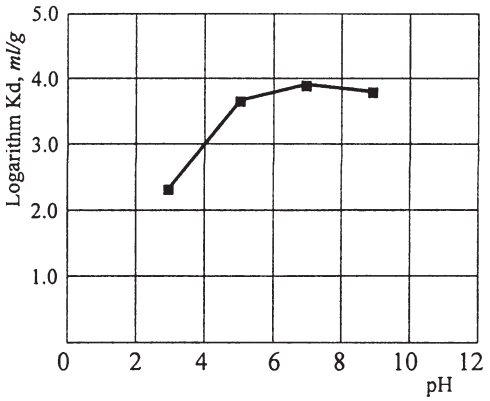

Fig. 1. Solution pH effect to the destribution coefficient (*Kd*) of silver (C 10 mg/l)

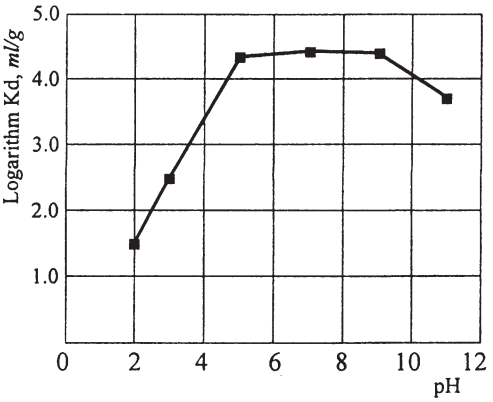

Fig. 2. Solution pH effect to the destribution coefficient of cadmium (C 10 mg/l)

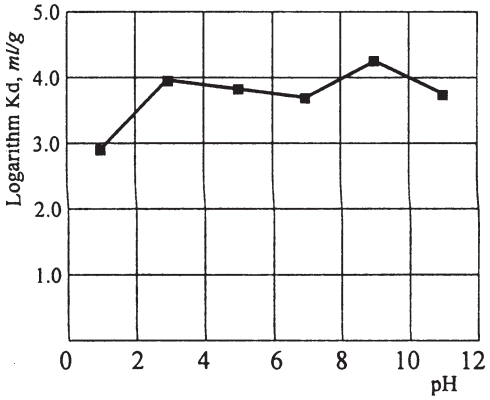

Fig. 3. Solution pH effect to the destribution coefficient of mercury (C 10 mg/l)

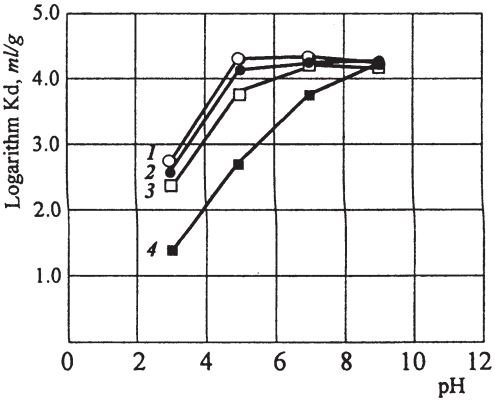

Fig. 4. Solution pH and concentracion effect to the destribution coefficient of zinc (C 5 mg/l -*1*, 10 mg/l -*2*, 20 mg/l -*3*, 40 mg/l -*4*)

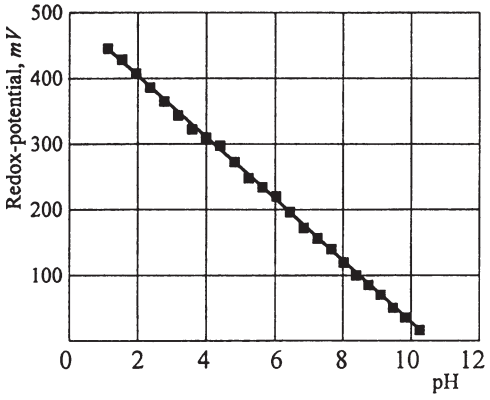

Fig. 5. Solution pH effect to redox-potential of Mycoton in the water system

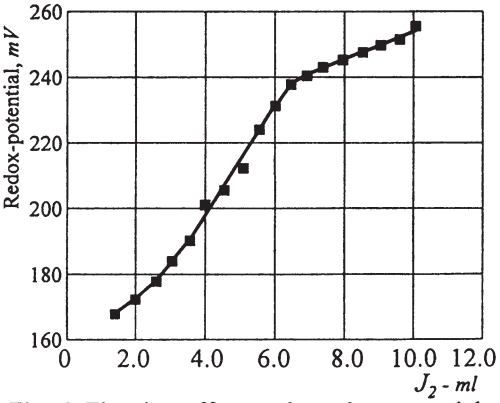

Fig. 6. Titration effect to the redox-potential of Mycoton (0.5% J_2 in 0.5 N *KJ*)

other fungi [3, 7, 12, 13]. Due to high sorption possibilities they are promising for application in the processes of heavy metals and radionuclides extraction.

When studying sorption of metals with the variable valence on the chitin-containing sorbents Mycoton it is necessary to take into account the redox properties of the system - metal ions may completely or partially be in valences differing from the initial ones.

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Comparative studies on molecular chain parameters of polyelectrolyte chains. The stiffness parameter *B* and the temperature coefficient of intrinsic viscosity of chitosans and poly(diallyldimethylammonium chloride)

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Summary
The empirical coefficient B, a measure of polyelectrolyte chain stiffness was estimated for two polycations: chitosans with F_A (mole fraction of *N*-acetyl-**D**-glucosamine units) ranging from 0.09 to 0.21 (determined by ^{1}HNMR method) and poly(diallyldimethylammonium chloride) (PDADMAC), in the presence of different counterions (Cl^-, Br^-, NO_3^-, ClO_4^-). The method relies on the measurements of the intrinsic viscosity, [η], of polyelectrolyte chains at different solution ionic strength (I). Thus, the B-parameter shows the response of the hydrodynamic volume of polyelectrolyte molecules on the salt concentration in solution (the so-called *salt tolerance*), referred to the [η] at I = 0.1M. The temperature coefficient of intrinsic viscosity of PDADMAC was also determined.

The comparison of the B-parameter and of the temperature coefficient of intrinsic viscosity for both polycations indicates the lower flexibility of chitosan than PDADMAC chains.

Introduction

Many of natural and synthetic cationic polyelectrolytes have found a wide spectrum of practical applications. Specially those water soluble are of significant importance in the field of environmental protection, for example as flocculates and coagulants, and also in paper making, petrol industry or in cosmetics and medicine [1–3].

Chitosan is commercially produced from chitin – the second-most important polysaccharide after cellulose in the world – using deacetylation processes [4, 5]. Because the deacetylation is not usually completed, chitosan can be considered as a binary heteropolysaccharide containing (1→4) linked 2-acetamide-2-deoxy-β-**D**-glucopyranose and 2-amino-2-deoxy-β-**D**-glucopyranose residues. The chemical composition of different chitosans is characterised by a F_A value (fraction of acetylated units) or by the deacetylation degree: $100(1 - F_A)$ %. Chitosan structure is shown in Fig. 1.

Poly(diallyldimethylammonium chloride) (PDADMAC) is a synthetic polymer (see Fig. 2) obtained by the radical polymerisation [6–8].

As chitosan and PDADMAC are polyelectrolytes (polycations), one may expect that intra- and intermolecular interactions of the electrostatic nature could influence the chain flexibility in a similar way. Moreover, both have a cyclic structure in the backbone.

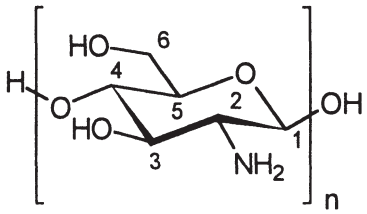

Figure 1. Structure of 100% deacetylated chitosan.

Thus, it was interesting to make some comparative studies on the chain stiffness (flexibility) of chitosan and PDADMAC. This was the aim of the present paper.

Figure 2. Structure of poly(diallyldimethylammonium chloride): a) *cis*-, b) *trans*-.

The relative chain stiffness (flexibility) of polyelectrolytes may be estimated on the basis of the *B*-parameter [9]. The use of this parameter as an indicator of the flexibility is based on the fact that stiffness of polyelectrolyte molecules is related to the salt tolerance in solution. The more flexible the chain, the higher the response of the intrinsic viscosity, $[\eta]$, on the change of solution ionic strength. Referring to the B-parameter: the higher the *B* value, the more flexible the polyelectrolyte chains. Determination of the *B*-parameter requires the knowledge of two dependencies: 1) $[\eta]$ vs. $1/\sqrt{I}$, where $[\eta]$ (dL/g), is the intrinsic viscosity of a polymer in a solution at a given ionic strength I (M), and 2) lg *S* vs. $\lg[\eta]_{I=0.1}$, where *S* is the slope of the plot obtained from:

$$S = d[\eta]/d\sqrt{I}^{-1} \tag{1}$$

The *B*-parameter is then related to *S* by equation:

$$S = B[\eta]^{v}_{I=0.1} \tag{2}$$

where *v* is a factor found to range between 1.2 for polyphosphates (flexible chains) and 1.4 for DNA (stiff chains) [9, 10]. The *v* factor can be obtained also as a slope, simultaneously with *B*:

$$\lg S = \lg B + v \lg[\eta]_{I=0.1} \tag{3}$$

when measurements with samples varying in molecular weight are performed.
Theoretical foundations for this procedure based on the theory of Fixman (1964) are discussed in [9]. The results obtained by Smidsrød and Haug for pectin and polyacrylate [9] support the idea that some variation in the stoichiometric charge density of polyelectrolytes does not lead to variation in B.

The temperature dependence of $[\eta]$, in general, can be written as follows [11, 12]:

$$d\ln[\eta]/dT = d\ln\Phi/dT + 1.5d\ln\langle\bar{S}^2\rangle_0/dT + 3d\ln\alpha_s/dT \qquad (4)$$

where Φ is the Flory viscosity function defined as $\Phi = [\eta]M/(6\langle\bar{S}^2\rangle)^{3/2}$, $\langle\bar{S}^2\rangle_0$ is the unperturbed mean-square radius of gyration and α_s^3 is the expansion coefficient of $\langle\bar{S}^2\rangle$. Thus, the quotient $d\ln[\eta]/dT$ is related to the temperature dependence of the unperturbed dimensions and to the excluded volume. The first term on the right-hand side of eq. (4), $d\ln\Phi/dT$, is a decreasing function of the macromolecular coil expansion. The second term, $d\ln\langle\bar{S}^2\rangle_0/dT$, can be positive (e.g. polystyrene [11, 13] or negative (e.g. polyethylene [11, 14] or cellulose diacetate [12]). The third term, $d\ln\alpha_s^3/dT$, governs the dependence of the $d\ln[\eta]/dT$ on molecular weight. The absolute value of $|d\ln\alpha_s^3/dT|$ is an increasing function of molecular weight. Thus, the resultant value of $d\ln[\eta]/dT$ depends on the sign and magnitude of the three terms in eq. (4) and, as a consequence, positive or negative values of $d\ln[\eta]/dT$ may be obtained.

Experimental

Chitosan was obtained from Fluka (Switzerland) and from Sea Fisheries Institute in Gdynia (Poland). Chitosan was degraded with H_2O_2 by the method determined at the Nicholas Copernicus University in Toruñ (Poland) (to be published). Chitosan samples not converted and converted to the chloride form by the method described elsewhere [15] were used. The F_A values were determined by the ^{1}HNMR method [16] using Jeol DPX 300 (300.13MHz) spectrometer. For the chitosan samples used in the present investigation F_A values were found to be: 0.21, 0.17 and 0.09.

Two sets (A and B) of commercial PDADMAC samples (high, medium and low molecular weight) were obtained from SIGMA (Germany), p. a. as 20% water solutions. Solutions of desired PDADMAC concentration were prepared by dilution of the stock, 20% water solution of PDADMAC.

Viscosity measurements were carried out using Schott-Geräte Ubbelohde capillary viscometer (type 531 01/0a), with flow-through time for solvents always above 200 seconds at 293K to 323K in acetate buffer (0.02M CH_3COOH/CH_3COONa, pH 4.5) in order to keep the same pH-value in all experiments and to have desired ionic strengths with NaCl, NaBr, $NaNO_3$ and $NaClO_4$ (simple electrolytes). The intrinsic viscosity values were calculated with respecting the Hagenbach correction. Chitosan samples dried to a constant weight were taken for measurements.

Results and Discussion

In order to obtain the B-parameter the intrinsic viscosities of three samples of chitosan chloride (F_A= 0.21, 0.17, 0.09) and three samples of w PDADMAC of low, medium, and high

molecular weight (MW), at the following ionic strengths: 0.02M, 0.05M, 0.07M, 0.1M, 0.5M, 1.0M in aqueous NaCl, at 293K, were determined. The Huggins and Kraemer equations were used to obtain intrinsic viscosities [17].

The dependence plotted as $[\eta]$ vs. $1/\sqrt{I}$ for chitosan chloride and PDADMAC is presented in Fig. 3.

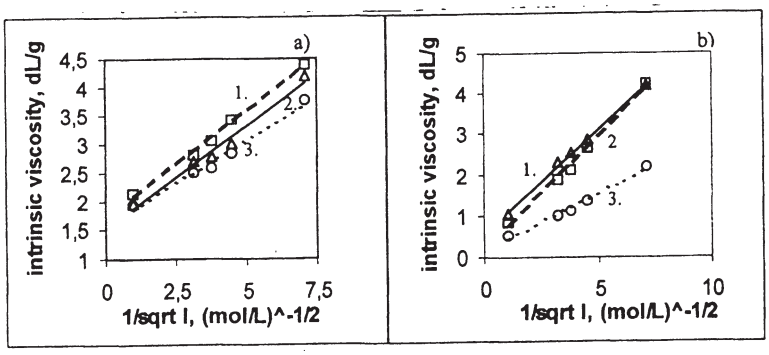

Figure 3. Determination of the *S*-parameter for a) chitosan chloride: 1. F_A = 0.17, 2. F_A = 0.09, 3. F_A = 0.21 and b) PDADMAC: 1. MW = $6.8 \cdot 10^4$, 2. $5.3 \cdot 10^4$, 3. $2.8 \cdot 10^4$. T = 293K.

The ν factor equals 1.3 was taken for calculation of the *B*-parameter of chitosan, whereas the values of ν = 1.2 and 1.3 were taken for PDADMAC. The obtained results of the intrinsic viscosities at different ionic strength, $[\eta]_I$, and the S and B-parameter of chitosan chloride and PDADMAC are depicted in Table 1. The obtained results confirmed that the *B*-parameter of

Table 1. Intrinsic viscosity at different ionic strengths, salt tolerance *S*, viscosity average molecular weight, $\overline{M}_v$, and experimental values of the *B*-parameter for chitosan chloride and PDADMAC.

	Chitosan chloride			PDADMAC (set A)		
$\overline{M}_v \cdot 10^{-4}$ 1)	4.6	5.6	4.7	2.8	5.3	6.8
F_A	0.21	0.17	0.09			
$[\eta]_{I=0.02}$	3.76	4.39	4.19	1.91	3.87	4.55
$[\eta]_{I=0.05}$	2.84	3.42	3.01	1.29	2.66	2.79
$[\eta]_{I=0.07}$	2.58	3.06	2.78	1.13	2.34	2.62
$[\eta]_{I=0.1}$	2.49	2.82	2.70	0.90	2.13	2.39
$[\eta]_{I=0.5}$	–	–	–	0.58	1.17	1.25
$[\eta]_{I=1.0}$	1.92	2.14	1.96	0.54	0.99	1.14
S	0.30	0.38	0.36	0.23	0.53	0.51
B_i 2)	**0.09**	**0.10**	**0.10**	**0.26**	**0.18**	**0.18**
B_i 3)	–	–	–	**0.26**	**0.19**	**0.20**
B_i 4)					**0.25**	

1) calculated according to Mark-Houwink-Sakurada equations in: [15] (chitosan chloride) and [6] (PDADMAC); 2) ν =1.3,3) ν =1.2; 4) from equation (3): lg S = −0.598+0.881lg$[\eta]_{0.1}$, R^2 = 0.996.

chitosan is not affected by the change of the F_A value in the range of F_A = 0.09 – 0.21. At a lower F_A value (F_A = 0.6) the value of B is decreasing to B = 0.02 [15], which means that chitosan molecules become less flexible. As the influence of the type of counter ions (Cl^-, NO_3^-) on the B-parameter of chitosan gave contradictory results [18–20], we measured the B-parameter of the mother chitosan samples, the same which were used for the conversion to the chloride form of chitosan.

Thus the measurements were performed with chitosan in its free amine form, in acetate buffer solution (pH=4.5), in aqueous NaBr, $NaNO_3$, $NaClO_4$ at 293K. For PDADMAC the B-parameter was additionally obtained in $NaNO_3$ solution. The results are collected in Table 2, together with the values of the B-parameter calculated by us on the basis of the reported data of $[\eta] = f(I)$ for chitosan [21] and PDADMAC [8, 22, 23]. The B values reported by other investigators for chitosan [15, 18, 21, 24–26] are also included. Our results showed (Table 2) that the influence of the type of counterions in the case of chitosan (Cl^-, Br^-, NO_3^-, ClO_4^-) as well as in the case of PDADMAC (Cl^-, NO_3^-) may be neglected. This is in line with the results of Rodriguez-Sanchez et al. [18, 19] for chitosan, who concluded that the conformational changes of chitosan molecules are independent on the nature of the counterions used. Thus, the hydrodynamic volume of both polycations does not change with the increase of the volume of the used, monovalent counterions.

Table 2. The B-parameter of chitosan and PDADMAC determined (this paper), calculated (with asterisk*) on the basis of data in the literature and the B value reported.

B-parameter of chitosan	*Experimental conditions*	*Reference*
0.06 – 0.09	free amine form, acetate buffer, $0.09 \le F_A \le 0.21$, counter ions: Br^-, NO_3^-, ClO_4^-	this paper
0.05 – 0.13*	free amine form, acetic acid/NaCl solution, $0.15 \le F_A \le 0.22$	[21]
0.02 – 0.10	chitosan chloride, $0 \le F_A \le 0.6$	[15]
0.08		[18, 24]
0.043 – 0.091	$0.12 \le F_A \le 0.52$	[25]
0.023	0.2 M acetic acid/0.1 M sodium acetate, F_A = 0.18	[26]

B-parameter of PDADMAC	*Experimental conditions*	*References*
0.21 (set B)	aqueous $NaNO_3$	this paper
0.28*	aqueous NaCl	[8]
0.22*	aqueous NaCl	[22]
0.17*	aqueous $NaNO_3$	[23]

It can be clearly seen that the B-parameter of chitosan has a lower value than that of PDADMAC. This indicates that chitosan molecules are less flexible than PDADMAC ones, being less sensitive for the change of ionic strength of solution. Due to its molecular structure, PDADMAC may occur in two isomeric forms: *cis-* and *trans-* (14% [7, 27]) and this may be the additional reason of creating more compact coils in reply to the change of I. There is also a possibility of rotation around methylene groups present between dimethylpyrrolidynium rings, what has an impact on the molecular flexibility of PDADMAC.

In the case of the chitosan, its macromolecules could only rotate around the glicosidic bonds, thus there is much less possibilities of changing the conformation when the change of I takes place. Moreover, presence of bulky acetamide groups does not facilitate the chitosan chain motion, which is manifested in the decreasing value of B (B = 0.02) at higher value of F_A (F_A = 0.6) [15]. Terbojevich et al. [25] suggested that this arises from the intramolecular hydrogen bonding between C(6')OH and C(2)$NHCOCH_3$ groups, which stabilises the more extended conformation of chitosan with higher values of F_A.

Values of the temperature coefficient of intrinsic viscosity were obtained for PDADMAC and compared to those reported for chitosan. The collected values of $d\ln[\eta]/dT$ (I) and $d\ln[\eta]/d(1/T)$ (II) (in parenthesis) are shown in Table 3.

Table 3. The values of the temperature coefficient, $d\ln[\eta]/dT$ (I) and $d\ln[\eta]/d(1/T)$ (II) (in parenthesis) of PDADMAC (this paper) and of chitosan (reported).

No	*I, M*	*The temperature coefficient of intrinsic viscosity* PDADMAC (set B)			
		(I)	(II)		$\overline{M}_v \cdot 10^{-4}$
1.	0.0032	$2.2 \cdot 10^{-3}$	(–211);	$R^2 = 0.828$	6.8
2.	0.0065	$2.2 \cdot 10^{-3}$	(–211);	$R^2 = 0.945$	2.8
3.	0.1	$3.3 \cdot 10^{-3}$	(–311);	$R^2 = 0.922$	6.8
4.	0.1	$3.6 \cdot 10^{-3}$	(–332);	$R^2 = 0.951$	2.8
5.	0.5	$3.5 \cdot 10^{-3}$	(–329);	$R^2 = 0.965$	6.8
6.	0.5	$3.6 \cdot 10^{-3}$	(–345);	$R^2 = 0.977$	2.8

No	*Experimental conditions*	*The temperature coefficient of intrinsic viscosity* Chitosan		
		(I)	(II)	*Molecular weight* $\cdot 10^{-5}$
1.	0.33M CH_3COOH/ 0.3MNaCl; $F_A = 0.2$ [29]	$-5.3 \cdot 10^{-3}$	(488)	1.34
2.	0.2M CH_3COOH/ 0.1M NaCl/ 4M urea $F_A = 0.21$ [30]	$-3.2 \cdot 10^{-3}$		1.35 – 4.92
3.	0.2M CH_3COOH/ 0.1M CH_3COONa $F_A = 0.21$ [30]	$-4 \cdot 10^{-3}$		1.35 – 4.92
4.	0.01M HCl $F_A = 0.17$ [28]		(1334–666)	0.78– 9.14

The data in Table 3 indicate that the essential difference between the temperature coefficients of chitosan and PDADMAC is its sign: the intrinsic viscosity of chitosan decreases with the increase of temperature, thus $d\ln[\eta]/dT$ is negative, whereas in the case of PDADMAC the increase of $[\eta]$ is observed, so $d\ln[\eta]/dT$ is positive.

It was established by R.H. Chen et al. [28] that the coefficient $d\ln[\eta]/d(1/T)$ decreases with the increase of molecular weight of chitosan, which means that the flexibility of chitosan chains is growing with its length. This is a general rule, well manifested in the case of stiffer macromolecules. It is known, moreover, that the conformation and hence the flexibility of polyelectrolytes depends on the ionic strength of the solution. This is also observed in chitosan.

Referring to the data in Table 3, Chen et al. [28] obtained the value of $d\ln[\eta]/d(1/T) = 1299$ for chitosan ($F_A = 0.17$) of MW = 148 kDa at I = 0.01M, whereas N.V. Pogodina et al. [29] obtained the value of $d\ln[\eta]/d(1/T) = 488$ for chitosan ($F_A = 0.2$) of MW = 134 kDa at I = 0.3M. The much higher value of $d\ln[\eta]/d(1/T)$ at the lower value of I, at other comparable experimental conditions (similar value of F_A and MW) showed that chitosan molecules are more stiff at lower value of I, because of the presence of intra- and intermolecular electrostatic interactions.

Referring to the results obtained by Lee [30] one may also conclude that the structure of water may influence the dimensions of chitosan chains. As it is seen (Table 3), at the same value of I (I = 0.1M) but in the presence of urea, known as a destroying agent of the „ice-berg" structure of water [31], the negative slope of $d\ln[\eta]/dT$ is less steep ($-3.2\cdot10^{-3}$) as without urea ($-4\cdot10^{-3}$). This means that the flexibility of chitosan molecules in aqueous urea solution is greater. Such conclusion may be an indication of the role of „ structured" water in the hydration process of chitosan.

As mentioned in the Introduction, the sign of $d\ln[\eta]/dT$ is a resultant of the three terms shown in eq. (4). Some of these terms are not determined for chitosan, but a comparison with the values obtained for other polysaccharides may be performed. This procedure is based on the observation that polyelectrolytes in solutions of relatively high ionic strength behave as nonionic polymers, thus their dimensions may be compared to the dimensions of nonionic polymers in organic solvents. For cellulose diacetate in acetone e.g. the term $d\ln\Phi/dT$ is positive and equals $(2.8\pm0.5)\cdot10^{-3}$ [12]. The term $d\ln\alpha_s^3/dT$ in the case of cellulose and its derivatives may be neglected [12]. Taking this into account, the negative value of $d\ln[\eta]/dT$ for chitosan is, as in cellulose diacetate, primarily determined by the negative value of $d\ln\langle\bar{S}^2\rangle_0/dT$. Noguchi [32] postulated that the decrease of chitosan chain dimensions with the rise of temperature may be, as in polyglucans, dextran etc., a result of loss of hydrogen-bonded water in the hydration sphere of chitosan.

The temperature coefficient of PDADMAC was determined for the sample of low molecular weight ($\overline{M}_v = 2.8\cdot10^4$ at I = 0.032M, 0.1M, 0.5M) and the sample of high molecular weight ($\overline{M}_v = 6{,}8\cdot10^4$ at I = 0.065M, 0.1M, 0.5M) (Table 3). As it was mentioned above, in the case of PDADMAC the slope of $d\ln[\eta]/dT$ is positive. Further, $d\ln[\eta]/dT$ of PDADMAC increases with the increase of I at the low range of I. This may be related to the higher value of the B-parameter (B = 0.26, Table 1) of PDADMAC sample of lower molecular weight (set A), indicating its higher chain flexibility. One may suggest, at present, that the unexpected results: the highest flexibility of PDADMAC (set A) with the lowest molecular weight, and the increase of the temperature coefficient of $[\eta]$ of PDADMAC (set B) in the range of ~0.065M $\leq$ I $\leq$0.1M (Table 3) may be caused by the chain branching of commercial PDADMAC [27, 33]. For comparison of the temperature coefficient of PDADMAC with that of chitosan, the PDADMAC sample of the higher molecular weight and at the higher value of I was taken. Following on the data in Table 3, the value of $d\ln[\eta]/d(1/T)$ of PDADMAC ($\overline{M}_v = 6.8\cdot10^4$, I = 0.5M) amounted (–329), whereas for chitosan (MW = $134\cdot10^3$, I = 0.3M [29]) it amounted 488. This comparison confirmed the earlier conclusion, drawn on the values of the B-parameter for chitosan and PDADMAC, that chitosan is less flexible than PDADMAC.

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Crystalline behavior of chitosan

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Summary
During storage of a chitosan monocarboxylic acid salt, a spontaneous removal of the acid accompanying dehydration of the salt was observed resulting in the occurrence of the anhydrous crystal of chitosan where chitosan takes up an extended two-fold helix. The salts with several acids showed fiber patterns typified to Type II salt, a hydrated crystal, where the backbone chitosan molecule takes up a less-extended two-fold helical conformation. The anhydrous crystals of chitosan used to be prepared by annealing a hydrated crystal in water at a high temperature leading to a little loss of orientation and to a partial thermal decomposition of the specimen. But, through Type II salts of monocarboxylic acids, the dehydrated crystals of chitosan could be obtained without any loss of orientation and decomposition of the chitosan specimen by the spontaneous removal of the acid. This crystalline transformation was accelerated when these salts were immersed in a mixture (3:1 v/v) of 2-propanol and water.

Introduction
Five crystalline polymorphs of chitosan have been found by X-ray diffraction measurements; four hydrated forms and one anhydrous. Hydrated polymorphs are called "Tendon" (the most abundant) [1], "Form II" [2], "L-2" [3] and "Eight-fold"[4] although the last polymorph is unstable. In the former three polymorphs, chitosan molecules take up an extended two-fold helix. Whereas, in the last, it had been predicted to take up an 8/5 helical structure [4]. However, our resent examinations have revealed that the last polymorph is a less-extended two-fold helix composed of tetramer of glucosamine residues as an asymmetric unit [5]. So that, we call "Less-extended" for the last polymorph. The anhydrous polymorph is prepared by heating a hydrated crystal of chitosan in water at a high temperature, such as 240 °C, depending on the molecular weight of chitosan [6, 7]. This polymorph is called an "Annealed" form where chitosan has an extended two-fold helix [6]. Recently, we have analyzed molecular and crystal structures of "Tendon" [8] and "Annealed" [9-11] polymorphs, and found that the transformation from the former to the latter occurred irreversibly [7] and involved a drastic change in the chain arrangement of chitosan although no change in the molecular conformation of each chitosan chain was observed [8]. Anhydrous crystalline chitosan does not dissolve in any aqueous acid solution, nor form a complex with any transition metal ion [7, 12]. Consequently, the anhydrous chitosan may be used to be an inert

material. However, the high temperature heating required for this crystalline transformation results in not only a little loss of the orientation but also in a thermal decomposition of chitosan to some extent, particularly, on the surface of the specimen. We found that a chitosan specimen of the hydrated "Tendon" polymorph changed to the anhydrous "Annealed" polymorph without any decomposition when it was stored in water at room temperature for 18 months [13], but the period is too long for practical application.

We have studied the crystals of various inorganic [14] and organic [15-17] acid salts of chitosan by X-ray fiber diffraction methods and have revealed that these crystals are classified into two types depending on the structure of the acid and sometimes on the temperature of salt preparation. One called Type I salts are mostly anhydrous, and in these crystals the backbone chitosan chains retain the extended two-fold helix of the unreacted chitosan molecule [8]. The other, Type II salt, is a hydrated crystal and has a less-extended two-fold helical conformation in the crystal [5]. Despite different anion sizes, all the Type II salts give not only similar fiber patterns to one another [14, 16] but also to the "Less-extended" polymorph of chitosan molecule [4]; that is, they have identical unit cell dimensions. These facts suggested that anions were not present regularly in the respective crystals of the Type II salts and consequently that only the backbone chitosan chains contributed to the fiber pattern [14]. A solid-state ^{13}C NMR study of these crystalline salts [18] revealed that the two types of helical structures were easily distinguishable by their spectra. In addition, other acid salts of chitosan including organic acid salts, $HClO_4$, HIO_4, H_3PO_4, HCOOH, and CF_3COOH, were also classified in the two types of conformation by the NMR study. Demarger-Andre and Domard have reported that anhydrous chitosan crystals can be obtained at room temperature from chitosan salts of several monocarboxylic acids (acetic, butyric or valeric acids) by spontaneous removal of the acids accompanied by dehydration [19]. We call it "spontaneous water removing action of acid".

In this report we develop new procedures to prepare the anhydrous "Annealed" crystal, the inert chitosan, without any decomposition, via Type II salts of many different acids using X-ray fiber diffraction measurements.

Materials and Methods

In order to prevent loss of molecular orientation a tendon chitosan was prepared by a heterogeneous *N*-deacetylation of a crab tendon chitin, *Chionecetes opirio* O. Fabricus, with 67% sodium hydroxide solution at 110 °C for 2 h under nitrogen atmosphere. Repeating the reaction twice led to complete *N*-deacetylation of resultant chitosan, as determined by FT-IR spectra and a colloidal titration. The viscosity average molecular weight of the tendon chitosan was 1.7×10^6 [7]. The preparation of all chitosan salts of inorganic [14] and organic acids [15-17] other than monocarboxylic acids have been reported previously. Since chitosan dissolves in aqueous acids leading to a loss of molecular orientation and crystallinity, the tendon chitosan was immersed in a mixture (3:1 v/v) of 2-propanol and an aqueous solution of respective acids at various temperatures for a given period depending on the kind of acid.

The X-ray fiber diffraction patterns were recorded using a flat film camera at 75% relative humidity in a helium atmosphere employing Ni-filtered CuKα radiation. Crystallinity of a chitosan specimen was calculated from WAXD intensity curves of a powder diffraction pattern using a microdensitometer.

Results and Discussion

As Demarger-Andre and Domard [19] have pointed out the spontaneous water removing

action of acid was observed with the monocarboxylic acid salts of chitosan only. All other acid salts [14-16] including dicarboxylic acids [20] studied so far have not shown the spontaneous action even after their storage at 100 % r.h. for one year or more. We have found from X-ray diffraction measurements on fiber diagrams that the action occurs in the crystals of chitosan acetic acid salt [17] which showed a diagram of typical Type II salt. A completely different fiber pattern regarded as the anhydrous "Annealed" polymorph of chitosan was observed when the salt specimen was stored at the room temperature and around 80% r.h. for three months. Measurements of density and the FT-IR spectrum of the specimen supported the transformation from the acetic acid salt to an anhydrous crystal of chitosan [17]. This change was accelerated when the salt was stored at higher humidity, e.g., at 100% r.h. for approximately one month.

All the monocarboxylic acids of chitosan studied were Type II salts, hydrated crystals, where the backbone chitosan chains took up the less-extended two-fold helical conformations [5]. The chitosan formic acid salt could be prepared at a lower temperature than the acetic acid salt, which may be due to stronger acidity of formic acid. Whereas, the temperature for salt formation was acetic > propionic > butyric acid regardless of the acidity, and the Type II salt of chitosan butyric acid could not be detected in the present X-ray study presumably because the water removing action of the butyric acid is too fast. The temperature dependence observed for salt formation may be due to the solubility of the monocarboxylic acid to water because the relative hydrophobicity of these acids is acetic < propionic < butyric, and because monodispersity (i.e., no molecular aggregation) of more hydrophobic material in water solution requires lower temperature [21].

Present results suggested that all the monocarboxylic acids studied were spontaneously removed accompanied with dehydration from the salts during storage for a given period of time. The period depended not only on the acid but also on relative humidity of the storage. When stored at 100% r.h., three months, one month and three weeks were required to produce the complete fiber pattern of the "Annealed" chitosan polymorph from formic, acetic and propionic acid salts of chitosan, respectively. In contrast, chitosan butyric acid salt gave the "Annealed" pattern of chitosan immediately after the preparation. These facts suggest that the water removing action of monocarboxylic acid is accelerated by the hydrophobicity in addition to p*Ka*, solubility and boiling point of acid as speculated by Demarger-Andre and Domard [19].

The mechanism of the spontaneous water-removing action of monocarboxylic acid has not been well defined yet, but may be attributed to the conformational stability of the chitosan molecule. The extended two-fold helix of chitosan is stabilized by strong O(3)----O(5') intramolecular hydrogen bonds, the distance of which is 0.275 nm [8, 9, 11]. Whereas, the distance of intramolecular hydrogen bonds of "Less-extended" two-fold helix are longer than those of the extended two-fold helix indicating that the latter conformation has weaker hydrogen bonds than the former. In addition, Cairns *et al.* have indicated that the "Eight-fold" ("Less-extended") polymorph is unstable and is easily converted to crystals of the extended two-fold helices. An axial rise per glucosamine residue is calculated from the fiber repeat divided by number of residues composing the repeat, is 0.52 nm for the extended two-fold helix [8, 9, 11] and 0.51 nm in average for the "Less-extended" two-fold helix [5]. It is reasonable to consider that the chitosan molecule having the extended two-fold helix in the hydrated crystal is twisted slightly by the formation of type II salt. The resulting "Less-extended" helix structure may be stabilized by an anion of the salt. However, if the interaction between the anion and chitosan is weak, the chitosan molecule tends to go back to the more stable two-fold conformation by throwing out the anion. The anhydrous "Annealed"

polymorph is more stable than the hydrated "Tendon" since transformation from the latter to the former is irreversible [7]. This fact indicates that the conformational change from "Less-extended" to the extended two-fold helix stimulates to be more stable "Annealed" crystal of chitosan, resulting in removing water molecules presented between chitosan chains. At present, we do not know why monocarboxylic acids have weaker interactions with chitosan molecules than other acids studied so far, and why the hydrophobicity of the acid and the humidity of storage accelerate the water removing action of the acids.

We have analyzed the crystal and molecular structures of hydrated "Tendon" [8] and anhydrous "Annealed" [9-11] polymorphs of chitosan where chitosan chains have a similar extended two-fold helix stabilized by O(3)----O(5') intramolecular hydrogen bonds in both crystals. In the hydrated crystal, adjacent antiparallel chitosan chains are bonded to each other by two interchain hydrogen bonds forming a sheet. Furthermore, the adjacent sheets are bonded by hydrogen bonds involving water molecules [8]. Whereas, in the anhydrous polymorph, intermolecular hydrogen bonds connect adjacent parallel chitosan chains to make a sheet, and adjacent sheets are antiparallel [9, 11]. These facts suggest that a drastic change in the arrangement of chitosan chains occurs during the transformation from the hydrated to the anhydrous crystal [8].

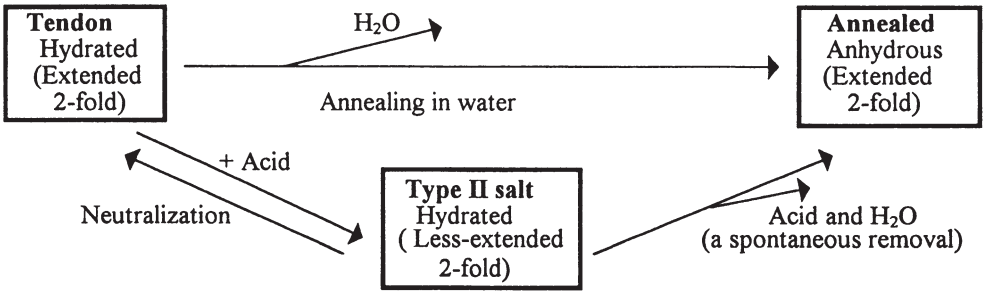

Figure 1. Two processes of water removing from the hydrated crystal of chitosan.

Two transformation procedures from the hydrated to the anhydrous polymorph of chitosan are illustrated in Figure 1. So far, the anhydrous chitosan has been prepared by annealing the hydrated crystal in the presence of water at a high temperature, such as 240 °C, although the temperature required depends on the molecular weight of chitosan [7]. The requirement for such a high temperature may be due to the drastic change in the chain arrangement of chitosan [8]. This results in a little loss of orientation and a thermal decomposition of sample specimen to some extent, particularly, on the surface. The decomposition is not appropriate for preparing the "Annealed" crystal of chitosan as an inert material. The present results indicate that the anhydrous polymorph can be easily obtained even at room temperature via Type II salts of monocarboxylic acids without any loss of orientation and thermal decomposition of chitosan molecules. At first, the hydrated chitosan reacts with a monocarboxylic acid to make a hydrated Type II salt where chitosan chain has an less-extended two-fold helix composed of tetramer of glucosamine residues as an asymmetric unit [5]. After that, during the storage of the salt, the acid evaporates spontaneously out of the chitosan chains accompanied by water molecules, resulting in the occurrence of the anhydrous polymorph of chitosan. Both transformations from hydrated to anhydrous polymorph are

irreversible although the Type II salt can be converted to the hydrated Tendon chitosan by neutralization with an aqueous alkali such as sodium hydroxide solution.

Practical preparation of the anhydrous polymorph of chitosan requires faster removal of acid and water molecules from a Type II salt. When each monocarboxylic acid salt of chitosan was immersed in a mixture (3:1 v/v) of 2-propanol and water at 25 °C, the anhydrous crystal of chitosan was obtained in much shorter time than the case of storage at 100% r.h.: 2 days for the formic acid salt and 3hrs.for the acetic. In case of propionic and butyric acid salts the removal of acid and water were too fast to detect by the present X-ray method. The crystallinity of resultant chitosan specimen was 82 % from the formate, 74 %, the acetate, 71 %, the propionate, and the butyrate, 61 %.

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The relationship between the crystallinity and degree of deacetylation of chitin from crab shell

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Summary
The N-deacetylation of chitin was performed under strong alkali condition at ambient temperature within different time of treatments and characterized by IR spectroscopy and HPLC method to determine degree of deacetylation. Different degree of deacetylation of chitin/chitosan samples were obtained for analysis and study of the crystallinity patterns by using X-Ray diffractometer.

Introduction
Chitin, a linear polysaccharide composed of β-(1-4)–linked–2-deoxy–acetamido-D-glucose unit (Glu-$NHCOCH_3$) is a microfibrillar materials with distinct crystal morphologies. Generally, chitin occures as a component of crustacean exoskeleton (shrimp, crab and cuttlefish), insect exoskeleton, fungel cell walls, and plankton.[1,2,3] The different sources of raw materialscontain different in both quality and quantity of chitin entire. Chitosan, a principle derivative of chitin can be obtained by the process of alkali deacetylation. Different conditions of alkali treatment yield different degree of deacetylation in the chitosan products. [3,4]

The terms chitin and chitosan do not refer to specific compounds but two ranges of copolymers, containing the two "monomer" residues, anhydro-N-acetyl-D-glucosamine and anhydro-D-glucosamine.The former is the predominat component in chitosan. The two ranges of copolymers form a continuum and classification of a sample which is normally done on basis of its solubility (chitosan) or insolubility (chitin) in dilute acid such as acetic acid, propionic acid, butyric acid etc.[5,6] Therefore, the study on degree of deacetylation will lead to chemical modification and application of chitin and chitosan.

The aim of this study is to observe the relationship between the increasing of degree of deacetylation and the crystallinity patterns of chitin from crab shells, during the deacetlation process. The crystallinity patterns were measured by X - Ray diffractometer while the degree of deacetylation is measured by IR–spectroscopy in comparison to the method of High Performance Liquid Chromatography (HPLC).[1,4]

Materials and Methods

The crude chitin was obtained from crab shell of *Carcinus* species. The crab shells were collected from the crab culture farm in Ranong province, sourthern part of Thailand. The deproteination of the shells was done in dilute alkali solution and followed by demineralization by dilute acid.

Deacetylation process of chitin was carried in 50%(w/w) NaOH solution at ambient temperature. The different times of treatment were varied at 24, 48, 72, 96, 120, 144, and 168 hours. The treated samples were then collected and throughly washed until reaching the neutral pH. After drying, the samples were ready for further analysis.

The measurement of degree of deacetylation was done by two different techniques namely IR-spectroscopy [1] and HPLC.[4] The X-ray diffractograms of different alkali-treated chitin were recorded by using Philips PW3710 Diffractometer using CuKα radiation and generated at 40 kV and 30mA. With scanning speed at 0.02 ^{0}2θ/min.

Results and discussions

Determination of Degree of Deacetylation : Figure 1 shows the IR-spectra of chitin and deacetylated samples. The observed band of the staring chitin at at 1661 cm^{-1} and 1559 cm^{-1} exhibit of the of $\nu_{C=O}$ and δ_{N-H} , respectively. However, the intensity of both two peaks decrease with the increase of treatment times. As expected for probe bands, the intensity of the $\nu_{C=O}$ and the δ_{N-H} shows decrease with the increase of treatment times, and it is important to note that the δ_{N-H} disappears faster than the $\nu_{C=O}$.

The %DD was mearsured from IR spectra compared with HPLC method. In Table 1, shown DD value calculated by the method of Alasdiar Boaxter[1] and the other was obtained by HPLC method of Ng Chuen How.[4] A good correlative of %DD is also found when IR data from time that 72 to 168 hours are compare with HPLC data as shown in Figure 3.

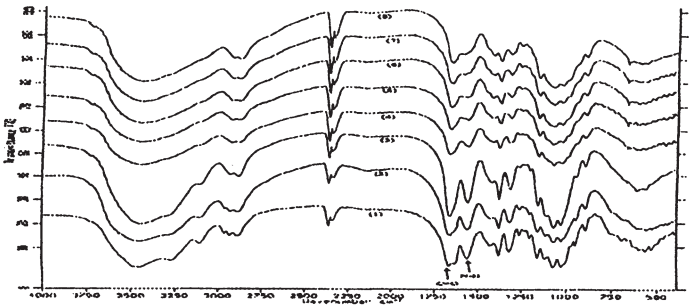

Figure 1. IR-spectra of starting chitin(1) and deacetylated chitin in 50%NaOH at ambient temperature and various of treatment times include 24hrs(2), 48 hrs(3), 72 hrs(4), 96 hrs(5), 120 hrs(6), 144 hrs(7) and 168 hrs(8).

The Observation of X-ray diffraction: In Figure 2 shows the diffractograms of starting chitin(1) with two major peaks at 9.46 and 19.18 ^{0} 2θ which have similar formation to the diffractograms of deacetylated chitin with the treatment times of 24(2) and 48(3) hours. Similarly, the diffractograms of deacetylated chitin samples at treatment times of 72(4), 96(5), 120(6), 144(7), and 168(8) hours show a similar formation and differ from starting chitin. And when the chitin sample was treatment with alkali condition, however, reduced crystallinity was observed.

The percent relative crystallinities of all samples was determined the ratio of crystalline fraction of chitin deacetylated to chitosan and calculated on the basis of the width at half-maximum intensity, which they are presented in Table 1. Comparison of the X-ray patterns in Fig.2 found that line broadening and width at half-maximum intensity of the composite at 19.18^0 (2θ) increased with increase of the treatment times. It is important to note that the percent relative crystallinity shows to increase when the treatment times is reach 168 hours as shows in Fig.3

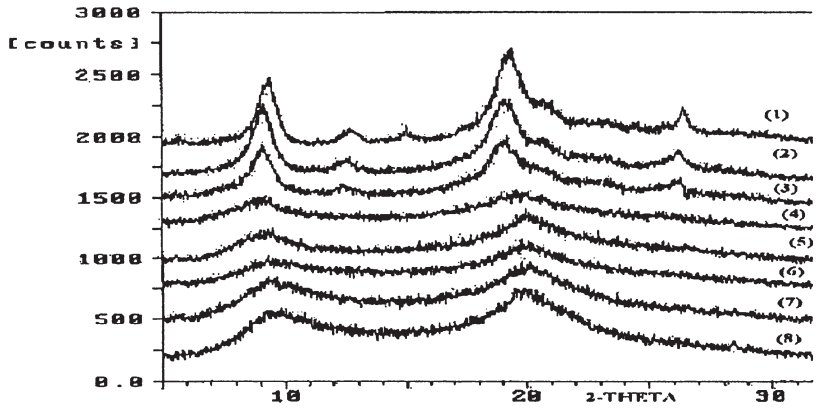

Figure 2. X-ray diffractometer pattern of starting chitin(1) and deacetylated chitin in 50%NaOH at ambient temperature and various of treatment times include 24hrs(2), 48 hrs(3), 72 hrs(4), 96 hrs(5), 120 hrs(6), 144 hrs(7) and 168 hrs(8).

Table 1. Chemical and physical characteristics of chitin and deacetylated chitin.

Deacetylation Times	Degree of Deacetylation(%DD)		Ash content	%Relative
(hour)	IR spectra	HPLC method	(%wt)	Crystallinity
0	16.5	8.6	9.69	100
24	36.11	17.5	0.54	80
48	45.4	22.6	0.52	66.6
72	51.9	51.5	0.35	36
96	58.2	60.4	0.27	46.7
120	68.3	69.9	0.30	36
144	68.9	73.5	0.25	60
168	72.3	76.1	0.16	70.7

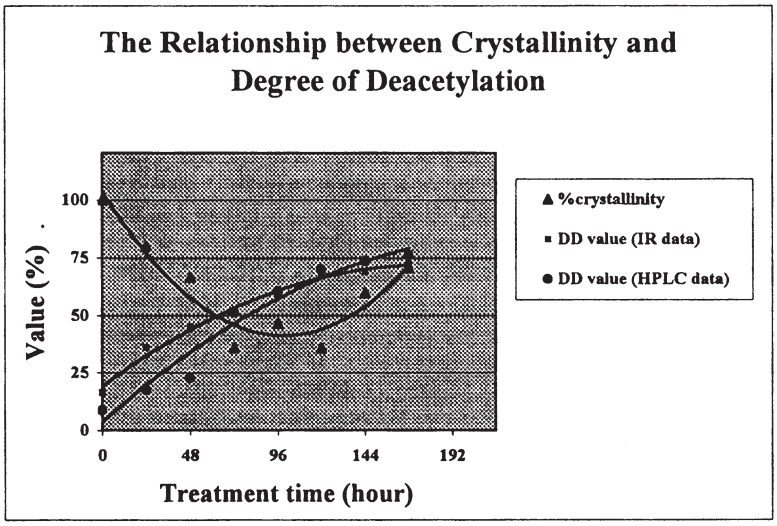

Figure 3. The relationship between %crystallinity and Degree of deacetylation.

Conclusion

The degree of deacetylation of chitin from crab shells was increasing along with the time of treatment. Meanwhile, the percentage of relative crystallinity of chitin was decreasing down to the transition period of chitin to chitosan which is about 51% of degree of deacetylation. Consequently, the crystallinity value was changed in the direction of gradually increasing along with the degree of deacetylation of chitosan.

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Reversible water-swellable chitin gel : modulation of swellability

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Summary
Reversible water-swellable chitin hydrogels were prepared by reacting chitin gel cast in 5% DMAc/LiCl solution with chloroacetic acid. By optimization of post reaction treatment with 1N HCl and 0.1N NaOH, a high degree of swellability can be achieved with good intractability of the hydrogel. Ultra structures of the gels were examined by scanning electron microscopy (SEM). The micrographs revealed that the surface morphology varied according to the sodium content of the gels as determined by Electron Dispersive Spectroscopy (EDS). In comparison, another ionic hydrogel was obtained by reacting chitin gel with triethyl amine. This hydrogel showed good water uptake profile and stability in water.

Introduction
In recent years reversible water-swellable gels have been of great interest because of their potential applications [1,2]. To enable more versatility in the utilization of chitin based hydrogels, we have reported the preparation of reversible water-swellable chitin gels by the "post-shape modification" process [3,4]. This process can impart different properties to different regions of the chitin substrate, particularly in multiple layer materials. In this further study, the swelling properties of chitin under various treatments after "post-shape modification" process was investigated.

The incorporation of substantial amounts of carboxylic group into the intractable chitin backbone produced a high swelling chitin hydrogel. Swelling characteristics of the chitin hydrogel was dominated by the hydrophilicity of the polymer. Treatment of the chitin hydrogel with acid or base influences the extent of polymer swellability. By treating with excess acid, the hydrogel absorbed less amounts of water. Conversely, by allowing sodium hydroxide to act on the polymer, an increase in swellability was observed until the gel became totally soluble. Apart from effecting the swellability, sodium hydroxide treatment also introduced brittleness to the chitin substrate. By increasing the swellability, the intractibility of the gel was sacrificed or vice versa. By controlling the degree of treatment the swellability and intractibility, the reversible chitin hydrogel could be modulated to a desirable stage for a particular end application.

Materials and Methods
Chitin was obtained from Polyscience, USA, purified by stirring in 1M NaOH at room temperature for 7 days and in 1M HCl for 1h. Degree of deacetylation was determined by FT-IR [8] and microanalysis to be 80%. All reagents used for the preparation of the chitin samples were of analytical grade unless stated otherwise.

Preparation of Chitin Solution: Anhydrous Lithium Chloride (9.6g) was dried at 130°C for about ½h, cooled inside a dessicator and dissolved in 200ml of N, N-dimethylacetamide (DMAc) by magnetic stirring. Chitin flakes (0.96g) was suspended in this solution and shake overnight at 4°C in a refrigerated shaking incubator to give 200ml of 0.5% (w/w) chitin solution in DMAc & 5% LiCl solvent system. The viscous clear solution was filtered through glass wool and stored in glass containers prior to casting.

Preparation of Chitin Gels: Chitin gels were cast by dispensing 200ml of 0.5% chitin solution into containers measuring 13cm x 21cm to give gels of average (n=5) thickness of approximately 0.1mm. The solutions were allowed to evaporate slowly in the fume hood, for three days. The gels were soaked in deionzed water for two days to ensure complete removal of DMAc. The chitin gels were blotted dry by filter papers, followed by vacuum drying in a dessicator. The dried gels were cut into smaller pieces measuring 2.5cm x 2.5cm.

Reaction of Chitin Gel with Chloroacetic Acid: Chloroacetic acid (10 mol-eq. of chitin) was added to propanol inside a round-bottomed flask, which is purged with nitrogen gas. After the dissolution of all the chloroacetic acid, chitin gels were added to the mixture. The chitin gels are previously activated by soaking into 1N NaOH for 6h. The reaction was carried out for 25mins under the inert nitrogen atmosphere and room temperature. The reacted gels are rinsed with acetone, soaked in deionzed water for 3h, subjected to various post reaction treatments and freeze dried.

Reaction of Chitin Gel with Triethylamine: Freeze dried chitin gels (4 pieces, weighed about 0.2g) was added to a stirring solution of triethyl amine (30ml). The reaction is carried out under nitrogen atmosphere at room temperature for 30mins. The reacted gels are rinsed with acetone, soaked in deionzed water for 3h and freeze dried.

Water Uptake Experiments: The freeze-dried gels were weighed and soaked into deionzed water. The gels are taken out at specific intervals, blotted dry with filter paper and weighed.

Swelling ratio = w/w_o where w : weight of swollen gel
w_o : weight of dried gel

Ultra Structure and Elemental Analysis of surface: Freeze-dried specimens were gold coated using a Fine Coat Ion Sputter JFC-1100 coater. SEM micrographs and EDS were obtained using a JEOL JSM-T220A scanning microscope at an accelerating voltage of 15kV.

Results and Discussion

Reaction of chitin gel with chloroacetic acid: The post treatment of reacted carboxymethylated chitin was shown in Figure 1. Reaction of the activated chitin with chloroacetic acid produced gel (B), with the attachment of sodium onto the carboxylic groups. Gel (C) was formed by soaking gel (B) in 1N HCl for 30 mins. The degree of neutralization was determined to be 80% by potentiometric titration. Gel (C) was further treated with 0.1N NaOH for 10mins to afford gel (D), with 40% of the carboxyl groups in the acidic form.

Water Uptake Profile: The water uptake profile for Gel (A)-(D) are shown in Figure 2. Gel (B) produced the highest water uptake at ca. 6.5x but is unable to maintain its intractability. This highly ionic gel starts to solubilize within the first 10mins of the uptake. The presence of the large number of ionic sodium carboxylate groups increases the hydrophilicity of the gel to the extent of solubilizing it in the aqueous medium. Although the initial swelling ratio is very high, it started decreasing after ca. 5mins. The gel disintegrated during the water uptake experiment due to its highly soluble state.

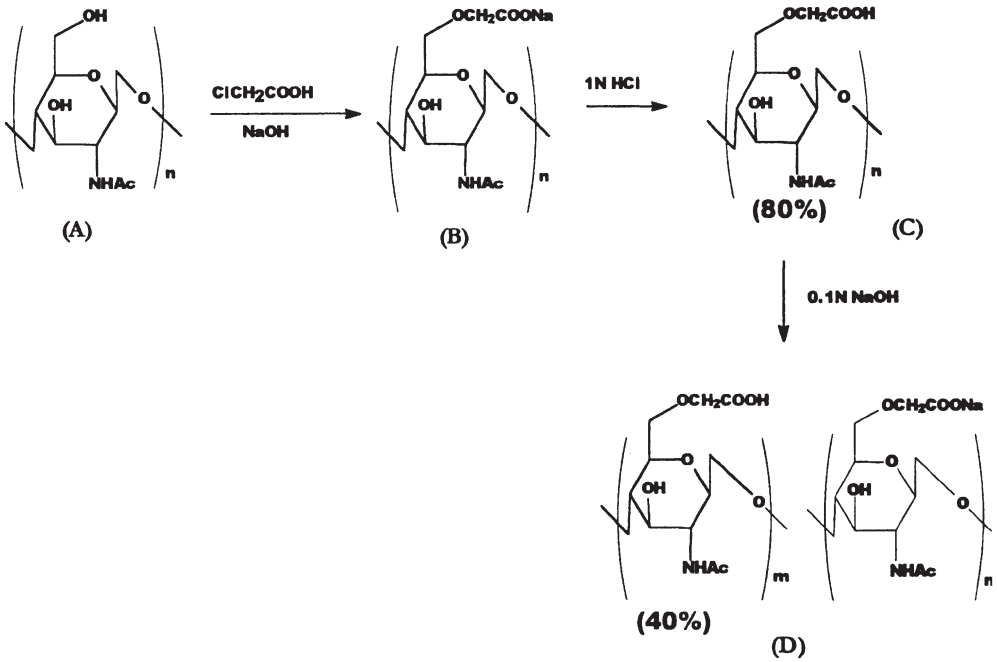


Figure 1. Post reaction treatment of carboxymethylated chitin gel.

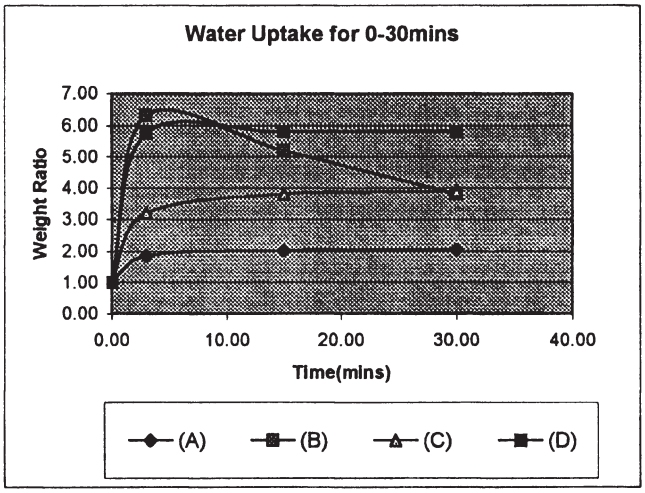
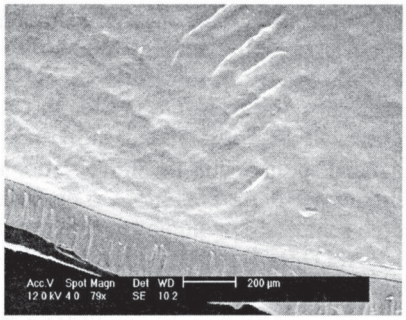


Figure 2. Water Uptake Profile of Gel (A) – (D).

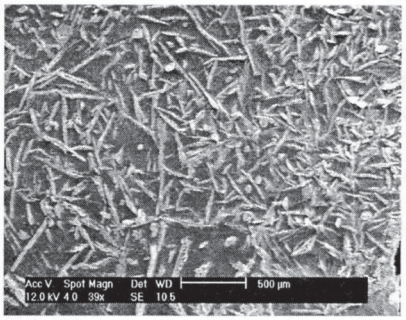
The intractability of the gels can be improved by acidifying the carboxylate groups.to produce gel (C) The acidified gel can maintained ca. 4-fold swelling up to about 48h. Since the rigidity of the chitin crystalline structure has been disrupted by the incursion of sodium ions, acidifying the gel will enhance the reformation of hydrogen bonds. This step is crucial since the acidified gel (C) is stable in the aqueous medium and still doubled the swelling ratio as compared to unreacted chitin.

In an attempt to further increase the swelling ratio of the gels, we have resorted to treat the acidified chitin gels with 0.1N NaOH. This step will increase the ionic character of the carboxymethylated chitin gel. Since a low NaOH concentration (0.1N) is used, the ionization process is limited to 40% of the acidic form remaining. This process has significantly increased the water uptake ratio to ca. 6-fold without losing the intractability of the gel even up to 48 hours.

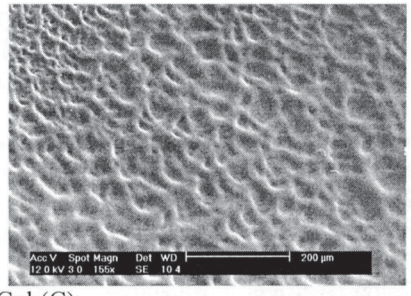
Scanning Electron Microscopy (SEM) & Electron Dispersive Spectroscopy (EDS): From the micrographs (Figure 3), it is visible that the morphology of the gels is affected by the sodium contents. Gel (B) showed large amount of sodium crystallites on the surface. This is also indicated by the high sodium count by EDS (102cps). This is in stark contrast to the unreacted chitin gel (A) that exhibits a non-porous structure without any visible sodium crystallites. The surface of gel (C) is very similar to gel (D) with rugged porous morphology. The porous structure is believed to have formed during the freeze-drying process that sublimates the ice crystals in the structures while drying the gel. This further supports the point that gel (C) and (D) have higher hydrophilicity than the unreacted chitin gel (A) but less sodium content than gel (B). The sodium content for gel (A), (C) and (D) is determined by EDS to be at 20, 34 and 63 cps respectively.



Gel (A)



Gel (B)



Gel (C)

Figure 3. Micrograph of Gel (A), Gel (B) and Gel (C)

Reaction of chitin gel with triethylamine: The treatment of chitin gel with triethylamine for 30 minutes produced a swellable gel with 60% substitution. The highly basic triethylamine deprotonate the hydroxyl group of the chitin gel to generate an ionic gel that has ca. 5-fold water uptake (Figure 4). The gel achieved the maximum uptake in about 15mins and has good intractability up to 96h in the aqueous medium. This reaction provides an alternative to the synthesis of charged chitin gel with good stability and hydrophilicity in the aqueous media.

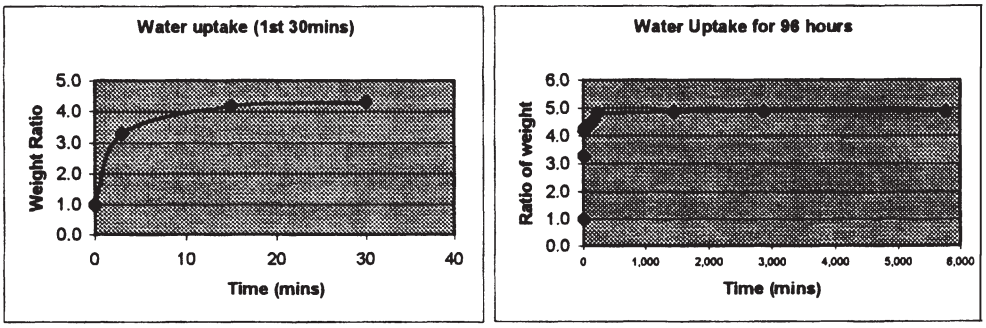


Figure 4. Water Uptake Profile for TEA chitin gel.

Conclusions

Variation in the swellability of hydrogel was achieved by controlling the polarity of the gel. Unfortunately, due to the high hydrophilicity, swellable gels are usually accompanied by low intractability in the swelling medium. By controlling the ionic groups in the chitin backbone we are able to optimize the intractability as well as the swellability of the gel.

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Syneresis aspects of chitosan based gel systems

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Summary
Chitosan (Hirano) gels were produced by homogenous *N*-acylation of chitosan in acyl-anhydride/acetic acid/co-solvents systems. The syneresis behaviour was investigated and monitored for up to 300 days. The effects of acyl anhydride concentration and co-solvent (diethyl ether):methanol ratio were studied. It was found that the addition of ether as a second co-solvent in the acetic acid/methanol system increased the reaction efficiency so decreasing the amount of acyl-anhydride necessary for gelation. The influence of the head-space volume on the rate of syneresis was also investigated.

Introduction

Chitosan can be homogeneously acylated to, for example, chitin in an acetic acid/methanol (MeOH) system with the respective anhydride as the reagent and the reaction system undergoing gel formation [1][2]. During the homogenous *N*-acylation of chitosan it was observed that gels prepared with a second co-solvent, ether, were much clearer than equivalent Hirano gels. The gels also exhibited much less syneresis than expected or has been reported in the literature [3]. One sample remained as a clear gel over a period of 18 months.

Materials and Methods

The chitosans employed were

a) Aber Technologies CHITOSAN MV – (Chit 98) Ref: A32E03 – (medium viscosity) le 06/07/95 F_A=[0.01], 65 cps (1% chitosan in 1% acetic acid) and
b) A sample of chitosan (low viscosity) prepared in the laboratory from Indian crab, F_A=[0.28], 16 cps (1% solution in 1% acetic acid).

Chitosan a) was used unless otherwise stated.
They were purified by filtration and reprecipitation.
General grade reagents and solvents were used as supplied.

Preparation of N-*acetyl chitosan*: For the *N*-acetylation of chitosan a 1 % polymer solution in 0.1M acetic acid was diluted 1:1 with MeOH and acetic anhydride was added in molar proportions ranging from 0.8 to 6.0 based on free amine groups [1][2]. The mixtures were prepared on stirring readily in appropriate moulds and covered with laboratory film unless otherwise stated.

Monitoring of syneresis: The weight of the initial system was determined after mixing. After a period of time the exuded solvent was discarded and the gels were carefully padded dry with laboratory tissue and weighed.The gels were not cut loose from the moulds as it has

been shown [3] that the difference in syneresis of a loose gel compared to an attached gel levels off in less than 24 h.

Determination of degree of acetylation: The degree of acetylation was determined via dye adsorption [4].

Results and Discussion

Gels series 0: Anhydride/amine proportion varying from 0.85 to 6.00: A series of 8 *N*-acetylation systems plus one reference system without reagent (acetic anhydride) was prepared using ether and the same solvent proportions as in the initial quantitative observation. In this series the effect of varying the proportions of anhydride to amine groups in a range of 0.85 to 6.00 (see table 1) on the syneresis behaviour of the gels was looked at.

As to be expected reference sample 9 without reagent showed the least weight loss, since there are only evaporation but no syneresis effects. Sample 1 did not gel and was thus discarded. While samples 2-4 show rather inconclusive behaviour* and no clear trend can be made out, there is a distinct increase in weight loss between the former group of samples and group 5 – 8. The latter group also shows a clear trend of increasing syneresis with increasing anhydride proportions.

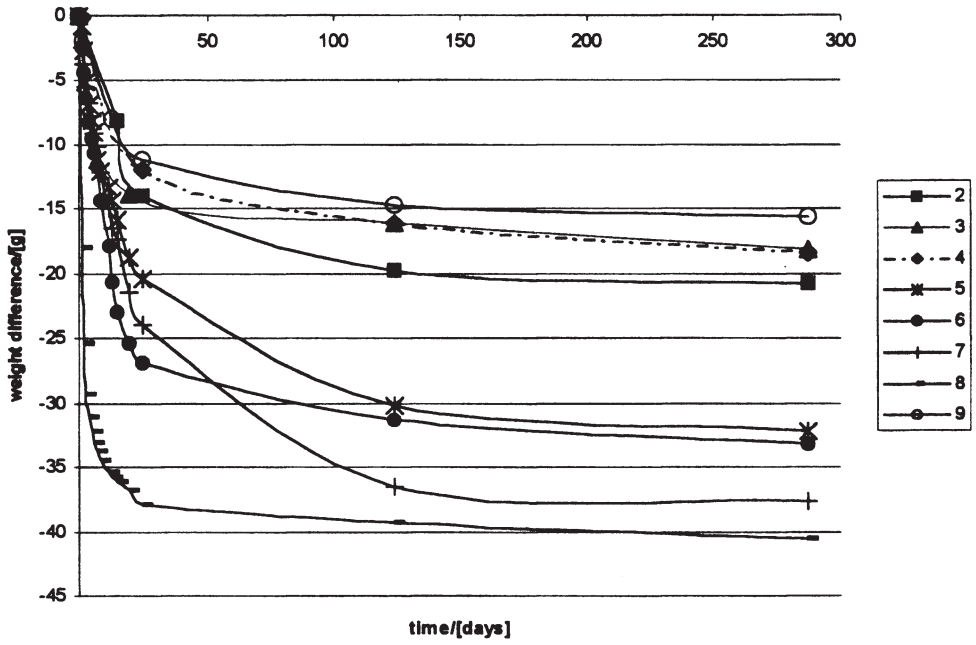


Figure 1. Range 0 - varying anhydride concentration

* This group synerised in the region of lower weight losses, which might have compromised the accuracy of the determination of differences in weight.

Table 1. Composition of series 0 and C

series 0 and C	1	2	3	4	5	6	7	8	9
chitosan solution/[g]*	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
MeOH/[ml]	18.4	17.6	16.7	16.1	15.5	14.9	14.3	8.6	20
ether/ml	10	10	10	10	10	10	10	10	10
Ac_2O/MeOH 1:10 / [ml]	1.6	2.4	3.3	3.9	4.5	5.1	5.7	11.4	-
molar proportion Ac_20/-NH_2	0.84	1.26	1.74	2.05	2.37	2.68	3.00	6.00	0.00
approx. (intended) proportion**	0.85	1.25	1.75	2.00	2.25	2.75	3.00	6.00	0.00
F_A for series 0		0.87	0.94	0.95	0.96	0.97	0.97	0.99	
F_A for series C			0.88	0.89	0.92	0.94	0.94	0.96	

*[medium viscosity (series 0)/low viscosity (series C)] chitosan

** 0.85 was chosen, since formation of a firm gel starts at approx. 80% acylation [5]

** 6.00 was chosen, since it has been reported [6] that syneresis in chitin gels reaches an equilibrium at above ratio

Gels series A: ether/MeOH proportion varying from 0.2 to 2: The influence of the ether concentration of the gelation systems was investigated by varying the ether/MeOH ratio from 1:5 to 2:1 (see table 2). No gelation occurred for a ratio >1:1 (gel 7) and gelation occurred readily at 1:5 but not at 1:3. Gels 3 to 5 show an increase in weight loss over time with increase in ether. Gel 1's weight decrease however is situated between gel 3 and 4. Gel 6 only formed a very slight gel and showed comparatively little syneresis, both indicating low cohesion forces within the gel and thus low ridgidity and contraction.

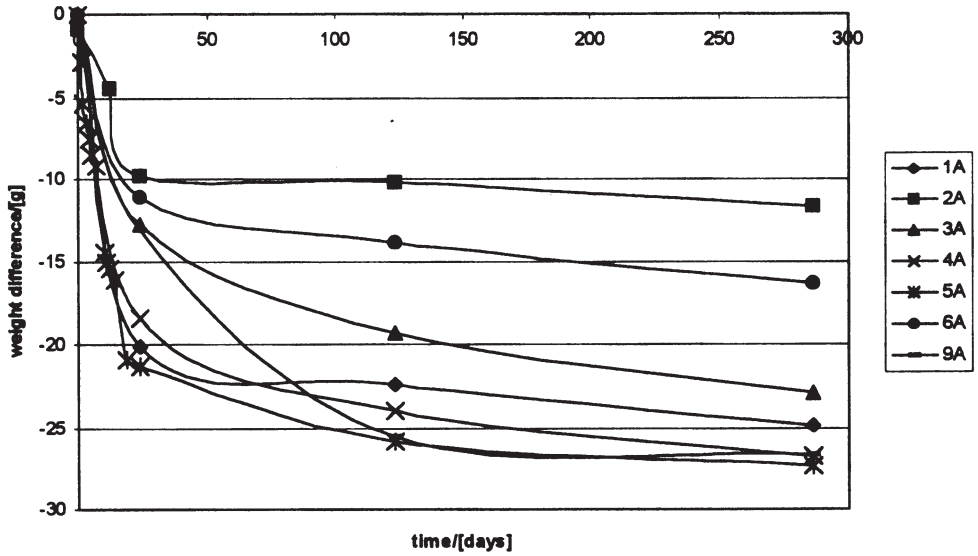


Figure 2. Range A – varying co-solvent ether concentration

Table 2. Composition of series A.

series A	1	2	3	4	5	6	7	8
chitosan solution/[ml]	30	30	30	30	30	30	30	30
MeOH/[ml]	20.5	18.5	16.5	14.5	12.5	10.5	8.5	5.5
ether/ml	5	7	9	11	13	15	17	20
Ratio ether/MeOH	1:5	1:3	1:2.3	1:1.7	1:1.3	1:1	1:0.8	1:0.5
Ac_2O/MeOH 1:10 / [ml]	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
F_A	0.94		0.95	0.96	0.92	0.88		
gelation	+	-	+	+	+	(+)	-	-

Gels B: varying head-space volume: The effect of head-space volume was looked at and the extend of syneresis of chitin gels with co-solvent ether was compared to Hirano gels. A matrix of gels was prepared of which groups of 3 each were prepared with ether/MeOH proportions 0:1 (=[*]), 0.4:1 (=[']) and 0.8:1 (=[~]). One of each group was left uncovered (nc), covered with cling film (cf) and covered with laboratory film (pf) (see table 3).

The weight loss of the gels is highest when uncovered and lowest when covered with laboratory film. When comparing the weight loss differences between the groups of different ether content it shows that the differences are narrowed down with increasing ether content.While the syneresis of the uncovered gels is fairly uniform across the range, the weight loss of the covered gels increases considerably with increase in ether concentration. When comparing the groups of gels with equivalent covering there is a surprising trend in the group of uncovered gels. Unlike the covered gels, which lose more solvent with higher ether content, the uncovered gels lose more solvent with decreasing ether content (see figure 3 (a, b, c)). An explanation for this observation would be, that contraction due to fast evaporation of ether-enriched solvent seals off the pores at the gel-gas phase boundary. This is not the case, when the gel is covered with film since the head-space volume is limited. An evaporation equilibrium is therefore reached and the gel surface stays in contact with solvent vapour phase.

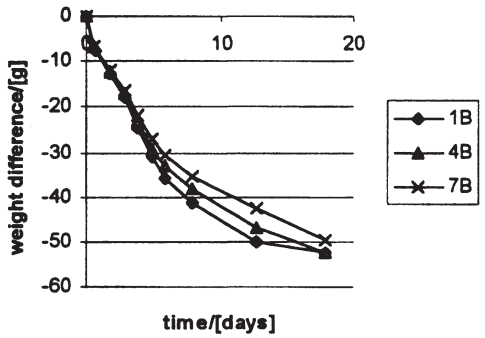
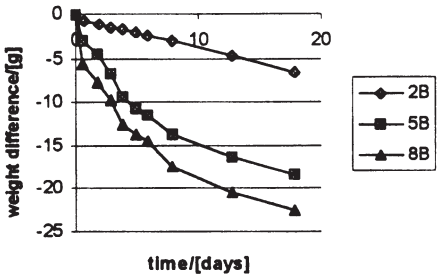


Figure 3a. uncovered gels .



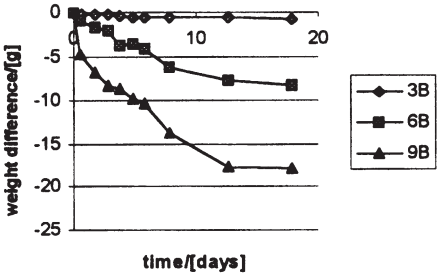


Figure 3b. gels covered with cling film.

Figure 3c. Gels covered with laboratory film .

Table 3. composition of series B

series B	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
chitosan solution/[ml]	30	30	30	30	30	30	30	30	30
MeOH/[ml]	26.7	26.7	26.7	17.7	17.7	17.7	13.7	13.7	13.7
ether/ml	0	0	0	9	9	9	13	13	13
Ac_2O/MeOH 1:10 / [ml]	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
covering	nc	cf	pf	nc	cf	pf	nc	cf	pf
F_A	0.87	0.87	0.88	0.91		0.92	0.91	0.92	0.92

Gels C: effect of molecular weight: A series of gels equivalent to series 0 was prepared using lower molecular weight chitosan in order to see how that would affect syneresis. System 1C and 2C did not show gelation, although the starting F_A was higher than in series 0.

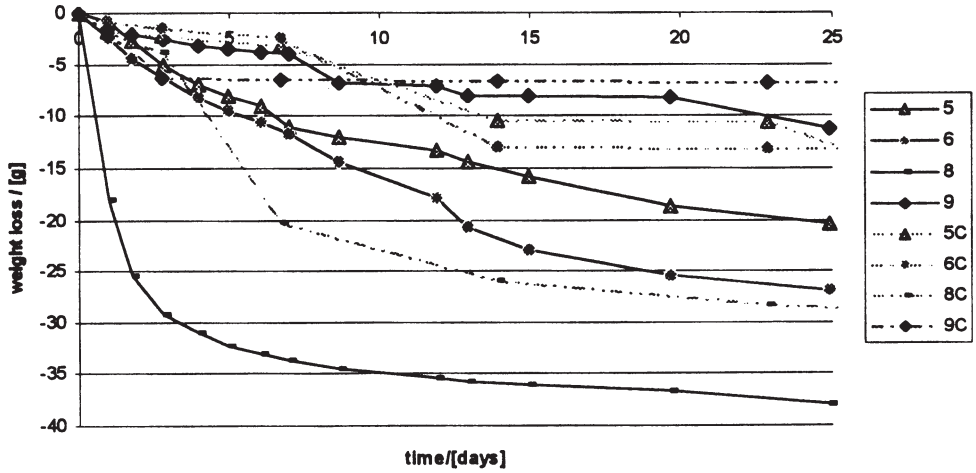


Figure 4. comparison of gels from two different MW chitosans.

The gels, with an exception of 4C, showed less syneresis than the analogous ones in series 0. This can be seen in Figure 4. Looking at the data for 9/9C the latter shows less solvent loss. This indicates a decrease of boiling point of the solvent system due to the increase in particles in the systems with lower MW chitosan. However the difference in weight loss between gels */*C is considerably greater and this is due to syneresis. The longer chain chitosan molecules possess higher intermolecular forces and thus the contraction of the gels with higher MW chitosan is greater.

Effect of co-solvent ether on reaction and gelation of Hirano gels: It was observed, that gelation occurred at lower acetic anhydride concentrations in the presence of ether. While system 3 formed a rigid gel, an equivalent gel prepared without ether only formed a very light gel indicating either higher acetylation or an influence of ether on the gel formation. Looking at the F_A values of series B (table 3) one can see that gels 1B-3B are lower than those of 4B-9B, which were prepared with ether. The F_A values of series A show maximum for ether/MeOH ratio of 1:1.17. This again suggests that the presence of ether makes the reaction more efficient and also, that there is an optimum ratio. This is a very promising result and is intended to investigate this further in the future.

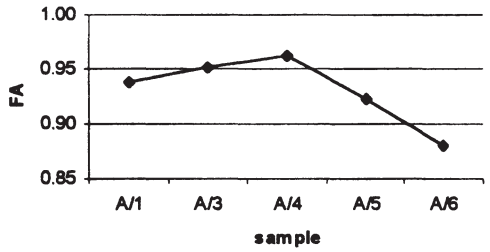


Figure 5. Degree of acetylation of series A.

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In situ chitosan gelation using the enzyme tyrosinase

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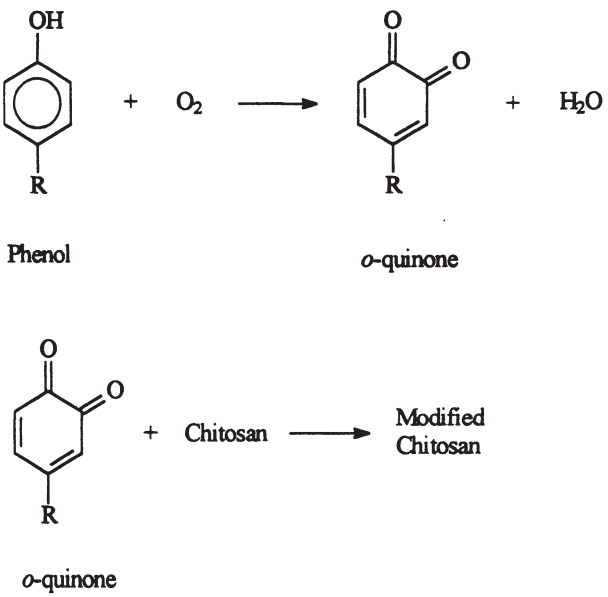
Summary
We observed that addition of tyrosinase and the simple phenol, *p*-cresol, to semi-dilute solutions of chitosan resulted in the *in situ* formation of chitosan gels. Specifically, homogeneous reactions were conducted with chitosan solutions (0.32 w/v %) at pH near 6 and with cresol levels of 0.6 molar equivalent (relative to chitosan amino groups). Oscillatory shear measurements showed that the enzymatic reaction resulted in large increases in the complex viscosity (η^*) and storage and loss moduli (G' and G''). These dynamic measurements indicate that the enzymatic reaction resulted in the conversion of the nearly Newtonian semi-dilute chitosan solutions into gels. The rheological behavior of these enzymatically-generated gels was compared to the behavior of acidic chitosan solutions and to solutions containing xanthan gum.

Introduction

We are studying a two step method to enzymatically functionalize chitosan. The enzyme in our study is tyrosinase (phenol oxidase) which uses O_2 to oxidize a broad range of natural and synthetic phenols. The product of tyrosinase-catalyzed phenol oxidation is an *o*-quinone which is a freely diffusible intermediate capable of reacting with the amino groups of chitosan. The advantage of this enzymatic approach is that it is simple, rapid, and can be exploited to confer a broad range of functional properties to chitosan. The disadvantage of this enzymatic modification approach is that quinones can undergo a variety of reactions that can be difficult to control, or even to characterize [1,2]. Our approach is



similar to one reported by Muzzarelli and coworkers [3,4] who chemically grafted phenolic moieties onto chitosan and then used the enzyme tyrosinase to react with these grafted phenols. In our initial studies, we demonstrated that tyrosinase-catalyzed oxidation products react with chitosan [5] and more recently, we have focused on demonstrating that these reactions can be exploited to confer important functional properties to chitosan. For instance, we have enzymatically-modified chitosan to generate base-soluble materials [6] and to form chitosan gels [7]. Here, we compare the rheological properties of these enzymatically-formed gels with the behavior of solutions containing xanthan gum. Xanthan gum is a commonly used industrial viscosifying agent.

Materials and Methods

The enzymatic method for forming gels is briefly described in the next section and more fully described elsewhere [7]. Dynamic rheological measurements were made on a Rheometrics DSR-200 stress rheometer with a 25 mm cone-and-plate geometry.

Results and Discussion

To compare enzymatically-modified chitosan gels, we first studied the rheological behavior of a 1% xanthan solution. As shown in Fig. 1 this xanthan solution is shear thinning (η^* decreases with ω), the elastic (G') and viscous (G'') moduli are of the same order-of-magnitude ($G' \approx 100$ and $G'' \approx 70$ dynes/cm^2), and the moduli are nearly independent of the strain frequency (ω). This behavior has been observed before [8] and is characteristic of weak gels.

The rheological behavior of two acidic chitosan solutions (0.32 and 1.4 %) were also measured and the results are shown in Fig. 1. For these chitosan solutions we observed the dynamic viscosity (η^*) was constant with ω, both G' and G'' increased with ω, and the loss tangent (tan $\delta = G''/G'$) was

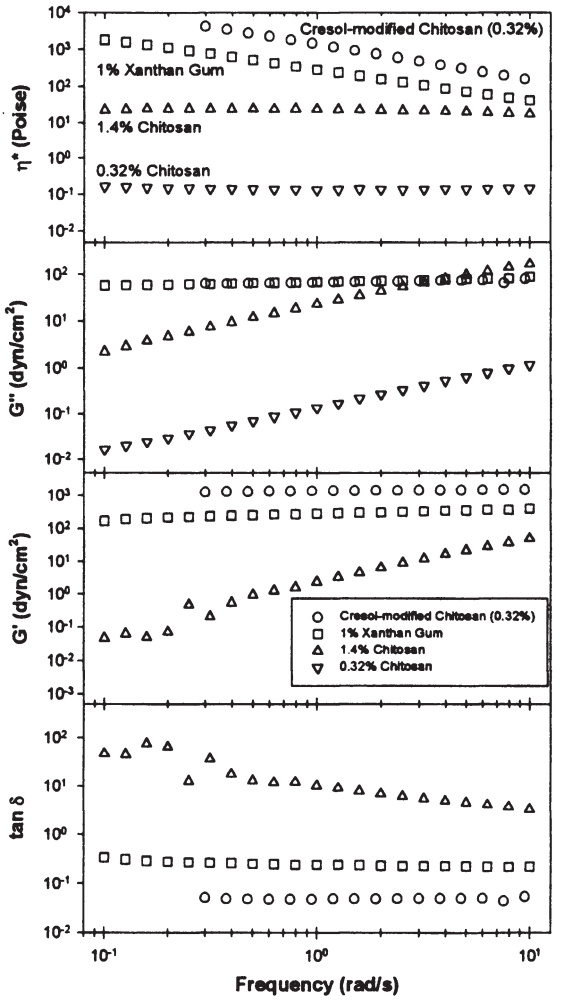


Figure 1

greater than one. [Note: G' for the 0.32 % chitosan solution was too low to be accurately measured.] These chitosan solutions show Newtonian behavior which is considerably different than the pseudoplastic behavior observed for the xanthan gum solutions.

To enzymatically generate chitosan gels, we used solutions containing 0.32 % chitosan at a pH of 6.0 and 12 mM *p*-cresol (0.6 molar equivalent relative to chitosan-N) as the phenolic reactant. In the reaction scheme shown on a previous page R=CH_3 for *p*-cresol. Over the course of 10 hours, the color of the solution was observed to change from colorless to black. Also, the solution viscosity was observed to increase significantly over the ten hour period. No viscosity increases were observed in control solutions that contained chitosan and *p*-cresol, chitosan and tyrosinase, or *p*-cresol and tyrosinase [7]. The latter control indicates that chitosan is required for the viscosity enhancement and that oligomeric or polymeric phenols are not solely responsible for this behavior.

Fig. 1 shows dynamic measurements that indicate enzymatic-modification of a 0.32% chitosan solution results in a shear thinning solution (η^* decreases with ω) with a G' of about 10^3 dynes/cm^2 which is nearly an order-of-magnitude higher than that for a 1 % xanthan solution. The loss tangent (tan δ) for this enzymatically-modified chitosan was observed to be less than 0.1 which is considerably lower than that of a 1% xanthan solution. At a molecular level, these results suggest that chitosan is being enzymatically crosslinked to create the network junctions which enhance elasticity (i.e. G'). It is well known that chitosan gels can be formed by chemical crosslinking [9,10] while some chemically modified chitosans can form physical gels through the formation of physical (non-covalent) network junctions [10-18]. In our work we are forming a gel without the need for reactive reagents (e.g. glutaraldehye or acid chlorides). Rather, we are using the enzyme for the *in situ* "activation" of a less reactive reagent (i.e. *p*-cresol). Practically, it is important to note that this enzymatically-modified chitosan has gel properties similar to those of a solution containing 3-fold higher xanthan levels.

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Preparation and characterization of controlling pore size chitosan membranes

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Summary
The different pore size values of chitosan membranes are prepared by the mixture of chitosan solution with some specific chemicals. Monosodium glutamate is one of the appropriate chemicals that used in different ratio of the chitosan casting solution to prepare the membranes within the average thickness of 30 micrometer. The physical characteristics of the membranes were measured both in wet and dry conditions. The specific pore-size-control membranes were characterized by the technique of solvent permeability. Molecular weight cutoff (MWCO) values of chitosan membranes were in the range of 5000 to 10000 dalton by using the standard dextran and cytochrom-C. The patterns of IR spectra, Crystallinity and SEM also have been determined.

Introduction
Chitin and chitosan are natural polymers of high availability on earth. The terms of chitin and chitosan do not refer to specific compounds but refer to two ranges of copolymers, containing the two monomer residues, which are anhydro-N-acetyl-D-glucosamine and anhydro-D-glucosamine.

The former is the predominant component in chitin and the later is predominant component in chitosan. Chitin and chitosan are the main structural components of the cuticles of crustaceans, insects and mollusks, and the cell walls of microorganisms. Chitosan is a unique cationic polysaccharide, which has positively charged amine groups on carbon number two of glucose unit. Chemically, chitosan is a poly (2-amino-2deoxy-D-glucose) which is mostly obtained from the deacetylation of chitin, a poly (N-acetyl-D-glucosamine). Chitosan is readily soluble in dilute aqueous organic acids such as acetic acid, propionic acid, formic acid and lactic acids. Chitosan also has highly chemical reactivity due to a large number of reactive hydroxyl (OH) and amine (NH_2) groups containing in the long chain polymer. In view of its hydrophilic property, high chemical reactivity, excellent film forming ability with good mechanical properties, including biocompatibility, chitosan can be excellent material for affinity membranes which are able to use in many areas of applications [1-5]. Although relatively high permeability of chitosan membranes were obtained [6], however, their reliable properties have not been completely satisfactory, and they need to be controlled by different techniques. Recently different techniques of controlling pore size of chitosan membranes have been reported by using chemical and physical modification such as the reaction of

gluteraldehyde, polyethylene oxide, potassium hydrogen phthalate including some other derivatizations and copolymerization. However, the results effect to the permeability and hydrophilicity of the chitosan membranes [5,6]. The preparation of macroporous chitosan membrane by using silica particle as porogen was reported and could be employed in affinity or ion-exchange bioseparation [7].

The aim of this study is to introduce a new kind of additive and a new simple process to prepare the possible controlling pore size of chitosan membranes by using different ratio of monosodium glutamate (MSG). The chitosan microporous membranes could be prepared by casting a chitosan solution in dilute acetic acid containing different percentages of MSG. The casting solutions were evaporated to dryness by the technique of phase-inversion. After neutralization and thoroughly washing, the CTS-MSG, membranes were obtained and kept for characterization.

Material and Methods

The chitosan sample was purchased from the local commercial products that mostly produced from shrimp shells of *Penaeus monodon*. The sample was analyzed for some properties such as moisture content of 10.00%, ash content of 0.50% and degree of deacetylation of 74.85%. The casting solution was prepared at 1.5% of chitosan in 1% acetic acid and stirred to obtain homogeneous solution.

The membrane formation of chitosan and monosodium glutamate (CTS-MSG) was controlled by maintain constant weight of chitosan solution and adding different percentage of MSG solutions were evaporated to dryness in the oven with control temperature at 30°C. After neutralization and thoroughly washing, the CTS-MSG membranes were dried and kept for characterization.

The average thickness of 30μm membranes were subjected to measure the tensile strength by Instron Universal Tester Instrument, Model 5583 [1]. The stress and strain values of CTS-MSG membranes were identified in both dry and wet conditions in compared with the control.

The wet CTS-MSG membranes were identified the pore size by subjecting in the filter holder with receiver apparatus for measuring the constant volume of 100 ml water, permeating through each membrane in a given time at constant pressure of 1 bar. The calculation was followed by Hagen-Poiseuille equation [3] which related to the solvent permeability method.

The very thin film of CTS-MSG membranes were subjected to the IR Instrument of Model EQUINOX 55/S to observe the interaction between chitosan and monosodium glutamate.

The pore-size diameter of 22nm CTS-MSG membrane was evaluated for the molecular weight cut off (MWCO) value by indirect measurement of the retention of known molecular-weight standards of dextran-5000, dextran-9400 and cytochrome-C 12384.

The comparative crystallinity pattern of CTS-MSG membranes in different pore-size diameter was measured by X-ray Diffractometry Philips Model PW 3710 BASED.

The structural morphology of the membranes was also studied under the Scanning Electron Microscope (SEM) within different magnifications.

Results and Discussion

The tensile strength of CTS-MSG membranes for both dry and wet conditions is shown in Table 1. It shows that the tensile stress and strain starts decreasing with the addition of MSG

until MSG addition was 5%. But it starts increasing with the more addition of MSG from 10% upto 30% resulting better tensile strength properties than the control.

Table 1. Tensile strength of CTS-MSG membranes in both dry and wet condition and pore size diameters

MSG	Stress (MPa)		Strain (%)		Pore Diameter
(%)	Dry	Wet	Dry	Wet	(nm)
0	92.12	22.15	79.37	107.05	57.54
1	81.64	4.27	36.28	40.17	39.04
5	57.57	5.38	14.35	61.52	39.12
10	100.73	11.76	41.17	100.65	35.12
20	117.56	16.93	34.10	132.80	22.02
30	103.50	20.32	33.17	138.35	20.32
40	94.76	8.37	36.00	114.77	17.92

Figure 1 shows the variation in case of wet condition. The pore size decreases significantly by addition of MSG where as the tensile strength showed decreasing and increasing alternately giving an highest values of stress and strain at 30% MSG addition.

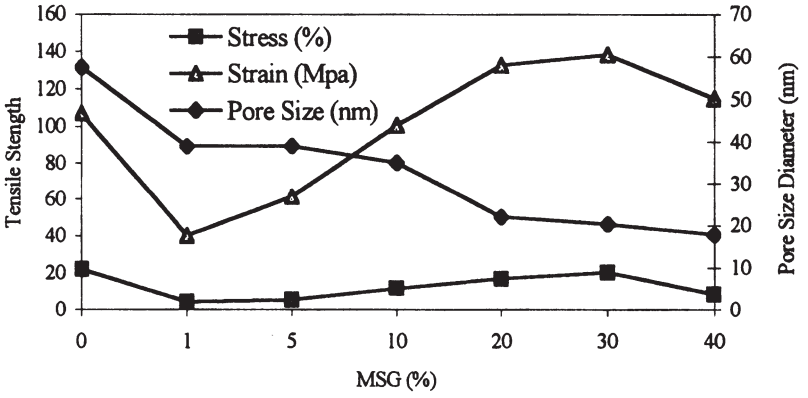

Figure 1. Variation of tensile strength (wet condition) and pore size with MSG addition.

The formation of CTS-MSG membranes were controlled the thickness and the concentration of the casting chitosan solution in order to reduce the effect on uniformity and permeability [4] which generally are important parameters on membrane properties.

It was found that the increasing quantities of MSG lead to increase the elution time which reflected the decreasing of pore size diameters. The addition of MSG into the membrane preparation showed that the significantly decrease in pore size diameters which can be categorized into two groups of pore diameter between 35.12 to 39.12 nm and another between 17.92 to 22.02 nm which corresponded to the addition of MSG between 1-10% and 20-40% respectively. The possibility of controlling the pore size of chitosan membranes for specific applications can be performed. The addition of highly soluble ionic salts such as monosodium glutamate to the polycationic nature chitosan solution might induce the charge arrangement in both inter and intra molecular polymeric chains which would enhance the

molecular order [4]. The physical interaction between chitosan and monosodium glutamate seemed to demonstrate in physical changes such as strength, elasticity and porosity. There is no significant chemical interaction appeared in CTS-MSG membranes as shown in IR spectrum patterns (Figure 2)

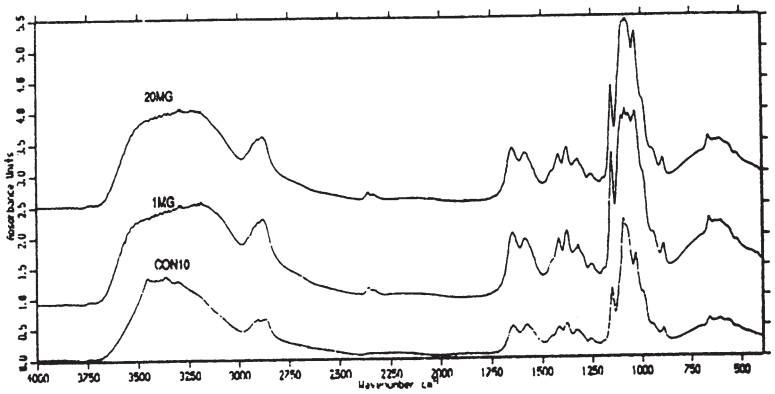

Figure 2. IR spectrum pattern in CTS-MSG membranes

Molecular weight cutoff of CTS-MSG membranes were measured indirectly as the retention of well characterized macromolecules of known sizes (dextran-5000, dextran 9400 and cytochrom-C 12384). With Cp as the permeate concentration and Cb the bulk concentration of macromolecules, the percent rejection, %R is defined:

$$\%R = (1-Cp/Cb) \times 100$$

From a complete sieving curve which was plotted onto a log molecular weight versus rejection, the molecular weight at 92.5% rejection were in the average of MWCO 5000 Da, which were in the range of application in ultrafiltration as shown in Figure 3.

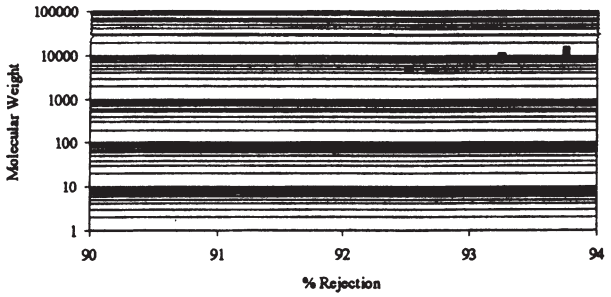

Figure 3. Molecular weight cutoff of CTS-MSG membrane (with 20% MSG).

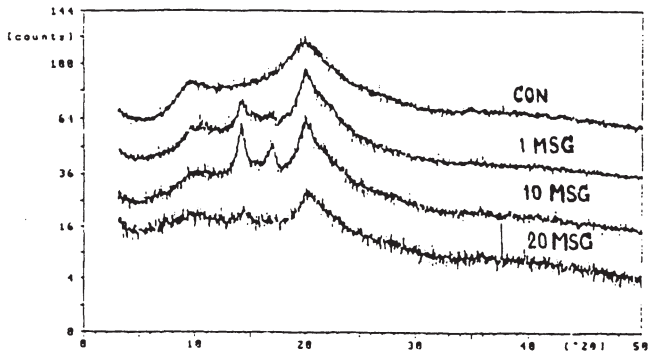

Figure 4. Comparative crystallinity pattern of CTS-MSG membrane.

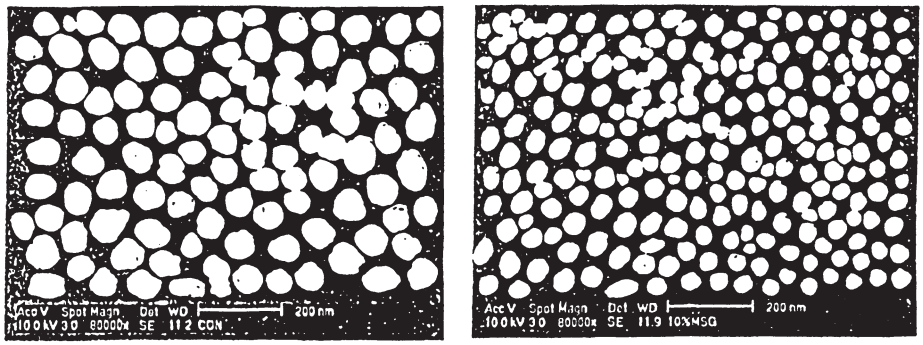

Figure 5. Scanning electron microscope (SEM) of CTS-MSG membrane.

Conclusion

Microporous chitosan membranes with different pore-size diameters were prepared from chitosan solution using monosodium glutamate as porogen. The desired pore-size diameter can be achieved by adding different quantities of monosodium glutamate. The MWCO of control pore-size membrane by using 20% MSG was below dextran 5000.The IR pattern of the membranes did not show the chemical interaction between chitosan and monosodium glutamate. The chitosan microporous membrane could be prepared with control porosity using monosodium glutamate as physically porogen.

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Fabrication of porous chitin matrices

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Summary
A series of porous chitin materials were prepared by freezing and lyophilization of chitin gels cast from 5% DMAc/LiCl solvent system. Ultra structures of the cross sections of the gels were examined by scanning electron microscopy (SEM). The micrographs showed that the pore size of the lyophilized gels varied according to the freezing temperature and gel density. A lower freezing temperature or higher gel density gives smaller pore dimensions. In comparison, non-porous foam was obtained from critical point drying of the chitin gels. The products of critical point drying were superior to that of lyophilization, with a 4-fold smaller elongation to fracture in both the dry and rehydrated state.

Introduction

Recent developments in cell and tissue transplantation have provided an alternative treatment to whole organ transplantation for failing or malfunction organs [1]. The potential of cell transplantation was investigated by various groups for the regeneration of several tissues such as nerve [2], pancreas [3], liver [4], cartilage [5] and bone [6]. Development of good biodegradable polymer that acts as a temporary scaffold determines the success of the cell transplantation therapy. Porous forms of biodegradable polymers are suitable for the above applications as they allow migration of host cells into the scaffold and growth of the cells into complete regeneration of the tissues.

In a paper on chitosan gels, Hirano et al mentioned the preparation of porous N-methylenechitosan gels for gel chromatography [7]. In this work, we have investigated the morphology of these porous chitin structures and factors that affect pore size and shrinkage of the gels following lyophilization. Critical point drying was performed as an alternative method to generate chitin foam. The tensile properties of both freeze-dried and critical point dried chitin were studied. The preparations of these porous chitin systems as scaffolds are useful in developing potential materials for tissue engineering.

Materials and Methods

Chitin was obtained from Polyscience, USA, purified by stirring in 1M NaOH at room temperature for 7 days and in 1M HCl for 1 hour. Degree of deacetylation was determined by FT-IR [8] and microanalysis to be 80%. All reagents used for the preparation of the chitin samples were of analytical grade unless stated otherwise.

Preparation of Chitin Solution: Anhydrous Lithium Chloride (9.6g) was dried at 130°C for about ½ hour, cooled inside a dessicator and dissolved in 200ml of N, N-dimethylacetamide (DMAc) by magnetic stirring. Chitin flakes (0.96g) was suspended in this solution and left overnight at 4°C, 150rpm in a refrigerated shaking incubator to give 200ml

of 0.5% (w/w) chitin solution in DMAc & 5% LiCl solvent system. The viscous clear solution was filtered through glass wool and stored in glass containers prior to casting.

Preparation of Chitin Gels: Chitin gels were cast by dispensing 5, 7.5, 10, 15ml of 0.5% chitin solution into molds measuring 5.7cm x 4.3cm to give gels of average (n=5) thickness of approximately 0.6, 1.25, 1.85 and 2.4 mm respectively. The solutions were allowed to evaporate slowly in the fume hood, for three days. The gels were soaked in deionzed water for two days to ensure complete removal of DMAc. The chitin gels were blotted dry with filter paper and dried in a vacuum desiccator.

Preparation of Chitin Sponges: (A) Freeze Drying (FD): Chitin gels were placed inside 150ml beakers and subjected to various freezing methods prior to freeze-drying (Table 1). Freeze drying was carried out in a Vertis Explorer Drying system. The pre-treated gels were transferred into a freeze-drying chamber, with the condenser temperature set at –40°C. The vacuum was maintained at about 0.1 Torr and freeze-drying was carried out for 24h.

Table 1. Freezing Methods and treatment time

Freezing Methods	Duration (mins.)
Dry Ice/ Acetone	20
Liquid Nitrogen	20
Freezer	20
No Treatment	Not Applicable

(B) Critical Point Drying (CPD): Critical point drying was performed for the purpose of comparison with the freeze-drying method. Chitin gels were transferred from water to ethanol by immersion in at least four changes of absolute ethanol over a period of about 20h. Exchange via a graded series of solvents was found to be unnecessary, as the average shrinkage ratio of the specimen dimensions after direct transfer to absolute alcohol was 0.99, i.e. little shrinkage had occurred. Critical point drying was performed on a Tousimis SAMDRI-780 Critical Point Drying apparatus with CO_2 as the critical fluid. The alcohol-exchanged specimens were placed in the CPD chamber with the intermediate fluid. The chamber was closed, cooled to 0°C and liquid CO_2 was allowed to fill the chamber. The temperature was maintained between 0-10°C for 15mins, after which the chamber was drained of CO_2. The empty chamber was refilled immediately with CO_2 and drained again after ½h. This process of filling and purging with CO_2 was repeated twice. The chamber was heated to 42°C in 7mins and maintained at this temperature for 4mins. The pressure was subsequently reduced at a rate of about 50p.s.i per 0.5 min, to yield the critical point dried samples.

Estimation of Pore Size: Freeze dried / critical point dried specimens were gold coated using a Fine Coat Ion Sputter JFC-1100 coater. SEM micrographs were obtained using a JEOL JSM-T220A scanning microscope at an accelerating voltage of 15kV.

Mechanical Properties: The dimensions of the dried specimens were approximately 3.3cm x 0.8cm x 2.0mm for the CPD and 4.4cm x 1.0cm x 1.6mm for the freeze-dried foam. These specimens were cut into thickness and widths of 1.6 x 3.5 mm for the CPD foam and 2.0 x 3.7 mm for the freeze-dried foam respectively. Tensile test were performed on an Instron tensile tester employing a gauge length of 20 mm and crosshead speed of 2mm/min, giving a strain rate of 0.1mm/min. To prevent slippage of the specimen at the grips due to the reduction in thickness upon clamping, a pair of rectangular cardboard pieces of thickness 1.5mm was glued to each end of the specimen. Hydrated foam samples were prepared by

immersing the dry samples into deionzed water for a period of between ½ to 1h prior to testing.

Results and Discussion

Effect of freezing temperature on the morphology of freeze-dried chitin gels: By subjecting the gels to freeze drying, porosity in the final dried state increases. This is in marked contrast to air-dried sample that only affords orderly-oriented fibrils with no apparent pore pattern (Figure 1). The pore structures on the surface of all the sponges collapsed after the freeze-drying procedure. The cross-section of the samples was obtained by manual cut using a razor blade. The pre-drying treatment of the gels also influences the pore size of the sponges. Sponges obtained from dry ice/acetone system (-38°C) produces the largest pore size at ca. 200-500μm (Figure 2). Freezing the gels with liquid nitrogen (-196°C) afforded a smaller pore size at ca.100-200μm (Figure 3). Gels that were allowed in the freezer (-20°C) for 20 minutes produces <10μm pores (Figure 4).

At lower freezing temperature, larger ice crystals are formed, leading to the formation of larger pore structures after freeze drying. Hence, a smaller pore dimension is obtained when the gels are subjected to liquid nitrogen compared to dry ice/acetone. In contrast, gels treated in the freezer produced sponges with much smaller pore size. It is believed that during the short freezing procedure of 20 minutes, incomplete ice crystallization occurred and resulted in the inability to form larger pores (<10μm). This is also seen in the air-dried gels that produce non-porous morphology. Therefore, larger pore dimension are obtained by subjecting the gels to a higher freezing rate and incomplete freezing produces smaller pores.

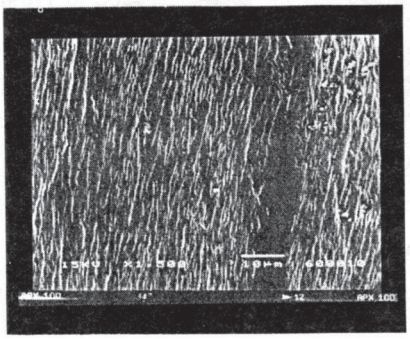

Figure 1. Air-dried chitin gel

Figure 2. Dry ice / acetone

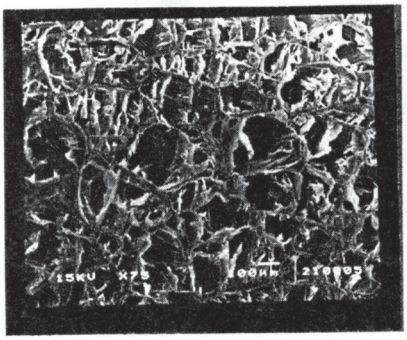

Figure 3. Liquid Nitrogen

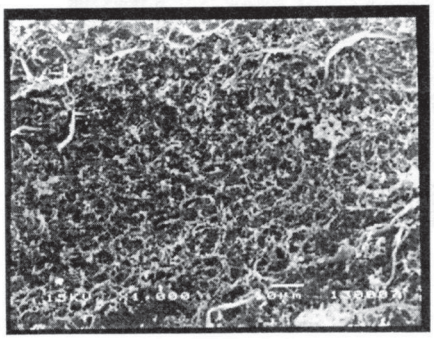

Figure 4. Freezer

Effect of drying by Critical Point Drying (CPD): Critical point dried samples are transparent light foams in contrast to the freeze-dried samples which are opaque. Furthermore, critical point dried samples exhibit a non-porous structure when viewed under the scanning electron microscope (Figure 6). The difference between these observations lies in the drying process itself. Freeze-drying involves sublimation of solids to gaseous. Formation of ice crystals during the freezing process will segregate the polymer network and water phases that lead to a more crystalline polymer structure with intramolecular hydrogen bonding.

In critical point drying, no solid to gaseous phase sublimation is involved. Instead, the liquid phase of the hydrogel is exchanged for a critical fluid (CO_2). The critical fluid is evaporated by careful control of temperature and pressure. Thus, no segregation of the liquid and polymer phases in the gel occurs. This leads to the microscopically non-porous, visually transparent appearance of the critical point dried sample.

The effect of chitin solution concentration on gel density and pore dimensions: The casting volume did not affect the final gel density significantly; however a greater degree of porosity could be achieved by freeze-drying chitin gels cast from lower concentrations of chitin solution. This was revealed by SEM, where the cross-sections of chitin gel cast from lower concentration were more porous than those cast from higher concentration (Figure 7(A), (B), (C)).

Figure 6: CPD specimen

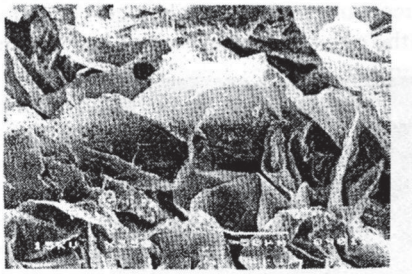

Figure 7(A): 0.125%

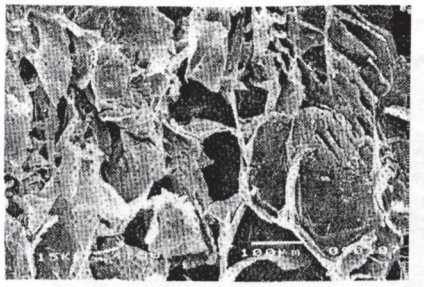

Figure 7(B): 1.0%

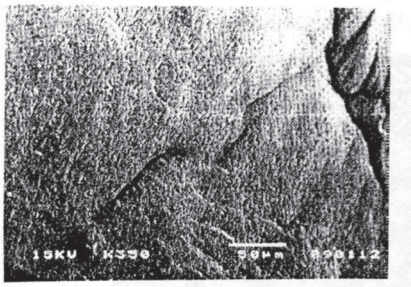

Figure 7(C): 2.0%

Tensile testing of dry and rehydrated materials: The CPD foam exhibited a larger value of elongation at fracture (16.7%) compared to FD foam (4.5%). The larger plasticity of the CPD samples may be due to its more amorphous polymer structure, which permits a certain degree of polymer flow in the direction of tension. In contrast, the higher crystallinity of the FD specimen resulted in higher brittleness that leads to crack initiation and propagation at a lower strain. The load-displacement curve of the rehydrated CPD foam (Figure 8) exhibits a phenomenon comparable to the densification that occurs for foam materials. At a tensile strain of about 1/3 (7 mm displacement), the polymer chains became substantially aligned with the tensile axis. Further strain requires the plastic extension of crystalline domains within the polymer, leading to an increase in modulus over this strain interval.

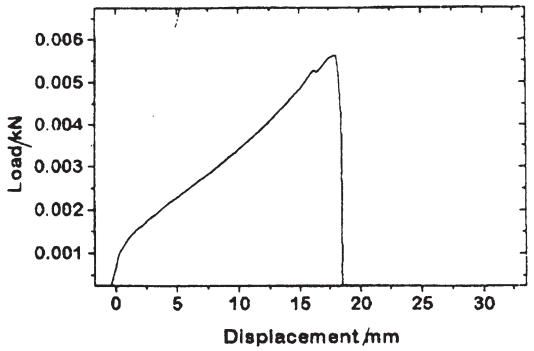

Figure 8. Load-displacement curve for rehydrated CPD foam.

Conclusions

The present studies describe the preparation and characterization of chitin gels to be used as potential scaffolds for cell growth and transplantation. The pore dimensions of the freeze-dried chitin forms can be controlled by two variables namely the freezing temperature and chitin gel density. Chitin foam obtained from critical point drying was found to possess physical properties that were more superior to the freeze-dried forms.

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Changes of polydispersity and limiting molecular weight of ultrasonic treated chitosan

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Summary
Chitosans with different degrees of deacetylation were prepared from shrimp processing waste by chemical method. One percent chitosan-acetic buffer solution was irradiated with ultrasonic wave at 220 W and 60 °C for different time spans. Changes in weight-average and number-average molecular weight of treated chitosans were determined by HPLC. The results indicate that ultrasonic treatment can be applied to degrade molecules and to lower the polydispersity of treated chitosans. Rate of molecular degradation is irregular during the time course of ultrasonic treatment. The limiting molecular weight of resultant molecules depend on degree of deacetylation, molecular weight of chitosan employed, power of ultrasonic wave used, as well as reaction environments such as solution temperature, pH, and ionic strength etc.

Introduction
Chitinous material is the 2nd most abundant biopolymer [1], and is considered to be the versatile enviromentally friendly raw materials [2]. It can be used in many areas such as food process, biochemistry, pharmaceuticals, medicine, agriculture etc. [3-5]. Functional properties of chitinous material such as viscosity [6], antimicrobial activity [7], immunoadjuvant activity [8], hypercholesterolemic activity [9], mechanical properties and porosity of the membrane [10-11], blood coagulation activity and wound-healing activity [12], metal binding capacity [13] etc. depend on the molecular weight and/or degree of deacetylation (dd) of chitosan. The molecular weight and/or dd of chitosan depend on the conditions of preparation. Alkali treatment at elevated temperature is very effective and common method used to produce various molecular weight and dd of chitinous material. Other methods such as extrusion [14], Sonification [15], γ-irradiation [16] has been proposed. The principle for applying the above physical or chemical means is to provide the energy needed to break the chemical bonds.

Molecular weight distribution in term of polydispersity (Mw/Mn) is a useful quantity to shed light on the degradation mechanism [17]. However, Casale and Porter [18] reported polydispersity may not be differ widely from different mechanism. Moreover, molecular weight distribution by itself can not reveal the degradation reaction fully. Because, degradation of polymer by ultrasonic is normally characteristized by a series of parallel-proceeding reactions. Since the original substance usually has a more or less broad molecular-weight distribution, the definite rate constant of degradation are therefore not obtained directly from kinetic studies because of their dependence on molecular weight.

This study explore the effect of ultrasonic treatment on polydispersity and limiting molecular weight of ultrasonic treated chitosan.

Materials and Methods

Chitosan preparation: Chitin was prepared from shrimp (*Solenocera prominentis*) waste by method of Chen et al [10,19] . Chitin powder was alkali treated (50% NaOH) at 90□ for 50 and 120 min to get ca. 60% and 75% dd chitosans.

Degree of deacetylation determination: The colloid titration method of Toei and Kohara [20] was followed. Degree of deacetylation was calculated with the following equations:

Degree of deacetylation = [(x/161)/(x/161 + y/203)] * 100

x = 1/400 * 1/1000 * f * 161 * V

y = 0.5 * 1/100 - x

V: milliters of N/400 PVSK used in titration

f: factor of N/400 PVSK solution.

Molecular weight determination: Molecular weight of degraded chitosans were determined by the size exclusion high performance liquid chromatography (SE-HPLC) method of Chen et al.[21]. A column (7.8 mm x 30 cm) packed with TSK gel G5000 PWXL (Tosoh Co. Ltd, Japan) was used. The mobile phase consisted of 0.2 M acetic acid/0.1 M sodium acetate, and 0.008 M sodium azide.

Calculation of polydispersion (molecular weight distribution): Polydispersity (molecular weight distribution) of ultrasonic degraded chitosans were calculated from ratio of Mw/Mn. Here Mw = $\sum$wiMi / $\sum$wi ; and Mn = $\sum$wi / $\sum$[wi / Mi]; w is the weight of species i of molecular weight Mi and can be obtained from SE-HPLC chromatography. Polydispersity was calculated by Chem-Lab. software.

Ultrasonic treatment: One percent (w/v) chitosan-0.2 M acetic acid /0.1 M sodium acetate solutions were prepared. Ultrasonic treatment was conducted at 220 W for 0, 1.5, 3, 6, 12, 24, and 48 h at 60 °C.

Results and Discussion

Effect on molecular weight and polydispersity of treated chitosan: Results in Table 1 show that chitosans of 62.8% DD and 74.3% DD were prepared by alkali (50% w/w) deacetylation at 99 °C for 50 and 120 min respectively. The molecular weight and polydispersity of obtained chitosans were 6.5 x 10^5 Da and 6.9, respectively, for chitosan treated for 50 min whereas, they are 4.1 x 10^5 Da and 4.8, respectively, for 120 min treated one. This indicates that prolonged alkali treatment not only resulted in increasing DD, but also resulted in progressively molecular degradation, as well as decreasing polydispersity of treated chitosans. The results of Muzzarelli and Rochetti [15]; Chen et al.[21] confirm the results in Table 1.

Table 1. Effect of alkali treatment conditions on degree of deacetylation, weight average molecular weight, and polydispersity of treated chitosan.

Temperature (°C)	Time (min)	Degree of deacetylation (%)	Weight-average Mw x 10^5 Da	Polydispersity
99	50	62.8	6.5	6.9
99	120	74.3	4.1	4.8

Effect on weight-average MW of treated chitosans: Results in Table 2 show that weight-average MW of 62.8% DD chitosan decreased dramatically from 6.5 x 10^5 to 1.6 x 10^5 Da after 1.5 h treatment. Changes slowed down and weight-average MW was around 1.2 x 10^5 Da at the end of the ultrasonic treatment. Weight-average MW of 74% DD chitosan decreased slowly from 4.1 x 10^5 to 3.1 x 10^5 Da during the first 12 h treatment then dropped to 1.0 x 10^5 Da during the next 12 h treatment. Changes become steady and weight-average MW was around 0.9 x 10^5 Da till the end of the ultrasonic treatment. The results in Table 2 show that ultrasonic treatment degraded chitosan molecules over time [21].

Table 2. Changes in weight average molecular weight, polydispersity, monomer and degradation percentage of chitosan of different DD by ultrasonic treatment.

Chitosan	Time (h)	Mw (w) x 10^{-5} Da	Polydispersity (Mw/Mn)	Monomer number	Degradation percentage (%)
63% DD	0	6.5	6.9	3704	
	1.5	1.6	3.2	889	76.0
	3	1.5	3.0	884	5.1
	6	1.4	2.9	782	7.3
	12	1.4	2.9	782	0
	24	1.3	3.0	731	6.5
	48	1.2	2.7	647	7.8
74% DD	0	4.1	4.8	2356	
	1.5	3.8	4.9	2187	7.1
	3	3.6	5.0	2070	5.3
	6	3.3	4.4	1896	8.4
	12	3.1	4.2	1780	6.1
	24	1.0	2.5	553	68.0
	48	0.9	2.5	533	3.6

DD: degree of deacetylation; Monomer number was calculated from weight average molecular divided by average molecular weight of the monomer. ; Degradation percentage is the decrease ratio of the monomer x 100 and is expressed as: (Nn-Nn-1/Nn) x 100.

The decrease in molecular weight is generally rapid at first, the reaction becoming slower until an apparent limiting molecular weight (M_{lim}) is reached. The limiting MW is ca. 1.2 x 10^5 Da or degree of polymerization (DP) of ca. 650 for 63% DD chitosan, whereas the limiting MW is 0.9 x 10^5 Da or DP of 530 for 74% DD chitosan. This may be attributed to the value of M_{lim} depends on mechanochemical conditions and chain flexibility of treated molecules. The more severe the ultrasonic action, the lower is M_{lim} in addition to that, flexible and entangled chains are more susceptible to be degraded by collapsing cavities during ultrasonic treatment. Because, tearing resulting from points of entanglement, collisions between polymer-polymer molecules, between polymer-solvent molecules, and intramolecular collisions are important mechanisms for the rupture of a chain molecules in the neighborhood of a collapsing cavity [18]. Koda et al. [17] studied the effect of chain flexibility on the degradation rate by using pullulan, PEO, poly (ethylene oxide), and PVP, poly (vinnyl pyrolidone). The apparent degradation rate constant (k) are 0.076, 0.032, and 0.030 min^{-1} for PEO, PVP, and pullulan respectively. The higher value of k for PEO means that scission of the PEO chain occurs more easily in comparison with the other two polymer. They concluded that flexibility of the polymer chain to be one of the most important factors determining the degradation rate. Chen et al. [22] reported chain flexibility of chitosan

depends on DD of chitosan, pH, and ionic strength of the solution etc. Chain flexibilities of high DD chitosans are flexibler than that of low DD chitosans. Tsaih and Chen [23] first reported the molecular weight-induced conformational change occurs in chitosan. Low molecular weight chitosans are tend to be in an extended conformation however, higher molecular weight ones are prone to be a random coil. In the same solution pH and ionic strength, molecules of 74% DD chitosan is inclined to be more entangled than that of 63% chitosan.

Effect on degradation rate of treated chitosans: Degradation rate is defined as changes of weight-average MW of treated chitosan with reaction time e.g. the slope of weight-average MW vs reaction time as shown in Fig. 1. Results show that degradation rate was very fast during the first 1.5 h for 63% DD chitosan. Afterward, degradation rate approached to zero (slope remained almost unchanged) till the end of the reaction. Degradation rate of 74% DD chitosan was moderate (8 x 10^3 Da/h) during the first 12 h treatment and increased remarkably to 18 x 10^3 Da/h during the second 12 h, then approached to zero till the end of the reaction. Results show that degradation rate of chitosan by ultrasonic treatment varied during the time course. Although, Ohta et al. [24] reported dextran degraded by ultrasonic was apparently a first order reaction and the apparent rate constant decreasing linear with decreasing molecular weight. As the molecular weight of dextran below 3 x 10^4, the apparent rate constant decrease gradually and approached at 0 at molecular weight equal to 7 x 10^3. However, Koda et al. [17] considered that the ultrasonic degradation process should be represented by a multiple –step reaction, only with a short ultra-sonication time the reaction may be assume to be first order reaction. The discrepancy between the results of Ohta et al. [24] and this report may be most probably due to different reaction time and/or due to different chain flexible among polymers, power of ultrasonic wave, and reaction conditions such as solution pH, ionic strength, temperature etc.

Effect on polydispersity of treated chitosans: Results in Fig. 2 shown polydispersity of 63% DD chitosan decreased pronouncedly from 6.9 to 3.2 during the first 1.5 h treatment. Polydispersity decreased gradually to 2.7 at the end of ultrasonic treatment for the elapsed time of 48h. For 74% DD chitosan, polydispersity decreased from 4.8 to 4.2 during the first 12 h treatment, then decreased to 2.5 during the second 12 h treatment. At the end of treatment, polydispersity remained at 2.5. Polydispersity decreased to 2.7 and 2.5 for 63% DD and 74% DD chitosan, respectively after 48 h ultrasonic treatment. Results indicate that

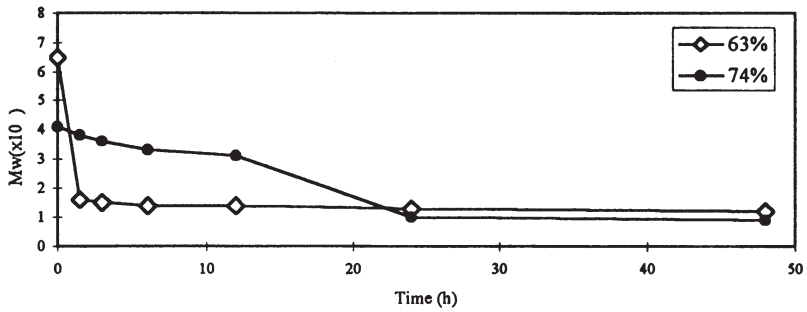

Figure 1. Changes of weight average molecular weight of treated chitosan with different degree of deacetylation during ultrasonic treatment.

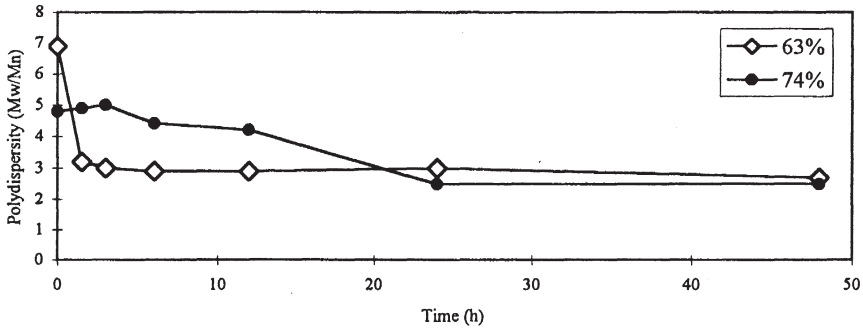

Figure 2. Changes of polydispersity of treated chitosan with different degree of deacetylation during ultrasonic treatment

ultrasonic treatment can narrow down the molecular weight distribution of treated chitosan. Polymer with low polydispersity will improve the mechanical properties such as glass-transtion temperature, melt viscosity, tensile impact strength, tensile elongation, tenacity, relaxation modulus and spinnability etc. [25]. Therefore, ultrasonic treated chitosans can be used to prepare membranes, fibers, or porous beads with better mechanical properties.

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A statistical evaluation of IR spectroscopic methods to determine the degree of acetylation of α–chitin and chitosan

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Summary

A statistical evaluation of the ratios $A_{PB(BL)}/A_{RB(BL)}$ resulting from the combination of all the probe bands, reference bands and base lines proposed so far to calculate the degree of acetylation of α-chitin and chitosan was carried out using the ^{13}C CP/MAS NMR spectroscopy as the standard technique. In spite of the complexities of the IR spectra, it was concluded that IR spectroscopy is a reliable technique to determine the degree of acetylation of those polymers and the best ratios for that purpose are $A_{1626(BL2)}/A_{2877(BL5)}$, $(A_{1663(BL2)}+A_{1626(BL2)})/A_{2877(BL5)}$, $A_{1561(BL2)}/A_{1074(BL6)}$ and $A_{1561(BL2)}/A_{1025(BL6)}$. Only the last two agree with previously used ratios. A new method to evaluate the absorbance ratios used to determine the degree of acetylation of chitin and chitosan by IR spectroscopy was also proposed.

Introduction

Chitin and chitosan, respectively poly-N-acetyl-D-glucosamine and poly-D-glucosamine, actually correspond to a family of polymers varying in the acetyl content measured by the degree of N-acetylation (DA).

Since the DA dictates the behaviour of these polymers, namely reactivity and solubility, the accurate determination of that quantity has been one of the major concerns over many decades.

The difficulty of determining the DA is well illustrated by the extensive number of different techniques, either modern instrumental or classic, which have been used for this purpose [1]. IR spectroscopy has been one of the most widely used techniques, both for soluble and non-soluble samples. In the IR technique the DA is determined using the base line (BL) method to measure the absorbances of the ratio of a probe band (PB) relative to a reference band (RB), A_{PB}/A_{RB}.

A quantitative IR analysis [2,3] of chitin and chitosan implies that:

i) a good PB should change its intensity with the variation of the DA and should lie in a region of the spectrum where other intense bands do not occur, particularly if their intensities also change with the DA. A good RB should not change its intensity with the DA. In each A_{PB}/A_{RB} ratio the PB and RB should lie in a spectral region for which the detector gives similar response for both;

ii) a calibration line must be estimated by plotting the ratios A_{PB}/A_{RB} against the DA values of samples chosen as standards. It is worth noting two important points. First, the standard samples should be similar and deacetylated using the same methods as those that will be used for the samples to be analysed. Second, the experimental calibration line is not always a straight line passing through the origin, as suggested by the Lambert-Beer law equation;

iii)the DA values of the unknown samples should be obtained using the calibration line by interpolation and never by extrapolation.

Although FTIR spectroscopy in particular seems to be an attractive technique to determine the DA (fast, sensitive, user friendly, found in almost all laboratories), the IR bands of chitin and chitosan do not always obey the conditions considered good, either for PB or RB, as will be discussed later. Furthermore, it is not easy to choose the suitable technique to determine the DA of samples to be used as standards. As a result, a great number of A_{PB}/A_{RB} ratios and BL have been proposed [4-9] and a great number of papers using a variety of techniques to evaluate those ratios have been published [4-16].

In previous studies [13,14,16] we have used ^{13}C cross polarisation magic angle spinning (CP/MAS) NMR spectroscopy to determine the DA of the standard samples. In addition, we have optimised the parameters (contact time and pulse delay) which determine the analytical reliability of the ^{13}C CP/MAS NMR. The option for this technique was based on the fact that, like IR, it is non-destructive, applicable to both soluble and non-soluble samples, and leads to absolute values of the DA. Some of the techniques previously used for the same purpose are labour-intensive and/or involve reactions the success of which depends on the particularities of the samples such as solubility and crystallinity.

Following on the same line of research, in this paper we performed a statistical evaluation of the ratios $A_{PB(BL)}/A_{RB(BL)}$ resulting from the combinations of all the PB, RB and BL used so far to calculate the DA of chitin and chitosan, and proposed an improved and accurate method to evaluate those ratios. Furthermore, we used the optimised ^{13}C CP/MAS NMR as the standard technique and at the same time imposed strict criteria on the selection of the best estimated calibration line which fits the experimental data.

Materials and Methods

Samples: Commercial chitin from crab shells (Sigma C-7170) was ground (grain size ≤ 100 mesh), in order to improve the quality of the IR spectra, and then purified with 1 M HCl for 3 h at room temperature and refluxing 2 M NaOH for 48 h. The NaOH solution was changed and the material washed with hot water every 6 h. Until neutrality after the final 6 h treatment. Purified chitin had 0.32 % ash and trace amounts of tyrosine, phenylalanine, lysine and histidine. Five samples were obtained after deacetylation of purified chitin with 12 M NaOH at 110 °C under nitrogen atmosphere [17], for 2, 4, 6, 8 and 13 h, washing with hot water and changing the solution after every 1 h treatment.

FTIR spectra: FTIR spectra of the samples in KBr disc form, which is suitable for soluble and non-soluble samples, were run in a Perkin-Elmer spectrometer 1725X with a resolution of 4 cm^{-1} and 32 accumulations. For each sample the spectra of five KBr discs were analysed. KBr discs were prepared in the usual way from very well dried mixtures of about 1 mg of the sample and 100 mg of KBr. The absorbance of the bands (band heights) was measured using WinFirst software.

^{13}C CP/MAS NMR spectra were run in a Bruker MSL 400 spectrometer with a spinning rate of 5000 Hz. The relevant parameters for signal acquisition were pulse delay = 5 s and contact time = 1 ms. The DA of the samples were determined according to Raymond *et al.* [18] and are 0.68, 0.50, 0.41, 0.29, 0.26, 0.16 (fraction of N-acetylated units).

Statistical evaluation of the ratios $A_{PB(BL)}/A_{RB(BL)}$[19,20]: The statistical evaluation of the IR methods was performed using the simple linear regression model.

The regression model assumes that only y-direction random errors occur, hence to estimate the "best" line it is necessary to seek the line which minimises the deviations in the y-direction between the experimental and the estimated points (y-residuals). Since some of these residuals will be positive and others negative, it is reasonable to seek to minimise the sum of the squares of the residuals (least squares method). The line thus calculated indicates how y varies when x is set to chosen values and has the algebraic form:

$$\hat{y} = \hat{b}\,x + \hat{a}$$

where $\hat{b}$ is the estimated slope, $\hat{a}$ is the estimated intercept, $\hat{y}$ stands for the predicted A_{PB}/A_{RB} ratios and x stands for the experimental DA ^{13}C CP/MAS NMR values.

To accurately choose the good regression lines among those we have studied, we need to work simultaneously with various criteria.

The criterion commonly applied is the coefficient of determination, R^2, which in the case of a straight line is equal to the square of the correlation coefficient, r^2, and varies between 0 and 1, the latter representing a perfect fit of a line to a set of data points. Although correlation coefficient values are simple to calculate, they can easily be misinterpreted. So, in addition, we evaluated the significance of the model through the powerful statistical technique Analysis of Variance (ANOVA), which applies the one tailed F-test to calculate the F-value defined by the ratio of two variances: mean square due to regression and mean square due to residuals. The greater the F-value, the more significant is the model.

The y-residuals are another criterion to provide valuable information on the success of the regression line. The residuals must be as small as possible if the line is to be a good fit to data points. In addition, we also calculated the 95% confidence limits for the slope and for the intercept, which must be as narrow as possible.

To summarise, the calibration line chosen as the best fit to the experimental data is the one obeying the last mentioned criteria for the slope and intercept confidence limits and with R^2 closer to one, the greatest F-value and the smallest residuals. The statistical calculations were performed using Excel software.

Results and Discussion

Figure 1 depicts IR spectrum of chitin showing the PB, RB and BL used to determine the DA.

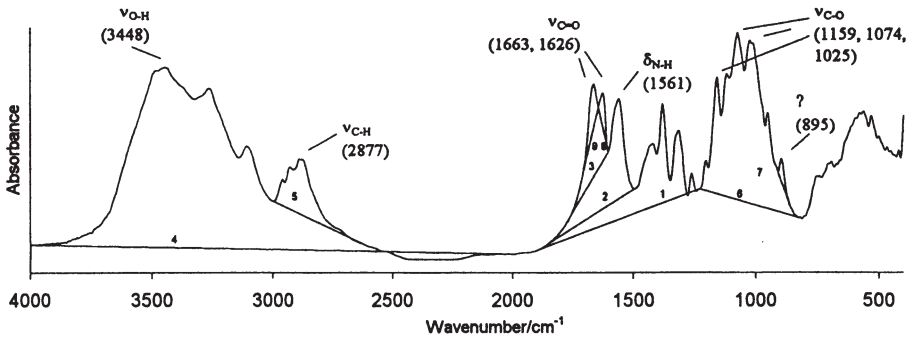

Figure 1. FTIR spectrum of the purified chitin showing the PB, RB and BL.

Figure 2 shows the evolution of PB and RB spectral regions with the decrease of the DA of the samples.

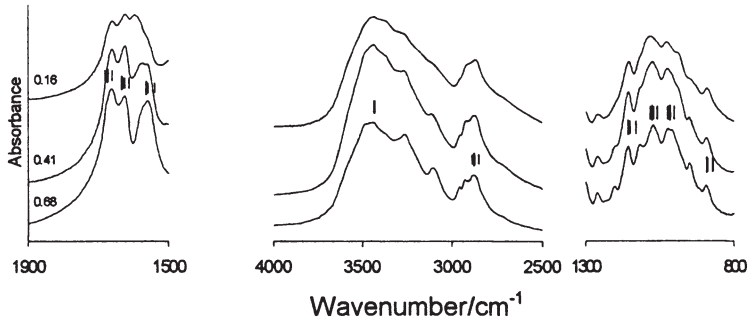

Figure 2. PB and RB IR spectral regions of samples with different DA.

The IR spectra of α-chitin and derived chitosan are difficult to analyse, either due to the complicated network of hydrogen-bonds in which OH, C=O and NH groups are involved [21, 22] or to the broadness of the IR bands typical of natural polymers. Further, when going from highly to less acetylated samples the IR spectra changes from typical secondary amide to IR spectra characteristic of a primary amine. In the latter case we might expect doublets in the NH vibrational region instead of a simple band. All these complications are responsible for the lack of an unequivocal assignment of the IR spectra of the polymers and for the difficulty of choosing the most suitable PB and RB, which will depend on the structure of the sample (α or β) or of the DA range. Thus, it is not surprising that researchers studying different samples might propose different ratios.

We can now analyse in detail the drawbacks of the PB and RB studied in this work which will probably be common to other chitin and chitosan samples with similar DA.

Probe bands

The position and intensities of amide I and amide II bands which have been used as PB [4-9] are affected by remained proteins and calcium binding to the chitin and chitosan matrix [23]. Moreover, it is difficult to choose the most suitable BL for these bands.

- *C=O stretching*, $\nu_{C=O}$, (amide I) at 1663 and 1626 cm^{-1}: This doublet is assigned to $\nu_{C=O}$ and arises from two types of hydrogen bonds in which C=O groups are involved [21]. Of these two bands the one at 1663 cm^{-1} has been the most widely used. Both of them may suffer interference from the OH bending, δ_{OH} at *ca.* 1640 cm^{-1}, either from the OH groups of the structure of chitin and chitosan or from H_2O if the samples are not well dried.

- *NH in plane bending*, δ_{NH} (amide II) at 1561 cm^{-1}: This band is proved not to be a good PB for highly deacetylated samples [15,24,25] but a reasonably good PB for the other samples. However, a band appearing at 1597 cm^{-1} with the decrease of the DA probably due to the doublet expected for the primary amines may complicate its use as PB.

Reference bands

- *OH stretching*, ν_{OH}, at 3448 cm^{-1}: This band has been considered by some researchers as a good RB [7,8]. Although it is not expected that its intensity will change with the DA, we think that it may suffer relevant interferences. One of them is due to a second OH stretching band at *ca.* 3480 cm^{-1} arising from another type of hydrogen-bonds in which the CH_2OH groups are

involved in α-chitin [21]. In fact, in the IR spectra of our samples we observed a shift of that band from 3448 cm^{-1} to 3436 cm^{-1} in the least acetylated sample. The second interference is due to the intense NH stretching, ν_{NH} at 3269 cm^{-1}, the intensity of which varies with the DA. In addition, the intensity of ν_{OH} bands will increase if the samples were not conveniently dried.

- *CH stretching*, ν_{CH}, at 2877 cm^{-1}: Some researchers have proposed the use of this band as RB [4,5]. The CH stretchings lie in a complex spectral region were at least five bands have been observed due to symmetric and asymmetric stretchings of CH from the ring and from CH_2OH and CH_3 groups [22]. Only the ν_{CH} from the latter groups changes its intensity with the DA. Although these bands are not unequivocally assigned, the IR spectra of our samples show that the intensity of the band at 2877 cm^{-1} does not change with the DA.

- *C-O stretchings*, $\nu_{C\text{-}O}$ at 1159, 1074 and 1025 cm^{-1}: More recently these bands were also proposed as RB [6,9]. The band at 1159 cm^{-1} is assigned to the C-O asymmetric stretching of bridge oxygen [22] and its intensity may change if depolymerisation occurs during the deacetylation treatment. As far as the other two bands are concerned, at least four bands have been observed in the same region due to the $\nu_{C\text{-}O}$ of the ring C-OH and of the C-O-C and CH_2OH groups, the intensities of which are not expected to change with the DA. In our IR spectra we observed a what seems shift in one of those bands from 1074 to 1083 cm^{-1} with the decrease of the DA. However, in an earlier paper [25] two bands were observed in this region in FTIR spectra of films of chitosan samples (at 1078 and 1098 cm^{-1}), the relative intensities of which change with the DA.

It is worthwhile to note that these bands lie in the fingerprint region of the IR spectra where frequently vibrational couplings occur. In general, the bands in this region suffer significant shifts even with small structural changes.

- *The band at 895 cm^{-1}*: This band proposed by Miya *et al.* [6] is not clearly assigned and is relatively weak and again lies in the fingerprint region.

Table 1 contains the statistical results of R^2 and F-values of the calibration lines obtained using all the ratios $A_{PB(BL)}/A_{RB(BL)}$ under study.

A cursory examination of the statistical results on Table 1 shows that in general to the highest R^2 values correspond the highest F values. A more detailed analysis required four sequential steps as follows:

i) to identify the R^2>0.9900 values associated with all PB(BL) in order to choose the best combinations. We concluded that they are all the PB(BL1 and BL2) and 1663(BL3);
ii) to identify the R^2>0.9900 values associated with RB(BL) in order to choose the best combinations. We concluded that they are 2877(BL5), 1159(BL6), 1074(BL6), and 1025(BL6), in accordance with the previous spectral analysis;
iii) to identify the R^2>0.9900 values associated with the previous chosen PB(BL) and RB(BL) sets (numbers in black);
iv) to choose the best absorbance ratios, $A_{PB(BL)}/A_{RB(BL)}$, based on the strict criteria: R^2>0.9900, F>1000, smallest residuals and confidence limits (numbers in bold).

The statistical evaluation performed in this study of all the $A_{PB(BL)}/A_{RB(BL)}$ ratios used so far, and some new ones allowed us to conclude that the best ratios to determine the DA of α–chitin and chitosan are $\mathbf{A_{1626(BL2)}/A_{2877(BL5)}}$, $\mathbf{(A_{1663(BL2)}+A_{1626(BL2)})/A_{2877(BL5)}}$, $\mathbf{A_{1561(BL2)}/A_{1074(BL6)}}$, $\mathbf{A_{1561(BL2)}/A_{1025(BL6)}}$.

It is worth noting that amongst the great number of ratios proposed so far, only the ratios $A_{1561(BL2)}/A_{1074(BL6)}$ and $A_{1561(BL2)}/A_{1025(BL6)}$ are in accordance with previous results [9]. Even the second best (numbers in black) do not agree with any previously proposed, and some of them extensively used, ratios.

Table 1. Coefficients of determination, R^2, and F-values of the significance tests

$A_{PB(BL)}$ \ $A_{RB(BL)}$	$A_{3448(BL4)}$ [7,8]	$A_{2877(BL4)}$ [4]	$A_{2877(BL5)}$ [5]	$A_{1159(BL6)}$ [6,9]	$A_{1074(BL6)}$ [9]	$A_{1025(BL6)}$ [9]	$A_{895(BL6)}$ *	$A_{895(BL7)}$ [6]
$A_{1663(BL1)}$ [7]	0.9790 186	0.9793 189	0.9907 426	0.9878 325	0.9949 778	0.9952 821	0.9623 102	0.9579 91
$A_{1663(BL2)}$ *	0.9745 153	0.9806 202	0.9949 782	0.9921 503	0.9926 537	0.9952 827	0.9613 99	0.9536 82
$A_{1663(BL3)}$ [5,6,8]	0.9800 196	0.9819 217	0.9855 272	0.9863 288	0.9933 593	0.9899 392	0.9750 156	0.9433 66
$A_{1663(BL9)}$ *	0.9528 81	0.9742 151	0.9433 66	0.9514 78	0.9669 117	0.9508 77	0.9031 37	0.7943 15
$A_{1626(BL1)}$ *	0.9811 207	0.9825 224	0.9922 506	0.9897 384	0.9944 712	0.9955 884	0.9462 70.4126	0.9325 55
$A_{1626(BL2)}$ *	0.9705 131	0.9856 273	**0.9972** **1436**	0.9948 759	0.9807 203	0.9856 274	0.9357 58	0.8872 31
$A_{1626(BL3)}$ *	0.9773 172	0.9830 231	0.9746 154	0.9747 154	0.9824 223	0.9760 163	0.9675 119	0.9181 45
$A_{1626(BL8)}$ [9]	0.9586 92	0.9722 140	0.9422 65	0.9426 66	0.9548 84	0.9423 65	0.9292 52	0.8451 22
$A_{1561(BL1)}$ [4]	0.9789 186	0.9773 172	0.9837 241	0.9808 204	0.9921 500	0.9893 369	0.9722 40	0.9488 74
$A_{1561(BL2)}$ [6,9]	0.9781 178	0.9784 181	0.9910 442	0.9888 354	**0.9969** **1279**	**0.9965** **1150**	0.9799 194	0.9565 88
$A_{1663(BL1)}+A_{1626(BL1)}$ *	0.9801 197	0.9809 206	0.9918 486	0.9891 362	0.9953 843	0.9959 978	0.9558 86	0.9488 74
$A_{1663(BL2)}+A_{1626(BL2)}$ *	0.9736 148	0.9832 233	**0.9970** **1317**	0.9943 694	0.9896 381	0.9934 597	0.9518 79	0.9334 56
$A_{1663(BL3)}+A_{1626(BL3)}$ *	0.9804 200	0.9834 237	0.9819 217	0.9827 227	0.9900 396	0.9852 266	0.9727 142	0.9325 55
$A_{1663(BL3)}+A_{1626(BL8)}$ [9]	0.9796 192	0.9833 235	0.9786 183	0.9802 198	0.9885 344	0.9826 225	0.9664 115	0.9194 46
$A_{1663(BL9)}+A_{1626(BL8)}$ *	0.9622 102	0.9759 162	0.9472 72	0.9529 81	0.9666 116	0.9527 81	0.9235 48	0.8310 20

*present study

The improved method proposed in this study to evaluate the ratios $A_{PB(BL)}/A_{RB(BL)}$ can be extended to different samples.

In spite of the drawbacks mentioned above for the PB and RB of the α–chitin and chitosan IR spectra as a final conclusion we hold that while FTIR spectroscopy is not an absolute technique to determine the DA of these polymers, it is nevertheless a reliable one, provided that the following measures are taken to eliminate the causes of errors:

- an optimised and accurate calibration technique must be used;
- conveniently purified and dried samples must be prepared;
- suitable $A_{PB(BL)}/A_{RB(BL)}$ must be used to plot the calibration line.

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Products of alkaline hydrolysis of dibutyrylchitin: chemical composition and DSC investigation

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Summary
Synthesis of dibuturylchitin (DBCH), DBCH fibres preparation and a process of alkaline treatment of DBCH fibres in 5% NaOH water solution at 50°C are described and products of alkaline hydrolysis are investigated. It was found that alkaline treatment carried out under heterogeneous conditions results in the loss of sample weight, due to loss of the bulky butyric groups, and in restoring of the hydroxyl groups of chitin. Degree of debutyrylation depends on the time of alkaline treatment. The final products of alkaline hydrolysis are pure chitin fibres. Partially debutyrylated chitin samples, released from non-reacted DBCH, are insoluble in the solvents of DBCH, and, like pure chitin, do not show the glass transition temperature in the range of 25-250° C, and start to decompose at the temperature above 240°C.

Introduction
In several monographs [1-9] and numerous original publications the extraordinary wound healing properties of chitin were described. Chitin could be used as an ideal material for wound dressings, but its insolubility in popular organic solvents strongly restricts any practical applications of chitin. Dibutyrylchitin (DBCH), a product of esterification of chitin with butyric anhydride [10-12], is easily soluble in several organic solvents and forms fibres [13, 14], non-wovens and other textile materials with comfortable application and good mechanical properties. Bioactivity of DBCH materials as a wound dressing is investigated presently.

It was found that alkaline treatment of the finished DBCH products leads to reconstruction of chitin without destroying their structure [15, 16]. This establishment gives a possibility to form a wide assortment of partially acylated chitin and pure chitin textile materials for biomedical applications. In the present work the results of alkaline treatment of DBCH fibres obtained by the wet method of spinning are discussed, and the structure and the thermal properties of the products of the alkaline hydrolysis of DBCH are described. Obtained textile materials with varied degree of butyrylation are now the subjects of investigation of their bioactivity.

Materials and Methods
Krill chitin, the product of the Sea Fisheries Institute, Gdynia, Poland, was additionally purified from residual calcium carbonate. Chitin had a degree of acetylation 0.98 and intrinsic viscosity value 18.0 dL/g.

Butyric anhydride, 98% (Aldrich), perchloric acid 70 to 72% (Merck), diethylether, acetone, N-methylpyrrolidone (NMP), LiCl, dimethyformamide (DMF), NaOH of normal laboratory grade were used without further purification.

Intrinsic viscosity values (IVV) of chitin and partially acylated chitin were determined at 25°C in 5%LiCl-NMP solutions using initial concentration of polymer ca 0.1 g/dL, IVV of DBCH was determined in acetone and in 5%LiCl-NMP solutions using initial concentration of polymer ca 0.5 g/dL.

Infrared spectra of chitin, partially acylated chitin samples and DBCH were recorded from films using Perkin-Elmer 2000 FTIR instrument. Chitin and partially acylated chitin films were prepared from 5%LiCl-NMP solutions, DBCH films were cast from acetone solutions.

Thermal analysis of DBCH, chitin and partially acylated chitin samples in fibre forms was made using differential scanning calorimetry (DSC) on Perkin-Elmer DSC 7 instrument. Dry N_2 was used as purge gas with a flow rate 30 cm^3/min, calibration was carried out with indium and zinc. The DSC curves were recorded at the scanning rate 20°C/min.

Results and Discussion

Preparation of dibutyrylchitin: Synthesis of DBCH was carried out at ca 20°C during 4 hours using 0,5 g of $HClO_4$ (70-72%) and 16 g of butyric anhydride (98%) per 1 g of krill chitin [13]. DBCH with intrinsic viscosity values 1.5 dL/g (determined in acetone solution) and 1.7 dL/g (determined in 5%LiCl-NMP solution) was obtained with the yield of 93%. Elemental analysis of DBCH gave: C – 55.80% (calc.value 55.98%), H – 7.51% (calc.value 7.29%) and N – 4.10% (calc. value 4.08%) suggesting that degree of chitin butyrylation was equal to 2.

Preparation of DBCH fibres: DBCH fibres were obtained using the wet method of spinning [14]. The dope containing ca 16% of polymer was prepared when DBCH was dissolved completely in DMF. The dope without pre-filtration was introduced into the dope reservoir of the laboratory scale spinning apparatus. DBCH fibres were spun into water coagulation bath, washed in water baths and finally were drawn in hot water. The fibres were wound up on the rollers and dried. Obtained fibres had yarn count 35.6 Tex, tenacity (in dry) 14.6 cN/Tex, knot strength 2.42 cN/Tex and elongation 9.7%.

***Alkaline hydrolysis of DBCH fibres*:** The samples of DBCH fibres were immersed in excess amount of 5% NaOH water solution at 50°C and allowed to be hydrolysed under heterogeneous conditions in different periods of time. After definite time the samples were put out, washed several times with water to remove any traces of alkali and dried. Dry products of alkaline hydrolysis were weighed and then treated with acetone to extract all soluble parts. The weights of the polymer extracted with acetone were determined after evaporation of the solvent, polymer remainded after acetone treatment was dried and weighed again. The intrinsic viscosity values of the residuum after acetone treatment were determined in the 5%LiCl-NMP solution at 25°C. Results of alkaline hydrolysis of DBCH fibres are collected in Table 1.

During the alkaline treatment of DBCH fibres, carried out under described above conditions, the loss of the bulky bytyric groups occurs, the hydroxyl groups of chitin are being restored and after ca 25 min ca 90% of initial DBCH has been hydrolysed The intrinsic viscosity values of hydrolysis products are increasing with the decrease of the butyric groups contents in the forming macromolecules (5%LiCl-NMP solution is thermodinamically better solvent for chitin than for DBCH and IVV of chitin is much higher than IVV of DBCH with the similar molecular weight).

Parts of the samples extracted with acetone were analysed by IR spectrometry and recorded spectra were compared with the spectrum of the initial DBCH (Figure 1). As it is seen in Figure 1, all polymers extracted with acetone from the products of DBCH alkaline treatment are the pure DBCH, which did not get in the reaction with NaOH in the time of alkaline treatment.

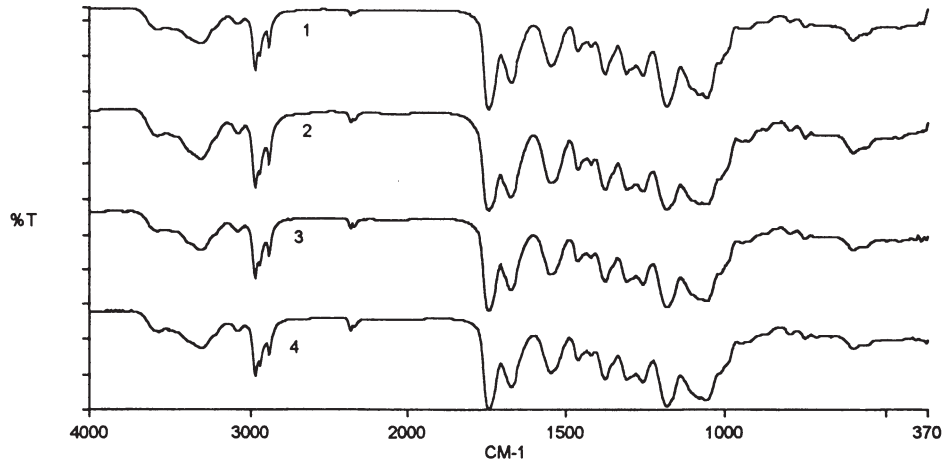

Figure 1. IR spectra of initial DBCH (1) and polymers extracted with acetone after alkaline treatment of DBCH fibres: 2 – 5 min, 3 – 15 min, 4 – 26 min.

Table 1. Results of heterogeneous alkaline hydrolysis of DBCH* fibres (5% NaOH, 50°C)

Sample No.	Alkaline treatment time, min	Loss of the sample weight after treatment with alkali, %	Composition of the products of alkaline treatment		Intrinsic viscosity values of the products insoluble in acetone, dL/g
			Part soluble in acetone, %	Part insoluble in acetone, %	
1	5	1.78	82.9	17.1	
2	10	8.53	65.3	34.7	3.1
3	15	13.76	57.3	42.7	3.8
4	20	22.11	34.0	66.0	4.4
5	26	28.12	7.5	92.5	5.2
6	40	35.41	2.9	97.1	
7	60	37.42	2.4	97.6	6.6
8	120	39.20	0	100	6.7

*Initial DBCH had the intrinsic viscosity value of 1.7 dL/g (in 5% LiCl-MNP solution).

Remainders after extraction of the non-reacted DBCH are insoluble in organic solvents dissolving DBCH, and are soluble in 5%LiCl-NMP solution. Films of these polymers prepared from 5%LiCl-MNP solutions were also analysed using IR spectrometry; obtained spectra are collected in Figure 2 and compared with the spectrum of krill chitin.

As there is seen in Figure 2, the absorbency due to carbonyl groups C=O at ca 1740 cm^{-1} (esters of fatty acid) decreases with the time of alkaline treatment, it is weak in the

spectrum of the sample obtained after 40 min and almost absent after 120 min of alkaline treatment. It means, that in the presence of the water solution of NaOH, when the process of debutyrylation of DBCH occured, varied butyrylchitins have been formed, and pure chitin as the final product of DBCH hydrolysis was restored.

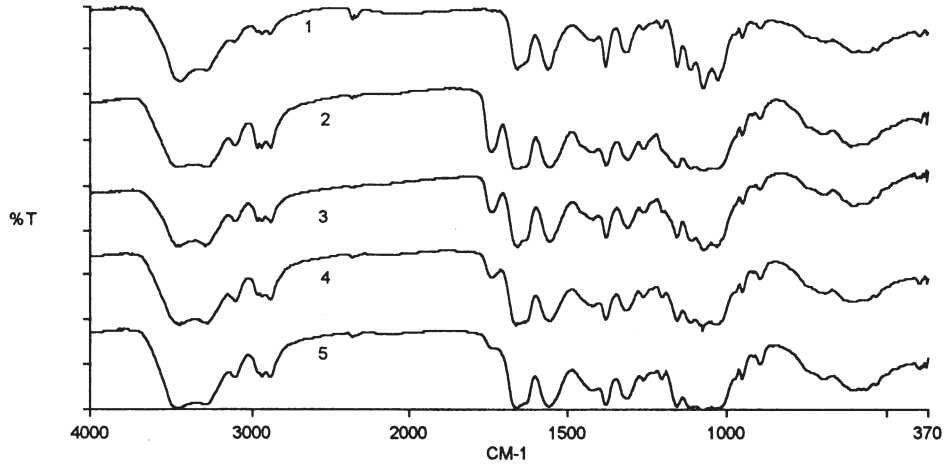

Figure 2. IR spectra of initial chitin (1) and products of alkaline hydrolysis of DBCH fibres: 2-after 15 min, 3 - after 20 min, 4 - after 40 min, 5 – after 120 min of alkaline treatment

The ratio of peak heights measured using base line at ca 1740 cm^{-1}, A_{1740}, (absorption due to ester groups) to ca 1555 cm^{-1}, A_{1555}, (absorption due to amide groups) and results of nitrogen contents determination by the elemental analysis are presented in Table 2. Degrees of debutyrylation of the products of alkaline hydrolysis of DBCH are calculated from the theoretical contents of nitrogen.

Table 2. Characteristic of the products of alkaline hydrolysis of DBCH fibres insoluble in acetone (hydrolysis in 5%NaOH, 50°C).

Sample No	Time of hydrolysis, min	A_{1740}/A_{1555}	N contents, %	Degree of debutyrylation, %
Initial DBCH	0	3.21	4.10	0
1	5		4.83	40
2	10		5.25	57
3	15	0.42	5.69	72
4	20	0.36	5.78	77
5	26	0.18	5.89	80
6	40	0.06	6.29	91
8	120	0	6.69 (calc.6.60)	100

The study of the hydrolysis of the DBCH fibres carried out under heterogeneous conditions resulted in following proceeding of this process: hydrolysis reaction starts on the surfaces of the DBCH fibres, then NaOH water solution penetrates into swollen fibres, NaOH reacts with further macromolecules of DBCH and with macromolecules of the formed partially butyrylated chitin, and the reaction is finished when pure chitin remains:

$$C_8H_{11}NO_5(C_3H_7CO)_2 + 2NaOH \rightarrow C_8H_{11}NO_3(OH)_2 + 2\ C_3H_7COONa \quad (1)$$

Previous investigations of this process [16] showed, that hydrolysis reaction carried out in the excess amounts of NaOH water solutions is well described by the equation of pseudo-first order reaction. The rate of the hydrolysis depends on the form of the used samples and on the concentration of NaOH and the temperature of the reaction. It was found also [13, 14], that mechanical properties of the chitin fibres obtained in the process of alkaline hydrolysis of DBCH fibres were better than those of the initial DBCH fibres.

Thermal analysis of the products of alkaline hydrolysis of DBCH fibres: Thermal analysis of the samples obtained after alkaline treatment of DBCH fibres as well as initial DBCH fibres was made using DSC method. The investigated samples in fibre form were cut into pieces of 1 mm of length and put into open-type DSC sample pans. Weights of the samples were in range 2-4 mg. The samples were heated after quenching in following scans: I – 25-180 °C, and then quenched to the room temperature; II – 25-200°C and III – 25-250°C.

In the first scan endothermic deviation in the sample baseline were observed due to vaporisation of water. The water content was determined from the area of evaporation endothermic peak. In the second scan of the samples of the initial DBCH and several products of alkaline treatment, containing certain amounts of non-reacted DBCH, the glass transition temperature was observed. This temperature was confirmed in the 3-rd scan.

The samples of the hydrolysis products released from non-reacted DBCH did not show glass transition temperature in the range of the measured temperatures, as well as the product obtained after 40 min of alkali treatment and the final product of alkaline hydrolysis (pure chitin obtained after 120 min). The glass transition temperature of DBCH lies in range of 155-160°C.

Water content in the products of hydrolysis increases with the increase of degree of debutyrylation due to increase the hydrophilic properties of the samples and is the highest in the case of pure chitin.

All samples start to decompose at the temperature over 240°C (pure chitin – over 250°C). The results of DSC investigation are collected in Table 3.

Table 3. Thermal properties and water contents in the products of alkaline hydrolysis of DBCH fibres (5%NaOH, 50°C).

Sample No.	Treatment time, min	Products containing non-reacted DBCH		Products released from non-reacted DBCH	
		T_g, °C	water contents, %	T_g, °C	water contents, %
2	10	161.0	5.1	no	
3	15	157.4	5.5	no	6.1
5	26	158.7		no	
6	40	no	8.5-9.2	no	9.6
8	120	no	11.9	no	11.9
Initial DBCH	0	155.5	1.4		

Conclusions

- The alkaline treatment of DBCH fibres carried out under heterogeneous conditions in 5% NaOH at 50°C leads to the loss of the bulky butyric groups and to the gradual restoring of hydroxyl groups.
- Samples obtained in the first 26 min of treatment still contain the pure DBCH, amount of which decreases with the increase of alkaline treatment time. DBCH shows the glass transition temperature in the range of 155-160°C and no melting up to 240°C, when DBCH starts to decompose.
- Pure DBCH, when it is necessary, can be removed in the process of extraction with acetone.
- Degree of debutyrylation during hydrolysis process increase with the time of alkaline treatment, and pure chitin fibres as the final product are formed.
- Products of DBCH hydrolysis, released from non-reacted DBCH, independently from the degree of debutyrylation are insoluble in solvents of DBCH, and, like chitin itself, do not show the glass transition temperature in the range of the measured temperatures.They start to decompose at 240-250°C, below the glass transition and melting temperature.
- The alkaline treatment of the DBCH fibres gives the possibility to obtain the wide assortment of the textile chitin products with varied degree of butyrylation and with defined physical and chemical characteristics.

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Chitosan emulsification properties

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Summary
The emulsification of sunflower oil by chitosan solutions with deacetylation degrees (DD) between 75 and 95 % were studied. The chitosan solutions were prepared in 0.2, 1.0, 2.0 % concentration with HCl 0.1 N, these concentrations allowed solutions with a wide range of viscosity.

The droplet size distribution, emulsion viscosity, emulsion stability and aging behavior were analyzed during 48 hours. Results were studied as function of the chitosan DD, solutions concentration and viscosity of them. Chitosan be an useful emulsifier and yields stable water/oil/water (w/o/w) emulsions.

Introduction
Stable emulsions are very important in many industrial processes [1] of colloidal systems such as emulsions, suspensions and foams.

Large surface-active molecules (macromolecules and polyelectrolytes) provide an interesting means for controlling both stabilization and type of emulsions. An efficient steric stabilization is achieved by adsorbing and controlling the conformation of the molecules at the interface between the disperse and continuous phases. Instead of most low-molecular-weight surface active agents operate only through electrostatic stabilization. Electrosteric stabilization [2-3] is the combination of both mechanisms.

Non adsorbing polymers such as synthetic or nature macromolecules have the viscosifying effect that influence emulsion stability by decreasing the rate of creaming.

Amphiphilic polyelectrolytes like chitosan combine both electrosteric and viscosifying stabilization mechanisms so they appear to be useful emulsifiers.

In account of the above considerations we studied the emulsification properties of chitosan in previous papers [4-5].

Chitosan is a useful emulsifier [4] that yields stable water-in-oil-in-water (w/o/w) multiple emulsions.

Multiple emulsions were described by Seifritz in 1925. These type of emulsions are complex system in which the droplets of the dispersed phase contain even smaller dispersed droplets of the continuous phase themselves.

Multiple emulsions have been prepared mainly by one-step emulsification and two-step emulsification [6]. The most common and better controlled preparation of double emulsions is based on the two–step emulsification process, where a hydrophobic emulsifier is used for the primary w/o emulsification, and a hydrophilic emulsifier is used for preparation of the o/w emulsion.

However, it was concluded that the classical double emulsion prepared with two sets of monomeric emulsifiers cannot provide long-term stability to the double emulsion, they are intrinsically thermodynamic unstable [7-8]. To increase the stability of these multiple emulsions macromolecules have been used with surfactants [9-10].

Chitosan produce w/o/w emulsions without adding any surfactant, it is possible because this biopolymer is composed of a mixture of molecules with different deacetylation degree (DD). Molecules with high DD promoted the formation of w/o emulsions, whereas those of lower DD promoted the emulsification of water inside the oil droplets. For that reason chitosan behaves as a mixture of surfactants with different HLB values.

In a first work we studied the emulsification properties of chitosan with an only DD (89 %). In a second study we reported the effect of chitosan DD in the emulsification. In both the solutions were prepared containing 1 % w/v chitosan in 1 % v/v aqueous acetic acid.

Following our studies in this field we have decided to examine the influence of chitosan DD, chitosan molecular weight (measured as apparent viscosity) and the concentration of this biopolymer on the emulsification properties when we used HCl instead of acetic acid.

Materials and Methods

For the experimental work we used chitosans with different DD, between 75 and 95 %, that were measured using a method described in the literature [11].

Stock solutions containing 0.2; 1.0 and 2.0 % w/v chitosan were prepared. Chitosans were stirred in HCl 0.1 N. After standing for 24 hours, undissolved chitosan was separated by filtration through a medium-porosity filter.

Emulsions were produced by adding 20 g sunflower oil to 80 g of different concentrations of chitosan in HCl, this chitosan/oil ratio was found previously. Samples were stirred in a food processor for 3 minutes.

To determine the droplet size distribution several samples of each emulsion were placed between slides in a microscope Olympus BH-2-UMA and were photographed with a camera Sony CCD IRIS/RGB. To avoid personal preferences in the selection of droplets for the determination of size distribution, four randomly drawn straight lines were placed , and the diameter of the droplets touching the lines were measured and counted. About 500 droplets were measured in each sample, giving a good confidence data [12].

Viscosities of the chitosan solutions and of emulsions were measured with a Brookfield model DV-IV+ viscosimeter. Measurements a different times during 48 hours were performed after gentle shaking to homogenize the emulsion in order to ensure the same volume fraction of droplets in all samples.

To determine the stability of the emulsions, the samples were left to rest and the time elapsed until the phase separation was recorded.

Results and Discussion

Following our studies in this field we decided to examine the influence of chitosan molecular weight (measured as apparent viscosity of chitosan solutions); chitosan deacetylation degree (DD) and concentration of the biopolymer on the emulsification properties when we used HCl instead of acetic acid to prepare the solutions.

Chitosan/oil work ratio: To determine the most efficient and economical proportion of chitosan/oil ratio we worked with all the samples prepared in three concentrations that we would use in this proposed study. The emulsions final weight were 100 g and we changed

both: chitosan solution and oil weights. We found the same behavior in every chitosan solutions as is shown in Table 1.

Table 1.

Chitosan/oil proportion (g)	Stability (1 h)
92 g chitosan/ 8 g oil	Two phases separation: Concentrated and dilute emulsion
86 g chitosan/ 14 g oil	**No phases separation**
80 g chitosan/ 20 g oil	**No phases separation**
70 g chitosan/ 30 g oil	Two phases separation: Concentrated and dilute emulsion
60 g chitosan/ 40 g oil	Three phases separation: Oil, concentrated and dilute emulsion
40 g chitosan/ 60 g oil	Three phases separation: Oil, concentrated and dilute emulsion

Best proportions that yield stable emulsions were 86 g chitosan solution/ 14 oil and 80 g chitosan solution/ 20 g oil. We selected as chitosan/ oil work ratio the last one because it was the most economical use of the emulsifier.

Droplet distribution: The distribution varies with the chitosan deacetylation degree (DD) and concentration of the chitosan solution, as can be seen in Table 2.

Table 2.

Chitosan	Deacetylation degree	Concentration	Droplet distribution
Sample 1	75 %	0.2 %	Unimodal
		1.0 %	Unimodal
		2.0%	Unimodal
Sample 2	76 %	0.2 %	Unimodal
		1.0 %	Polymodal
		2.0%	Unimodal
Sample 3	78 %	0.2 %	Polymodal
		1.0 %	Polymodal
		2.0 %	Unimodal
Sample 4	85 %	0.2 %	Polymodal
		1.0 %	Unimodal
		2.0 %	Unimodal
Sample 5	93 %	0.2 %	Polymodal
		1.0 %	Polymodal
		2.0 %	Unimodal
Sample 6	94 %	0.2 %	Unimodal
		1.0 %	Polymodal
		2.0 %	-
Sample 7	95 %	0.2 %	Unimodal
		1.0 %	Unimodal
		2.0 %	Unimodal

The found distribution was unimodal at low DD (< 76%) and at high DD (>93%) for all concentrations used. At intermediate DD distribution was unimodal only when we used the most concentrated solutions.

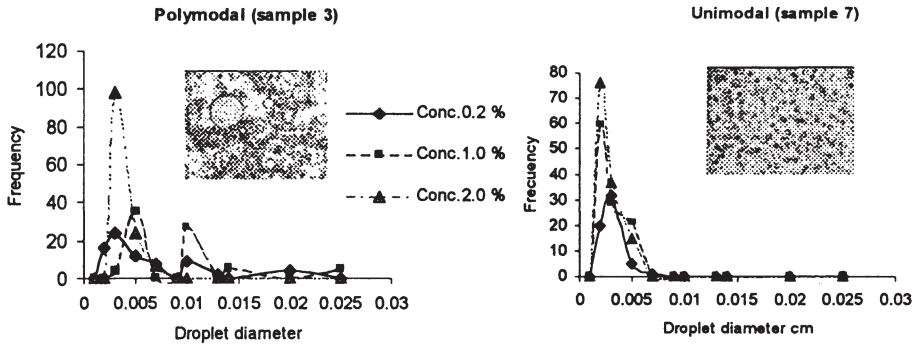

Figure 1.

On the other hand, droplet size distribution was independent of both: chitosan solution viscosity and emulsion viscosity, but it was dependent on the biopolymer concentration. The chitosan emulsification properties were proportional to chitosan concentration and most efficient work concentration was 2%. Two droplet size distributions and microscopic photographs are shown in Fig. 1. For all studied distributions a minor mean diameter of 50μm belonged to the large proportion of droplets, so the emulsion appearance were "milky".

Table 3

Chitosan	Deacetylation degree	Conc.	η_{CH}	η_E	x
Sample 1	75 %	0.2 %	3	10	3.33
		1.0 %	174	362	2.08
		2.0 %	999	999	1.00
Sample 2	76 %	0.2 %	1	6	6.00
		1.0 %	3	98	3.27
		2.0 %	999	999	1.00
Sample 3	78 %	0.2 %	3	12	4.00
		1.0 %	38	75	1.97
		2.0 %	264	242	0.92
Sample 4	85 %	0.2 %	2	9	4.50
		1.0 %	65	105	1.62
		2.0 %	648	915	1.41
Sample 5	93 %	0.2 %	3	24	8.00
		1.0 %	16	40	2.50
		2.0 %	63	116	1.84
Sample 6	94 %	0.2 %	2	7	3.50
		1.0 %	26	48	1.85
		2.0 %	-	-	-
Sample 7	95 %	0.2 %	1	5	5.00
		1.0 %	3	8	2.67
		2.0 %	7	17	2.43

Emulsion viscosity: Continuous phase viscosity is the most important factor that set up the rheological behavior of an emulsion. The equation $\eta = \eta_o x$ expresses a direct proportion between emulsion viscosity (η) and continuous phase viscosity (η_o), "x" is a factor that represents the other properties that have influences in the emulsion viscosity. Chitosan solution and emulsion viscosity had dependence on biopolymer concentration. A relationship between the viscosity of freshly prepared emulsions and respective chitosan solution viscosity was found with no dependence on deacetylation degree.

In this work we verified that when chitosan concentration increased "x" factor decreased for all DD used. Conclusion agrees with the fact that the increment of chitosan concentration produces increment of the continuous phase viscosity (secondary emulsifier). Data that demonstrate the above mentioned are given in table 3, figure 2 and figure 3.

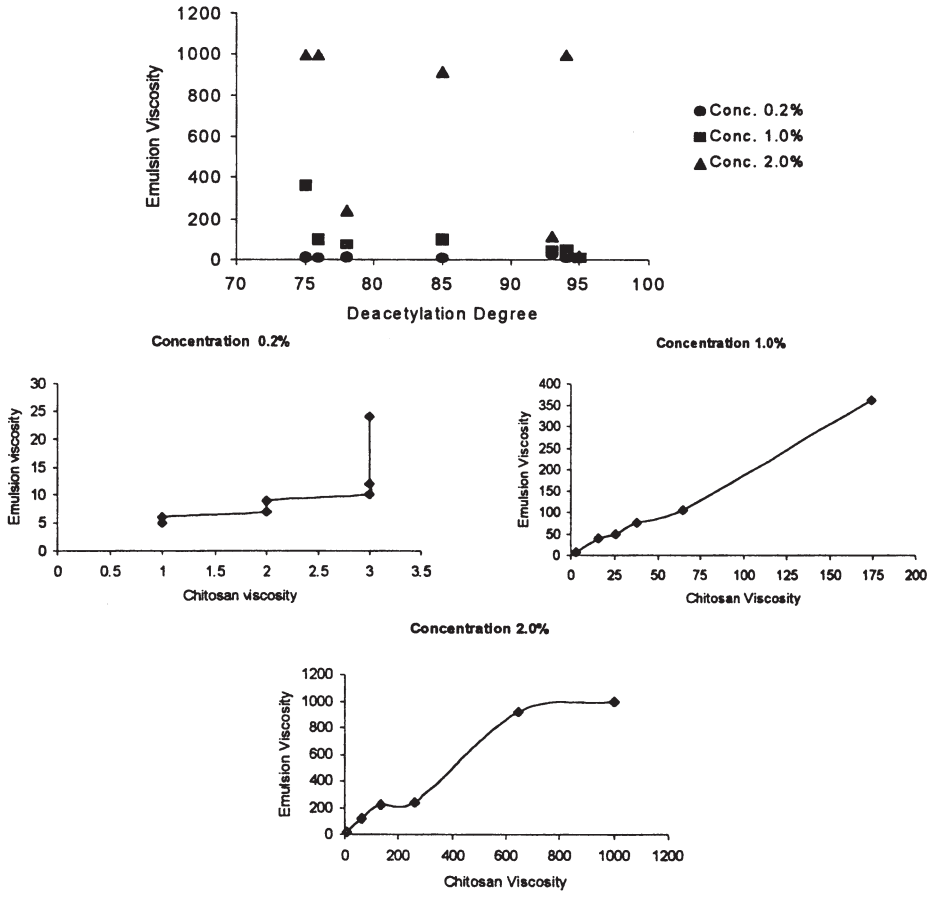

Figure 2.

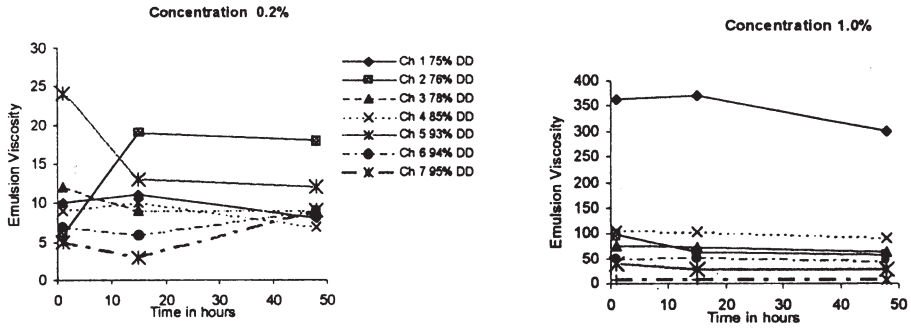

Figure 3.

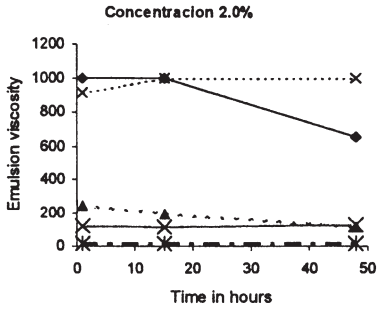

Figure 4.

As well as the preceding conclusions, the aging behavior had the same dependence on chitosan concentration and no dependence on chitosan DD. When chitosan concentration increased emulsion viscosity as function of time was more stable.

Emulsion stability: Emulsion stability depends on the chitosan concentration. After 4-6 hours we observed that when chitosan concentration was 0.2 % phases separation in dilute emulsion at the bottom of the tube (2/3) and concentrated emulsion at the top (1/3) was the same for all DD used in the experience. Emulsions prepared with chitosan 1.0 % showed that time separation was not similar for different DD but it depended on the emulsion viscosity. More concentrated emulsions (chitosan 2.0%) had good stability, except when the biopolymer viscosity was low. In that case the separation time was accelerated. There were no cases where we detected top oil separation.

Conclusion

With results obtained in this work we conclude that chitosan solutions in HCl stabilize double emulsions w/o/w and this property depends on the viscosity and concentration of them but no depends on chitosan deacetylation degree.

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Chemically modified chitinous materials: preparation and properties

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Summary
Soluble derivatives of chitin have been prepared and evaluated as precursors for the facile introduction of side chains. Partially deacetylated water-soluble chitin and 6-*O*-triphenylmethyl-chitosan are suitable for the introduction of substituents at the amino groups in aqueous solution or in organic solvents, respectively. *N*-Phthaloyl-chitosan is a superb organosoluble precursor for regioselective introduction of various branches into chitin. Fully trimethylsilylated chitin in another interesting derivative, which allows efficient chemical modifications as exemplified by triphenylmethylation and glycosylation.

Introduction

Although structurally similar, chitin and cellulose are quite different in many respects. Because of the presence of acetamide groups at C-2, chitin exhibits various unique biological and physicochemical properties [1-5]. It is considered to be a specialty biopolymer and quite important not only as an unutilized biomass resource but also as a novel type of functional polymer. However, the utilization as well as basic studies generally encounters some problems associated with the intractable nature of this amino polysaccharide.

In the process of thorough development of the high potentials of chitin, structural optimizations to meet requirements for advanced utilizations would be indispensable. Special attention has thus been paid to the chemical modifications to design sophisticated molecular environments. Practical modifications of chitin are, however, generally difficult because of the lack of solubility in appropriate solvents and multi-functionality. The reactions under heterogeneous conditions are usually accompanied by problems such as poor extents of reaction, difficulty in regioselective substitution, structural ununiformity of the products, and partial degradation due to severe reaction conditions.

To solve the problems in chemical modifications of chitin and thereby to prepare a wide variety of derivatives in efficient and, hopefully, site selective ways, it would be necessary to carry out reactions under homogeneous or almost homogeneous conditions. In this concept, a significant progress is being made in various modes of modification reactions based on appropriate precursors to prepare derivatives with well-defined structures.

Effective destruction of the crystalline structure stabilized by strong intermolecular hydrogen bonding has been confirmed to bring about solubility of this rigid polysaccharide, either in water or in organic solvents depending on the structures. We have evaluated some of these derivatives as precursors for facile and controlled modifications. Here we discuss the preparation and typical modification reactions of some soluble chitin derivatives to introduce side chains.

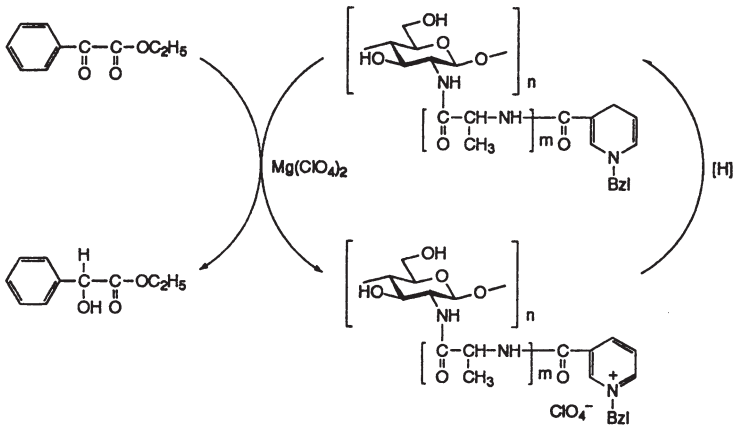
Results and Discussion

Modifications of the amino group

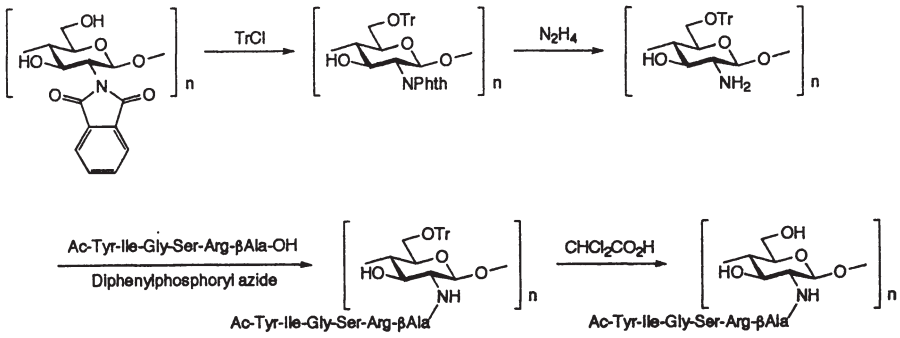
Efficient introduction of side chains may be possible at the amino group formed by deacetylation of chitin. Some substituents are thus readily introduced at C-2.

Partial deacetylation of chitin to about 50% in homogeneous alkaline solution or, alternatively, partial N-acetylation of chitosan imparts solubility in neutral water [6], which has made possible various reactions in aqueous homogeneous solution or in a highly swollen state in organic solvents. Treatment of the water-soluble chitin with N-carboxy anhydrides (NCAs) of amino acids in water/ethyl acetate results in the graft copolymerization of the NCAs to incorporate polypeptide side branches [7]. Despite the high susceptibility of the NCAs to hydrolysis, the grafting efficiency is remarkably high, 70-90%. This supports that the NCAs are attacked preferably by the amino groups even in aqueous solution under these conditions.

The grafted peptide branches have a free amino group at the terminal and can be used for further modifications. For example, the oligoalanine chains are useful as spacer arms for immobilizing active species such as dihydronicotinamide moieties leading to polymer-supported asymmetric reducing agents [8]. A similar polymeric reagent having an alanine residue as a spacer arm can also be prepared by acylation of the amino groups of chitosan [9].



In order to conduct modification reactions at the amino group efficiently in organic solvents, an organosoluble chitosan derivative is desirable. Chitosan is thus transformed into 6-*O*-triphenylmethyl-chitosan (trityl-chitosan) by *N*-phthaloylation of fully deacetylated chitosan followed by tritylation and dephthaloylation [10]. The resulting trityl-chitosan is soluble in polar organic solvents such as dimethyl sulfoxide (DMSO) and *N,N*-dimethylformamide (DMF) and allows facile *N*-substitution. Poly(ethylene glycol) side chains can be introduced by acylation of the amino groups with the corresponding carboxylic acid in DMF. Detritylation gives chitosan/PEG hybrid materials, which show interesting aggregation behavior in water [11]. Another example is the preparation of chitosan having Tyr-Ile-Gly-Ser-Arg (YIGSR), a partial peptide sequence of laminin, by coupling of trityl-chitosan with Ac-Tyr-Ile-Gly-Ser-Arg-β-Ala-OH, where β-Ala is a spacer arm, and the subsequent detritylation [12]. The resulting chitosan-YIGSR conjugate exhibits higher inhibitory effect on cancer metastasis than the parent oligopeptide YIGSR. Although direct coupling of chitosan with the oligopeptide was not successful, the N-acylated chitosan with glycine is suitable for the coupling to afford a similar conjugate [13].

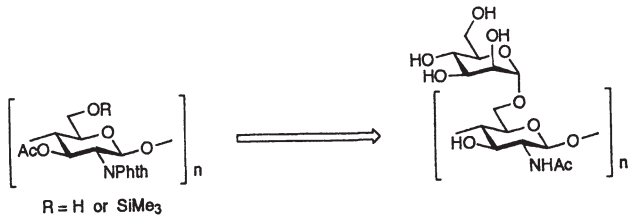


Branching at the C-6 position

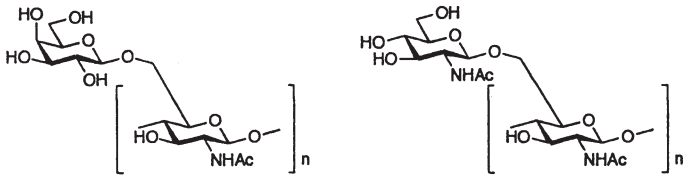
N-Phthaloyl-chitosan is prepared from chitosan and phthalic anhydride. It is soluble in polar organic solvents and, moreover, deprotected to regenerate free amino groups. The N-phthaloyl group is thus crucial for protection of the amino group as well as for solubilization in organic solvents. With this precursor, regioselective substitutions have become possible to afford various derivatives having well-defined structures [10].

Synthesis of nonnatural branched chitins is a typical example of modifications. A derivative having a free OH group at C-6 is prepared from N-phthaloyl-chitosan by a series of reactions including tritylation, acetylation at C-3, and detritylation. The product having reactive groups only at C-6 has enabled regioselective branching to prepare nonnatural branched polysaccharides having α-mannoside branches [14].

Other sugar branches introduced in a similar manner include galactose [15], maltose, and glucosamine [16]. The branched chitin having *N*-acetylglucosamine and branched chitosan having glucosamine are particularly interesting, since they have the same sugar units in both the main chains and branches.



The branched chitins are characterized by high solubility in neutral water in sharp contrast to the insoluble nature of the original chitin. The branched chitosans may be effective as water-soluble antimicrobial agents.



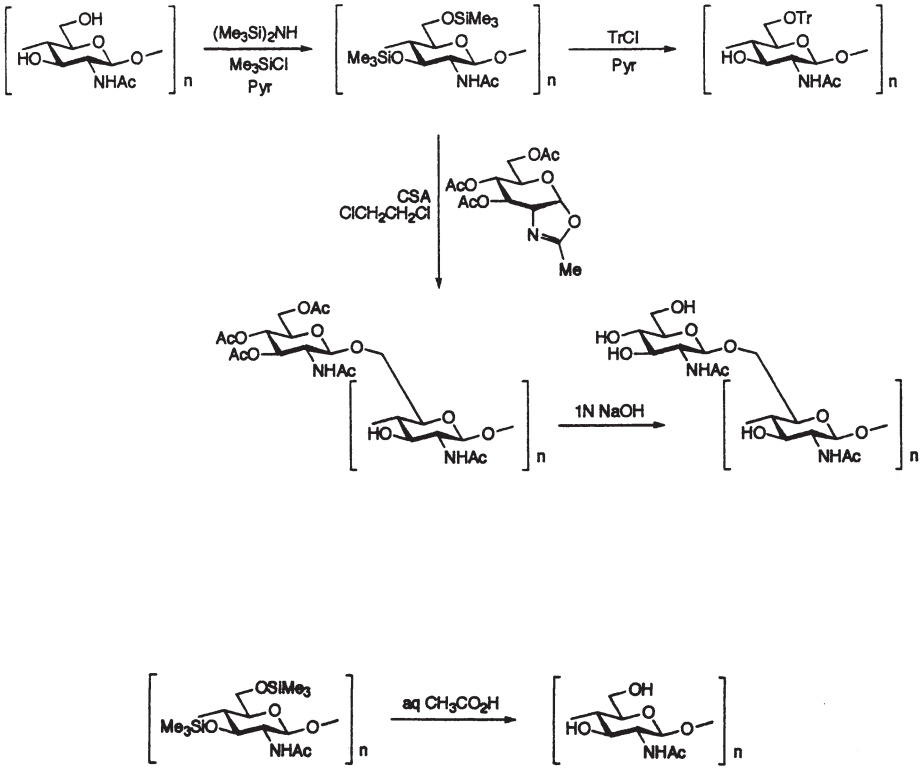
Trimethylsilylation

Silylation of chitin may be another way for solubilization, while retaining the considerable reactivity of hydroxy functionalities. The product is a novel type of derivative possibly characterized by some favorable properties: high solubility in common organic solvents, considerable reactivity, and easy regeneration of chitin structure. Silylated chitin is therefore expected to be important as an organosoluble derivative, which will be useful for further controlled manipulations as well as film casting.

The possibility of quantitative introduction of trimethylsilyl groups into chitin has been examined in detail, and full trimethylsilylation is attained with a mixture of hexamethyldisilazane and chlorotrimethylsilane. Although β-chitin is more easily silylated, α-chitin can also be fully substituted. The silylated product is soluble in acetone and pyridine.

Trimethylsilyl-chitin is now being evaluated as an organosoluble precursor for modifications under mild conditions. Besides the improved solubility in common organic solvents, it exhibits high chemical reactivity. Tritylation proceeds efficiently to give a fully tritylated product. An important modification reaction of the silylated chitin is glycosylation; when it is treated with an oxazoline synthesized from glucosamine, branching takes place smoothly [17]. This branching process based on the silylated chitin is superior in simplicity and overall yield than that with *N*-phthaloyl-chitosan described above.

Because of the solubility in acetone, silylated chitin films are prepared easily by solution casting. Furthermore, the introduced silyl group is removed under acidic conditions at room temperature. Silylated chitin films can therefore be converted into chitin films quite readily by treating with aqueous acetic acid.



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Progress on the modification of chitosan

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Summary
Progress on chitosan research and development in Poland in several areas such as modification, biotechnological aspects as well as applications in medicine, agriculture and technics is presented. At the same time the last results related to progress on chitosan modifications are discussed. The molecular characteristic changes occurred during modification, in connection with a turn of the super-molecular characteristic of chitosan structure, are shown.

Introduction

The modification of chitosan enables to produce the several types of chitosan derivatives through its chemical treatment as well as the several types of chitosan with adjusted structure through its physical-chemical treatment [1-3]. The suitable changes of structure characteristic are also occurred during the chemical modification of chitosan [1]. The modification of chitosan, both physical – chemical and chemical, produces the valuable chitosan modificates and derivatives for several applications [1-3]. This process is also useful for the creation of assumated structure of chitosan and its derivatives, especially valuable for the standarized chitosan [1].

The aim of this paper is to present progress on chitosan modification conducted out to the products with controlled molecular and super-molecular structure characteristics. At the same time the present status of the chitosan research and development in Poland related to the modification of chitosan is discussed.

Present status of chitosan R & D in Poland.

The chitosan research and development in Poland has been concentrated on the three main areas

- chitosan modification,
- chitosan biotechnology,
- chitosan applications [4].

Microcrystalline chitosan prepared by the physical – chemical modification using an aggregation technique [4-5] has been studied since several years [1,4] Dibutyrylchitin obtained on the base of chitin and butyric anhydrate as well as its modificates has been also continuously investigated [4,6]. At the same time the preparation of chitosan using the marine organism shells to obtain the product with different properties was studied [4,7].

A preparation of chitin and chitosan by the biotechnological methods using a biosynthesis with different types of fungi was carried out at the Institute of Chemical Fibres, Lodz and Technical University of Warsaw [4,8]. A great research related to the degradation of

chitosan and its derivatives, both hydrolytic and enzymatic, has been continuously carried out in several centers including the Technical University of Lodz, the Sea Fishering Institute in Gdańsk or the Institute of Chemical Fibres in Lodz [4,9-12].

A main trend on the chitosan applications has been related to three areas as medicine, agriculture and technics [4]. The woundhealing dressings, dentistry, pharmacy and antitumor agents are the most important examples of medical uses of chitosan and its derivatives studied in the different scales [4,13].

The agricultural applications of chitosan based agents are generally related to the microcrystalline chitosan and chitosan salts to be used for the plant protection and plant growth biostimulation [14-15].

The technical uses of chitosan and its derivatives comprise the waste water treatment using the microcrystalline chitosan and chitosan beads [4]. Another important studies were concerned with chitosan fibre spinning and textile treatments [4].

The chitin R & D in Poland is generally supported by the National Committee for Science and Research with cooperation of the industrial enterprises. At the same time the Polish Chitin Society is continuously organizing the Polish chitin cooperation, including yearly Workshops [4].

Preparation of chitosan modificates with controlled structure.

A preparation of the chitosan modificates with controlled structure, including molecular, super-molecular and morphological, seems to be very important for special applications exploiting their biological activity. At the same time this process enables to create the chitosan based products with standarized properties required especially for medical and agricultural uses.

The initial chitosans purchased from Vanson Co. (USA), Chemopol Co. (India) and donated by Sonat Co. (Russia) were used in these studies.

A microcrystalline chitosan in a form of gel-like dispersion and air spray dried powder by the original method was prepared [5,16]. The lyophilized chitosan salts were obtained using own elaborated method [16].

All used analytical methods were the standard techniques applied previously in this type of research [1,4-5].

Microcrystalline chitosan prepared according to the agglomeration method [5] in a form of aqueous gel-like dispersion was characterized with properties presented in Table 1.

Table 1. Some properties of microcrystalline chitosan gel-like dispersion.

Symbol of sample	Polymer content %	$\bar{M}v$ kD	DD %	WRV %	CrI* %
MKCh / I	1.93	248	77.4	1520	12.1
MKCh / II	1.58	224	68.0	1200	15.8
MKCh / V1	3.14	480	85.6	920	16.5
MKCh / 2	3.00	43	85.6	870	16.1

* - in film form

Microcrystalline chitosan powder produced by air-spray drying using its aqueous dispersion was distinguished with properties shown in Table 2.

The lyophilized chitosan salts properties are presented in Table 3. These chitosan salt powders were completely water-soluble (Tab.3).

Table 2. Some properties of microcrystalline chitosan powder*.

Symbol of sample	Polymer content %	$\bar{M}v$ kD	DD %	WRV %	CrI %
MKCh P/C	92.3	341	79.3	240	41.2
MKCh P/K	93.4	329	82.9	200	44.6
MKCh P/P	89.8	303	72.7	200	41.3
MKCh P/MK	89.2	208	70.0	210	45.0

* - air spray drying

Table 3. Some properties of chitosan salt powder*.

Symbol of sample	Chitosan salt type	Polymer content %	$\bar{M}v$ kD	DD %	CrI %
SO3 / M	lactate	88.4	287	89.2	27.8
SO3 / O	acetate	89.5	295	89.2	21.4
SO3 / HCl	hydrochloride	89.8	14	89.2	0

- lyophilized

A physical–chemical modification of initial chitosan by the agglomeration of glucosamine macromolecules occured their degradation was resulted in a preparation of microcrystalline chitosan, both gel-like dispersion and powder, with assumated molecular, super-molecular and morphological structure characteristics (Tabs. 1-2). Generally, the deacetylation degree of modificates obtained during above process was the same as for the initial chitosan. However, it is possible to prepare the microcrystalline chitosan, using different initial chitosans, with similar properties (Tab.1). This method of modification enables to prepare the microcrystalline chitosan with assumated structure and standarized properties.

The same initial chitosan (Sonat Co.) used for preparation of lyophilized chitosan salts was resulted in the powders of chitosan acetate and lactate characterized with similar average molecular weight values and different super-molecular structure characteristic (Tab.3) At the same time the chitosan hydrochloride obtained as a completely amorphous powder was usually distinguished with a low average molecular weight caused by a strong hydrolytic degradation process occurring during this salt preparation (Tab.3).

It can be concluded on the base of above results that the structure of chitosan modificates is controlled by the processing conditions and a type of modification process. These parameters are affecting on the modificates molecular, super-molecular and morphological characteristics. The use of suitable modification methods is resulting in a preparation of the products with assumated structure and standarized properties.

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The graft copolymerization of chitosan with methyl acrylate using an organohalide - manganese carbonyl coinitiator system

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Summary
This work has begun investigating the vinyl graft copolymerization of chitosan with methyl acrylate by means of an organohalide - manganese carbonyl ($Mn_2(CO)_{10}$) coinitiator system. The nature of the coinitiator system could reduce homopolymerization due to its more specific macroradical formation. $Mn_2(CO)_{10}$ can react photochemically with organohalides to yield carbon radicals upon removal of the halide substituent. A trichloroacetylated (TCA) chitosan derivative has been prepared to serve as the organohalide coinitiator. With the addition of $Mn_2(CO)_{10}$, TCA chitosan was grafted with methyl acrylate (according to IR spectroscopy) upon exposure to 436 nm light.

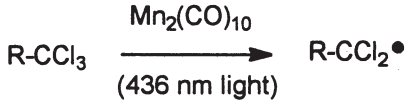
Introduction
Chitin and chitosan continue to be under utilized renewable resources. Within the United States, the seafood industry produces thousands of tons of shell fish waste annually, that has been shown to be an economical source of these polymers [1]. Chitin is essentially a homopolymer of 2-acetamido-2-deoxy-β-D-glucopyranose, although some of the glucopyranose residues are in the deacetylated form as 2-amino-2-deoxy-β-D-glucopyranose. When chitin is further deacetylated to about 50%, it becomes soluble in dilute aqueous acids and is referred to as chitosan. Thus chitosan is the N-deacetylated derivative of chitin, although the N-deacetylation is almost never complete.

Due to chitosan's ability to be dissolved in aqueous acidic solutions (chitin however is considered the more intractable derivative), various physical forms of chitosan have been produced, namely powders, films, fibers, and fibrids, [2,3] that exhibit interesting and potentially useful properties. Work has also been conducted on the chemical modification of chitosan by graft copolymerization with vinyl monomers using a wide variety of initiating systems such as the ceric ion [4,5], Fenton's reagent and variations thereof [6,7], γ-radiation [8,9], AIBN, $(NH_4)_2S_2O_8$, and H_2O_2 [10]. While all of the initiating systems referenced above are successful in producing a grafted derivative, homopolymer was an unwanted side product of the grafting reaction that had to be removed. Several of the initiator systems cited create free radicals first in solution that can subsequently abstract hydrogens from the trunk polymer, thereby creating free radicals on the polymer backbone that are capable of initiating a grafted polymer chain [6,7,10].

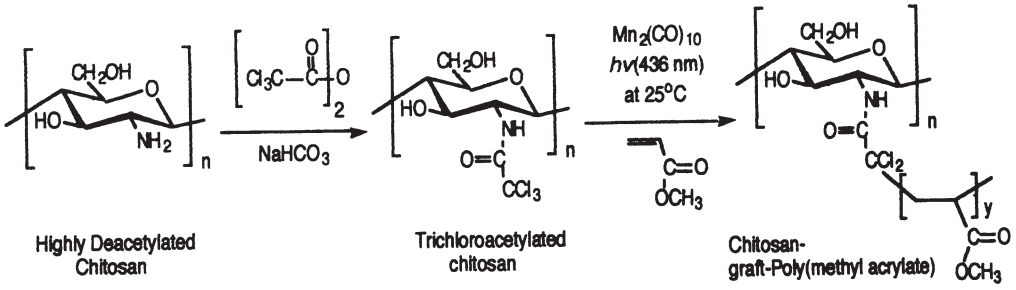
The likelihood of homopolymer production is increased with this mechanism since the first radicals are produced in solution in the presence of vinyl monomer. Grafting with γ-rays

can also lead to further homopolymerization because radicals are created not only on the trunk polymer backbone, but also in solution during irradiation of the medium.

The focus of this work is to heterogeneously graft chitosan with vinyl monomer (in particular, methyl acrylate), allowing potential modification of powders, films, and fibers, using a more specific initiation method in an attempt to reduce homopolymerization. Ceric initiation is capable of specific macroradical generation on chitosan [11], however this method generally requires aqueous acidic conditions in order to maximize yields, thus eliminating it as an initiation technique capable of maintaining a certain physical form of chitosan during the graft copolymerization. Reviews by Bamford describe different organometallic complexes which react with various organohalide sites to yield carbon based radicals capable of initiating vinyl polymerizations which do not operate under acidic conditions [12,13]. In particular, manganese carbonyl ($Mn_2(CO)_{10}$) can react with trichloro sites under photoinitiating conditions (436 nm light) producing carbon radicals upon removal of a halide substituent as shown below.



This work describes the heterogeneous graft copolymerization of chitosan powders with methyl acrylate applying the organohalide (trichloro site via trichloroacetylation of chitosan) - $Mn_2(CO)_{10}$ coinitiator system based on the sequence of reactions shown in Scheme 1.



Scheme 1. Synthetic scheme for graft copolymerization of chitosan with methyl acrylate using a trichloroacetyl-$Mn_2(CO)_{10}$ coinitiator system. O-trichloroacetylation of chitosan is not shown in the scheme for simplicity.

Materials and Methods

FTIR: All infrared spectra were obtained using a Nicolet 510P FT-IR Spectrophotometer with the following data collection parameters: Bench type: 510P, Detector: DTGS, Gain: 1, Resolution: 2.0 cm^{-1}, Scans: 64. The FTIR sample chamber was continually flushed with dry air. Samples were prepared as KBr pellets and were scanned against a blank KBr pellet / air background.

Preparation of Highly Deacetylated Chitosan: Chitosan, approximately 80-85% deacetylated, was received from Pronova Biopolymer, Inc. as a ground course, yellowish flake that was obtained from crab shells. To help remove this color, one part of chitosan flake was

combined with 10 parts of a 20%(w/w) NaOH solution. The slurry was well mixed and kept at 5°C for at least 24 hours. The mixture was then filtered, washed with distilled water until neutral to pH paper, and then allowed to air dry.

The chitosan was subsequently deacetylated by adding one part of chitosan flake to twenty parts of 50%(w/w) NaOH. The slurry, while under a continual nitrogen purge, was vigorously mixed at 120°C for two hours. Afterwards, the chitosan was filtered, washed with distilled water until neutral to pH paper, and allowed to air dry. This procedure was performed twice on the chitosan in order to obtain a high degree of deacetylation. The degree of deacetylation for the processed chitosan was determined to be approximately 96.6 ± 0.3% by means of a potentiometric titration method [1].

Trichloroacetylation of Highly Deacetylated Chitosan Powder: A 3.0 L three necked flask was charged with 4.0 g of ground (60 mesh) highly deacetylated chitosan (dried at 60°C under vacuum for over 10 hours), 40.3 g of sodium bicarbonate, 220 mL of trichloroacetic anhydride, and 500 mL of methylene chloride. The 3.0 L flask was fitted with a condenser and a half-moon stirrer, and the mixture was refluxed (43-45°C) for 24 hours. At the end of the 24 hour period, the product was filtered, placed in 2.0 L of a 1.375 M sodium bicarbonate (aq) solution, and shaken for approximately 10-20 minutes. At this point, the product was filtered, washed with distilled water until neutral to pH paper, and dried.

Methyl Acrylate Purification: Methyl acrylate was obtained from Aldrich and washed with an aqueous solution of 5% (w/w) NaOH / 20% (w/w) NaCl with subsequent washing with distilled water, followed by drying over $CaCl_2$. The washed methyl acrylate was then vacuum distilled at 160 mm Hg (37°C) taking the middle 60% as purified monomer and storing it over CaH_2 and in an argon atmosphere in a brown glass bottle in the refrigerator. The purified monomer (20 mL) was freshly vacuum distilled again just before polymerization over CaH_2 at 160 mm Hg (37°C).

Manganese Carbonyl Purification: Manganese carbonyl was obtained from Aldrich at approximately 97% purity and was sublimed under vacuum at 50°C. It was stored under Ar and refrigerated until needed.

Graft Copolymerization of Trichloroacetylated Chitosan Powder: Grafting was performed by charging a 10 mL test tube with 50 mg of trichloroacetylated chitosan powder, 15 mg of $Mn_2(CO)_{10}$, and 2 mL of methyl acrylate. The contents were stirred in the dark under an argon purge for 30 minutes before the light source (Oriel instruments light source (500 Watt Mercury Arc Lamp: Model 68810) equipped with a 436 nm interference band pass filter and dichroic mirror) was turned on at 150 W for 30 minutes while continuing to purge with argon at room temperature. At the end of the reaction time, 5 mL of 0.05 M hydroquinone in ethyl acetate was added to the flask to quench the reaction. A weighing bottle and extraction thimble (cellulose thimbles were extracted with ethyl ether overnight before use to remove natural waxes) were dried at 70°C for 3 hours under vacuum and weighed (W_1). The product was precipitated in ethyl ether, filtered through the thimble, then Soxhlet extracted for approximately 16 hours with ethyl ether to remove monomer and any manganese compounds. Once the extraction was complete, the sample was dried and weighed (W_2). The sample was next Soxhlet extracted with acetone, removing homopolymer, until a constant weight was achieved (W_3). The graft and homopolymer yields were calculated as follows:

$$\%G \text{ (on weight of chitosan)} = (((W_3-W_1)-50 \text{ mg})/50 \text{ mg}) \times 100\%$$

$$\%H \text{ (on weight of total polymer formed)} = (W_2-W_3)/(W_2-W_1-50 \text{ mg}) \times 100\%.$$

Results and Discussion

Although not shown in this paper, the FTIR spectrum of the highly deacetylated chitosan showed a strong absorbance at 1600 cm^{-1} (NH_2 deformation) and no absorbances at either 1645 cm^{-1} (C=O stretch of N-acetyl) or 1550 cm^{-1} (N-H deformation of N-acetyl). Figure 1 provides the FTIR spectrum of the trichloroacetylated (TCA) chitosan powder heterogeneously synthesized from highly deacetylated chitosan. Evidence of trichloroacetylation is shown by new absorptions at approximately 1770, 1700, 1530, 845, 820, 760, and 670 cm^{-1}. The absorption at 1770 cm^{-1} is assigned to the carbonyl stretch of the O-trichloroacetyl. Absorptions at 1700 and 1530 cm^{-1} are assigned to the carbonyl stretch and the N-H deformation of the N-trichloroacetyl, respectively. Due to the facts that C-Cl bonds generally absorb in the range of 900-600 cm^{-1}, the peaks at 845, 820, 760, and 670 cm^{-1} are assigned to the CCl_3 moiety.

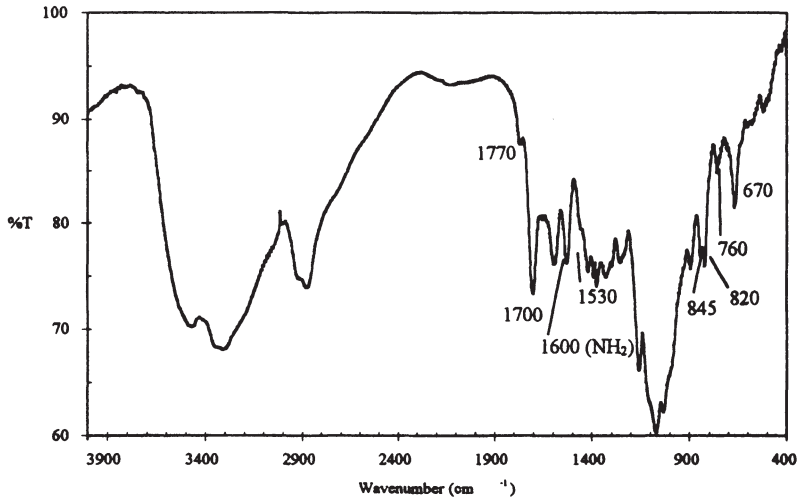


Figure 1. FTIR spectrum of trichloroacetylated (TCA) chitosan powder.

Degrees of N and O trichloroacetylation were estimated to be 0.09 and 0.03, respectively, from N and Cl elemental analysis of TCA chitosan and of a sample of TCA chitosan exposed to 0.01 M $NaOCH_3$ in methanol at room temperature for 48 hours (in order to cleave ester linkages).

Under photoinitiating conditions (436 nm, 25°C), TCA chitosan was grafted using $Mn_2(CO)_{10}$ coinitiation in bulk methyl acrylate. Large amounts of homopolymer (%H > 90%) were obtained from the product by extracting with acetone to constant weight (50% of monomer charged was homopolymerized). Evidence of grafting is observed in the FTIR spectrum shown in Figure 2 of the extracted sample due to the absorbence at 1735 cm^{-1}, characteristic of the carbonyl side group of poly(methyl acrylate). The final weight of grafted chitosan was actually 5% less than the weight of TCA chitosan charged suggesting removal of surface grafted polymer during extractions. No polymerization was observed when methyl acrylate was exposed to photoinitiating conditions in the presence of $Mn_2(CO)_{10}$ without any halide suggesting that homopolymerization resulted from chain transfer of growing grafted chains.

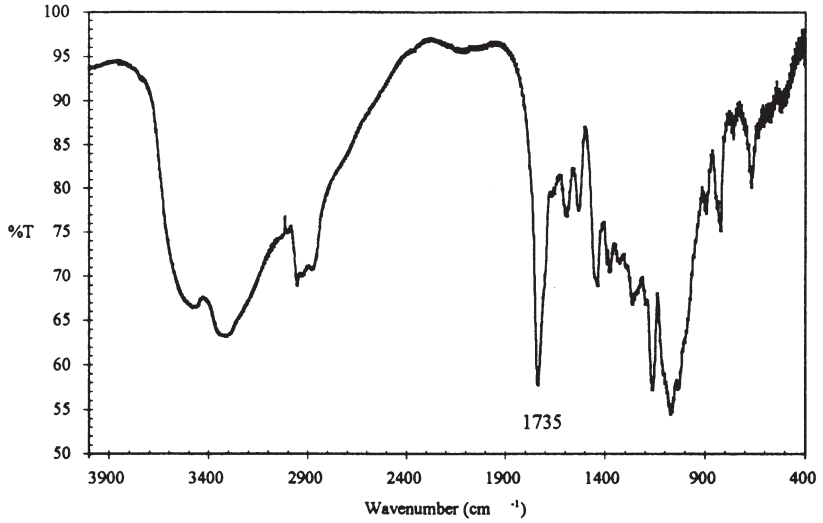


Figure 2. FTIR spectrum of TCA chitosan powder heterogeneously grafted with methyl acrylate under photoinitiating conditions (436 nm, 25°C) using $Mn_2(CO)_{10}$ coinitiation.

Conclusions

Highly deacetylated chitosan powder was heterogeneously trichloroacetylated with trichloroacetic anhydride using $NaHCO_3$ to neutralize acid by-products during the reaction. The TCA chitosan powder was heterogeneously graft copolymerized with methyl acrylate using $Mn_2(CO)_{10}$ coinitiation under photoinitiating conditions. The grafting yield is unknown due to apparent removal of surface grafted polymer during extractions. Homopolymer yields were unexpectedly high, presumably from chain transfer of growing grafted chains since the method of macroradical formation was specific to the chitosan backbone. Future work will vary the concentrations of monomer and $Mn_2(CO)_{10}$ to investigate their effects on graft and homopolymer yields.

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Grafting of 4-vinyl pyridine, maleic acid and maleic anhydride onto chitin and chitosan

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Summary
4-Vinyl pyridine (4VP) was grafted onto chitin and chitosan and maleic acid (MA) was grafted onto chitosan by redox initiation using cerium (IV) ammonium nitrate (CAN) as initiator. Grafting of maleic anhydride (MAn) onto chitosan backbone was achieved by free radical initiation using benzoyl peroxide as the initiator. The products were characterized spectrometrically in the solid state using Fourier Transform Infrared (FTIR) and C-13 CP/MAS Nuclear Magnetic Resonance (NMR) techniques.

Introduction

Grafting various monomers onto its backbone can diversify potential applications of chitin and chitosan.

There are several articles on graft copolymerization of vinyl monomers such as acrylamide [1], acrylic acid [1], 4-vinyl pyridine [2], methyl methacrylate, acrylonitrile and vinyl acetate [3] onto chitin or chitosan by using different initiation methods including (CAN), iron (II)-hydrogen peroxide (Fenton's reagent), potassium persulfate, and AIBN.

Poly (4-vinyl pyridine) (P4VP), similar to chitosan behaves as a cationic polyelectrolyte in aqueous acidic solutions and is a good metal-chelating agent. Poly (vinyl pyridines) have also been found to be promising anti-bacterial agents due to their cationic property [4]. Poly (maleic anhydride) (PMAn), on the contrary is an anionic polyelectrolyte in aqueous solution. It is a biocompatible synthetic polymer. Poly (maleic anhydride) and its derivatives have found applications in drug releasing systems. Especially the copolymers of PMAn are known to exhibit antiviral, antimicrobial and antifungal activity [5].

Possibilities to modify chitin and chitosan with 4-vinyl pyridine and maleic anhydride were investigated since the products promise to enhance metal-chelating ability and enrich biomedical and pharmaceutical applications of chitin and chitosan.

Materials and Methods

Chitin (Sigma), 4-vinyl pyridine (Aldrich), cerium ammonium nitrate (Aldrich), benzoyl peroxide and ceric ammonium nitrate (Aldrich), maleic anhydride (Sigma), acetic acid (Merck) and sodium hydroxide (Merck) were used as supplied.

Preparation of chitosan from chitin: Chitin was deacetylated to chitosan by being treated with 50% w/w NaOH solution at 109°C for 3 hours under reflux. At the end of 3 hours chitosan samples were washed with distilled water until neutral, and dried in air.

Grafting of 4VP onto Chitin by CAN Initiation: Grafting of 4VP onto chitin was carried out under nitrogen atmosphere by stirring at 50 rpm and at 70°C. 0.20g chitin was treated with 20 mL $8.68x10^{-3}$M solution of 4VP prepared in 0.15 M HNO_3 . CAN concentration in the reaction medium was $3.61x10^{-2}$ M. At the end of one-hour grafting time the product was washed with water and extracted in ethanol in soxhlet apparatus for 48 hours to remove any P4VP formed.

Grafting of 4VP onto Chitosan by CAN Initiation: 0.20g chitosan dissolved in 15 mL 1% acetic acid solution was treated with CAN and the monomer at 50°C for one hour under nitrogen atmosphere, stirring at 50 rpm. Initiator and monomer concentrations in the reaction medium were $5.10x10^{-2}$ M and $1.04x10^{-1}$ M respectively. The product was precipitated in acetone and extracted with ethanol for 48 hours to remove any P4VP formed.

Grafting of MA onto Chitosan by CAN Initiation: Grafting of MA onto chitosan was achieved by adding monomer (MAn hydrolyzed in aqueous medium to MA), and initiator to a solution of chitosan, which was prepared by dissolving 0.10-g chitosan in 10 mL 1.0 % w/w HAc solution. The initiator and monomer concentrations corresponded to $9.12x10^{-2}$ M and $2.60x10^{-1}$ M, respectively. Grafting reaction was carried out under nitrogen atmosphere and at a constant temperature of 70°C stirring at 50 rpm. After three hours, the product was precipitated with acetone and further washed and extracted with water to remove any poly (maleic acid) (PMA) formed.

Grafting of MAn onto Chitosan by Benzoyl peroxide Initiation: Grafting of maleic anhydride onto chitosan was carried out under nitrogen atmosphere at 80°C. After adding 0.200 g chitosan onto 2.00g molten maleic anhydride 0.044g BPO was added. Reaction was carried out at constant temperature for two hours stirring at 50 rpm. Grafting was stopped and 100 mL toluene was added to the reaction medium. The product was then filtered and the product was extracted with DMF in a soxhlet apparatus for 48 hours to get rid of any PMAn formed.

Characterization of the products:

Determination of degree of deacetylation of chitosan: The degree of deacetylation of chitosan was found to be 60 % when hydrogen bromide salt of chitosan was titrated with 0.1M NaOH.

Determination of Molar Mass of Chitosan: Chitosan was characterized by dilute solution viscometry at 30°C in 0.1M acetic acid / 0.2M sodium acetate buffer solution. The equation given below [6] was used to calculate the viscosity average molecular weight of the sample prepared:

$[\eta] = 1.04x10^{-4} M^{1.12} (ml/g)$. $\overline{M}_v$ was calculated to be 1.24 x 10^6 g/mol.

Fourier Transform Infrared Spectrophotometry (FTIR): The samples were analyzed by FTIR spectrophotometry using a Mattson 1000 FTIR Spectrometer.

C-13 Nuclear Magnetic Resonance (NMR) Spectrometry: The samples were studied in the solid state by the ^{13}C *CP/MAS* technique at a spinning rate of 8 kHz, repetition time 3 seconds, contact time 1 millisecond and number of scans 15000. Line integrals were normalized at C1 signal of chitosan at 105 ppm.

Results and Discussion

Grafting of 4VP onto Chitin and Chitosan:

Infrared Analysis: FTIR spectra of chitin, P4VP and grafted chitin are shown in Figure 1 (a), (b) and (c) respectively. Characteristic absorption bands of chitin such as the pyranose

ring absorption band around 1060 cm^{-1} are clearly observable in the grafted product. Aromatic C-H stretching band of 4VP is also observable at 820 cm^{-1}. Additionally, it can be observed that the C-N stretching band at around 1380 cm^{-1} in chitin appears in an enhanced form in the grafted product, indicating that the small number of amine groups present on the chitin backbone should be preferred grafting sites.

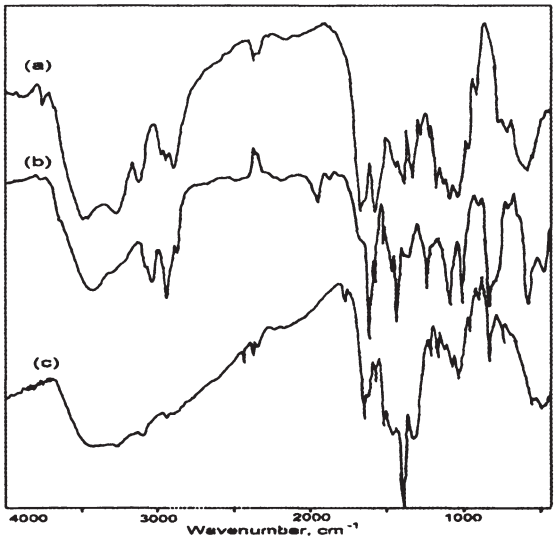

Figure 1. FTIR Spectra of (a) Chitin, (b) P4VP (c) Grafted Chitin

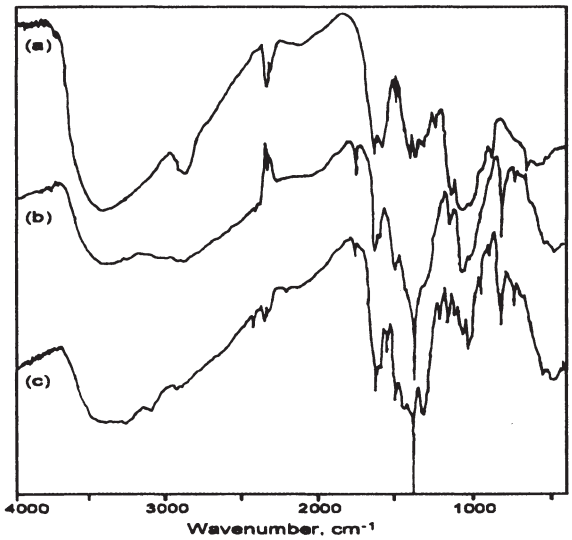

Figure 2. FTIR Spectra of (a) Chitosan, (b) Grafted Chitosan, (c) Grafted Chitin

FTIR spectra of chitosan, grafted chitosan and grafted chitin are given in Figure 2 (a), (b) and (c) respectively. It is possible to make a similar analysis on grafted chitosan as given

above for grafted chitin. Characteristic peaks mentioned above are observable in Figure 2 as well supplying evidence for grafting of 4VP onto chitosan. Still the amine groups seem to be the preferred sites of grafting. Reaction of amine groups on chitin/chitosan backbone by the vinyl group of 4VP through Ce(IV) initiation results in formation of new C-N bonds.

Grafting of MA onto Chitosan:

Infra-Red Analysis: Figure 3 (a) and (b) show FTIR spectra of chitosan and maleylated chitosan. Amide I band of chitosan at 1667 cm^{-1} appears only as a shoulder in maleylated chitosan. The peaks that appear due to the C – O stretching of ether linkages on chitosan backbone are also observable at 1050 – 1060 cm^{-1}. However, the peak at 1060 cm^{-1} in the product is much sharper. Incorporation of maleic acid into the structure of chitosan is indicated by the absorption at 1523 cm^{-1} and 1340 cm^{-1} interpreted as stretching of olefinic carbons bonded to the carbonyl group and by the peak which appears at 940 cm^{-1} as a result of the C–H bending vibrations of olefinic carbons of maleic acid.

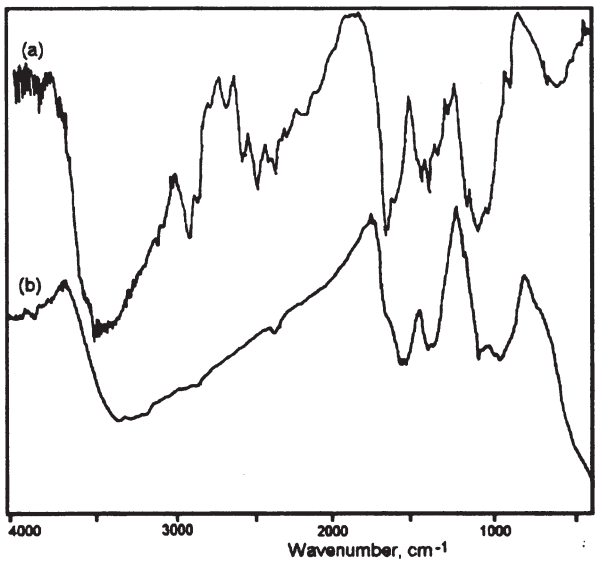

Figure 3. FTIR Spectra of (a) Chitosan, (b) Grafted Chitosan.

^{13}C CP/MAS Analysis: Figure 4(a) shows ^{13}C CP/MAS spectrum of chitosan. Carbonyl carbon and methyl carbons of N-acetyl group appear at 174 ppm and 24 ppm respectively. The peaks at 106, 84, 76, 60 and 56 ppm belong to C-1, C-4, C-5 and C-3, C-6 and C-2 carbons of the pyranose ring of chitosan respectively.

Figure 4 (b) belongs to the grafted product. It shows that maleylation takes place in 3 hours at the expense of acetyl groups since the area of the peak at 24 ppm belonging to methyl groups of N-acetyl groups decreases. However, the area of the peak at 174-ppm belonging to the carbonyl groups increases significantly. Additionally a broad peak at 136 ppm belonging to C=C indicating incorporation of maleic acid into chitosan appears. In contrast to grafting of 4VP onto chitosan, MA seems to be incorparated onto chitosan backbone through a reaction at amide nitrogens but not the amine groups.

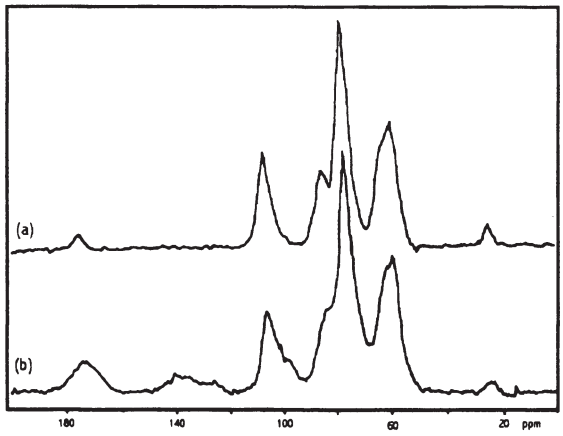

Figure 4. ^{13}C CP/MAS of (a) Chitosan, (b) Grafted Chitosan

Grafting by Benzoyl Peroxide Initiation: In Figure 5 (a), (b) and (c) FTIR spectra of chitosan, chitosan grafted with maleic anhydride and PMAn are given. In addition to characteristic bands of chitosan, olefinic carbon stretching at 1562 cm^{-1} and C-H bending vibrations of the olefinic carbons at 890 cm^{-1} due to the presence of maleic anhydride unit can be identified.

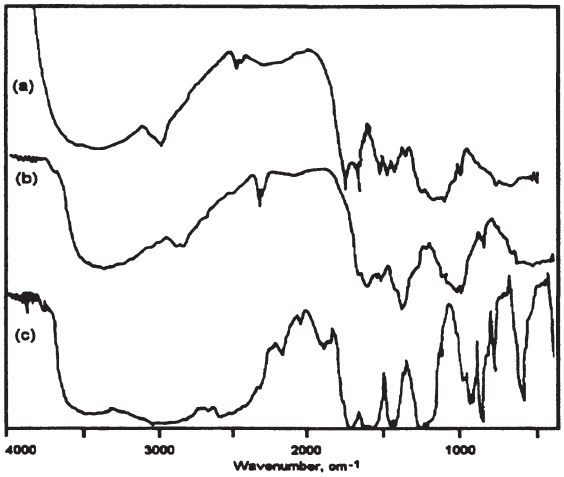

Figure 5. FTIR Spectra of (a) Chitosan, (b) Grafted Chitosan,(c) PMAn

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Peptide synthesis on chitosan/chitin

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Summary
The approach of using chitosan/chitin as a support for solid-phase peptide synthesis in batch strategy is described and illustrated by production of three peptides using different cleavable linkers: 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy-acetic acid; 4-hydroxy-methyl-3-methoxyphenoxy-acetic acid; 4-methylene-3-methoxy-phenol. Those peptides were designed as putative Ras protein farnesyl transferase inhibitors: Fmoc-Cys-Aib-Aib-Met-NH_2; Fmoc-Cys-Aib-Aib-Met-OH; Fmoc-Cys-Val-Gly-Met-NH_2. This work shows that chitosan/chitin in its derivatized form can be utilized as a support for peptide synthesis.

Introduction
Chitin, known to be complexed or covalently bound to proteins, rarely occurs alone in nature. This property may be attributed to the fact that not all the amino groups of the majority of chitins are *N*-acetylated. In the present study, we wanted to use those amino groups in more deacetylated forms of chitin as attachment sites for synthesis purpose. The Merrifield resin, chloromethylated styrene-divinylbenzene copolymer, is a commonly used support for solid-phase peptide synthesis. Numerous other materials, including cross-linked polyamides, polyacrylic resin and various polyglycol-based materials, have been also used in solid-phase synthesis as supports. These materials were developed in response to the need for supports with different physical and chemical properties. The hydrophilic support would be superior for synthesis of certain peptide sequences. In this group, naturally occurring polymers like cellulose with proper derivatization have been used as a support. The use of chitin as a support for solid-phase peptide synthesis as well as serving as an immunological carrier at the same time [1] has been described. Peptide synthesis on chitin in a continuous-flow synthesizer was recently reported [2]. Chitosan, a deacetylated form of chitin, contains free amino groups, which could be used as sites for peptide synthesis. To derivatize chitosan with cleavable linkers we used "Rink Amide" linker, linker H, vanillin (Fig.1) and they are accordingly: 4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy-acetic acid; 4-hydroxymethyl-3-methoxyphenoxy-acetic acid; 4-hydroxy-3-methoxy-benzaldehyde. Chitosan used for peptide synthesis should be brought to soft form in order to expose its surface to interface reactions. Peptide synthesis on chitosan/chitin –linker complex made in Fmoc strategy gives yield and quality of the crude peptides comparable to commonly used supports. In this work, we demonstrate the use of chitosan/chitin in peptide synthesis, using three peptides as examples. Those peptides were expected to be Ras protein farnesyl transferase inhibitors: Fmoc-Cys-Aib-Aib-Met-NH_2; Fmoc-Cys-Aib-Aib-Met-OH;

Fmoc-Cys-Val-Gly-Met-NH_2. This work shows that chitosan/chitin in its derivatized form can be utilized as a support for peptide synthesis.

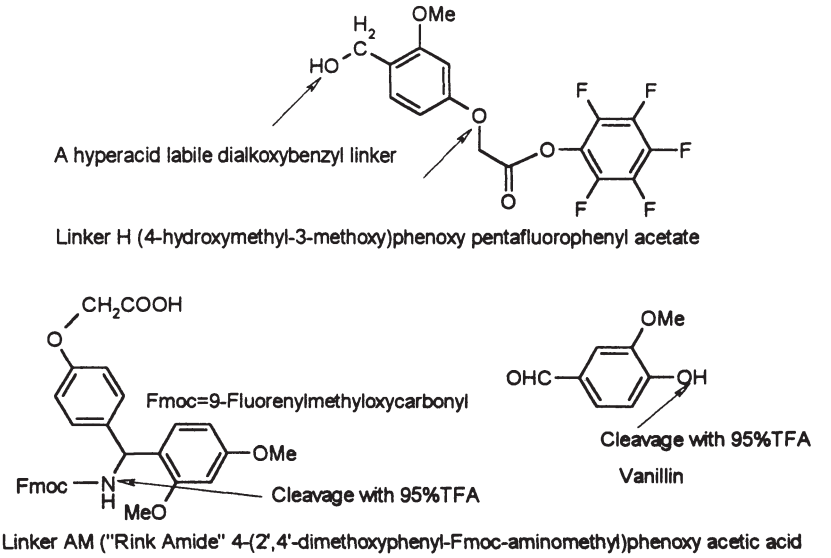


Figure 1. Structures of linkers used for derivatization of chitosan.

Materials and Methods

Chemicals. Chitosan, at ~80% deacetylation degree from squid was supplied by Sea Fisheries Institute, Gdynia, Poland. The crude chitosan was dissolved in 1N acetic acid and a viscous solution freeze-dried to give a white, very soft material. Linker H was purchased from Millipore, Mississauga, ON. Rink-type linker was purchased from Bachem Biosience Inc., King of Prussia, PA. Vanillin, DEA, NMM were purchased from Aldrich Chemical Comp.Inc., Milwaukee, WI. TBTU, HOBt were from Novabiochem, La Jolla, CA. Fmoc amino acids were purchased from Chem-Impex International, Wood Dale, IL. Solvents were from Anachemia, Montréal, QC.

Chitosan derivatization and peptide synthesis. Chitosan dissolved in 1N acetic acid and freeze-dried to give a white soft material is functionalized with cleavable linkers. These linkers allow to couple the first amino acid, and subsequently to build the whole peptide sequence on it. The Rink linker was attached (Fig.2) to chitosan acetate in *N*-methyl-2-pyrrolidinone (NMP) with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetra-methyluronium tetrafluoroborate (TBTU) in presence of *N*-hydroxybenzotriazol (HOBt) and adjusted to pH~9 with *N*-methyl morpholine (NMM) in a manually controlled peptide synthesizer (Applied Biosystems model 430A).

The uncoupled amino groups were capped to chitin form with 20% acetic anhydride in 30% diisopropylethylamine (DEA) in methylene chloride. Fmoc protecting groups were cleaved with 50% piperidine in NMP. The coupling of amino acids on this support was performed with TBTU/HOBt/NMM activation in acetonitrile and completion of the reaction was controlled by Kaiser test.

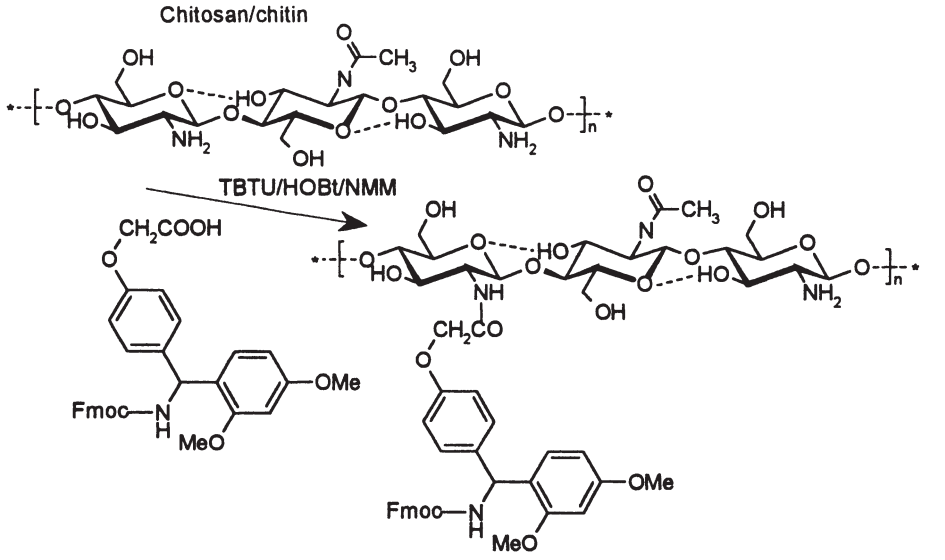


Figure 2. Coupling of Rink amide type linker for *C*-terminal peptide amides.

The linker H was coupled (Fig.3) to chitosan acetate in NMP as a pentafluorophenyl active ester after adjusting pH to 8 with NMM and shaking over night in batch peptide synthesizer (Applied Biosystems model 430A).

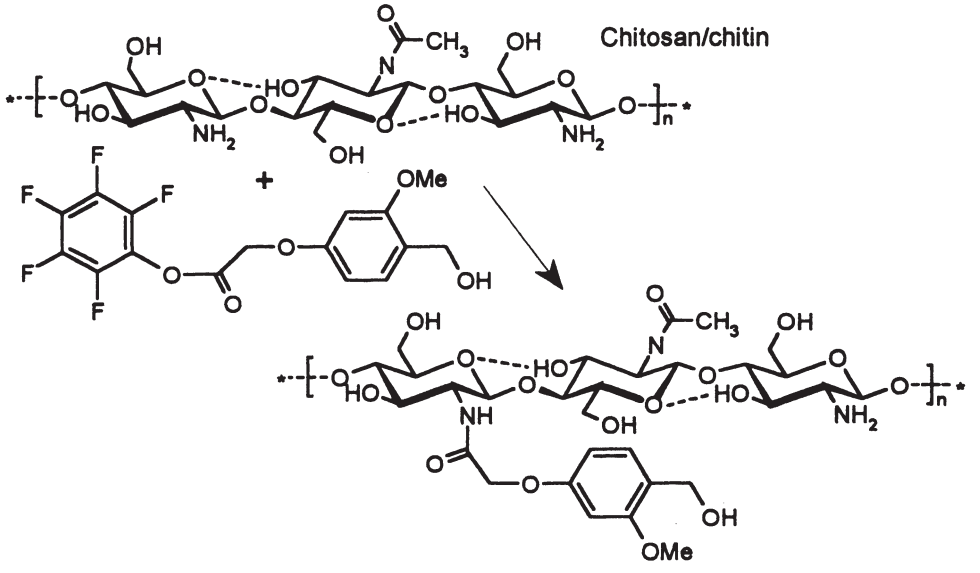


Figure 3. Coupling of active ester linker H for *C*-terminal peptide acids.

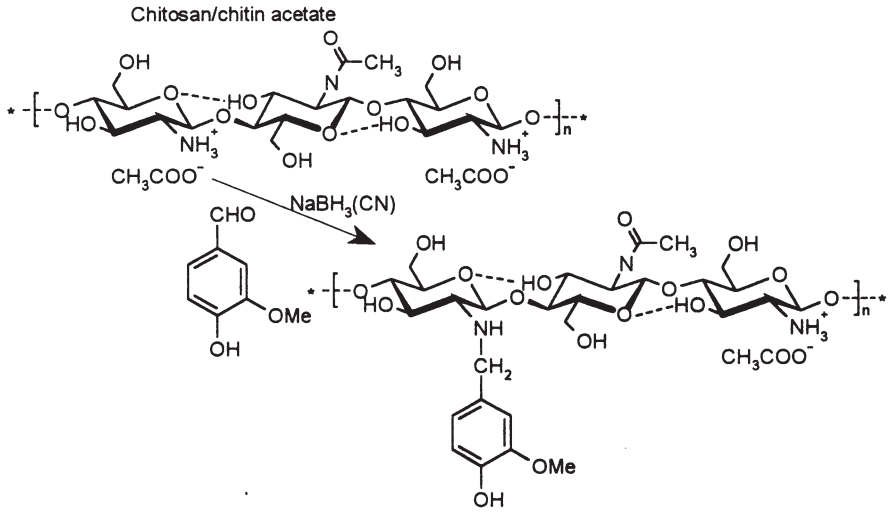
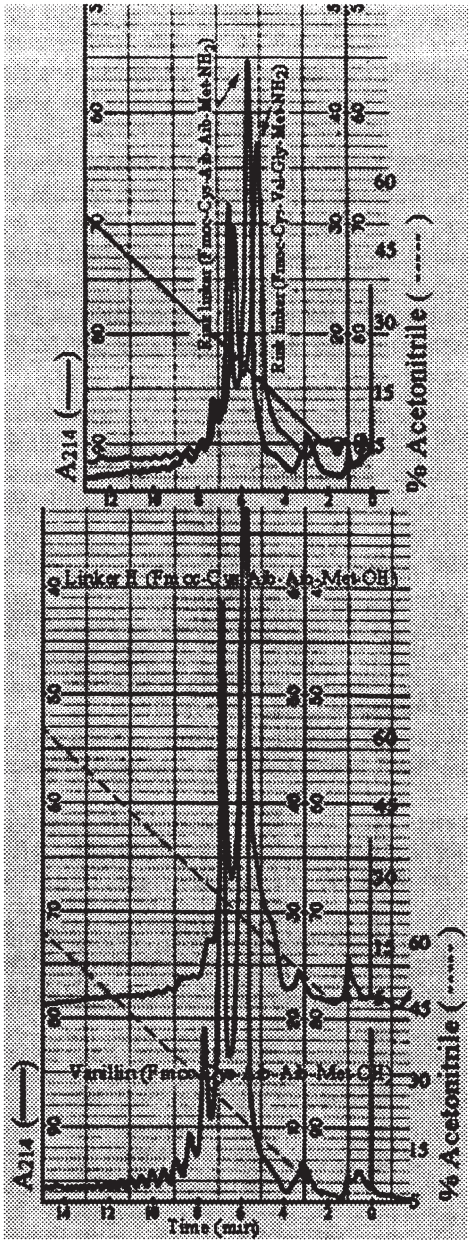


Figure 4. Coupling of vanillin linker for *C*-terminal peptide acids.

Coupling of the peptide sequence, on this chitosan/chitin linker complex was performed as in the Rink linker example. Derivatization of chitosan [3] with vanillin (Fig.4) was formed by reductive *N*-benzylation of chitosan acetate with the aldehyde group of vanillin (3.0 equiv.) in presence of $NaBH_3(CN)$ (10 equiv.) [4] in DMF (25mL) containing 1% acetic acid at ambient temperature for1h. Coupling of the peptide sequence on this chitosan/chitin linker complex was performed as in the Rink linker example. All synthesized peptides were left with Fmoc protecting groups on the *N*-terminus. Peptides were cleaved from the chitin complex (0.25g) by shaking with 20mL 95%TFA for 0.5h and filtered. The filtrate was dropped to methyl-*t*-butylether. Normally, peptides are expected to precipitate in ether but some more hydrophobic peptides and still protected with a bulky group like Fmoc did not precipitate. Ether and acid were therefore evaporated and the peptides were suspended in water and freeze-dried. Crude peptides were purified on reverse phase HPLC. Peptide identities were verified by mass spectrometry.

Results and Discussion

Results. Rink amide, linker H and vanillin can be used to functionalize chitosan/chitin for peptide synthesis. Due to their chemical nature of attachment sites (a carboxyl group, an active ester and an aldehyde), suitable ways of anchoring were applied. Coupling of the linkers was performed with 4-fold excess of activated linker form to saturate all possible sites for the reaction. Coupling of the Fmoc-protected amino acids was accomplished with 2-fold excess of its TBTU/HOBt/NMM activated form. As a solvent for coupling of amino acids, acetonitrile proved to be the best with his hydrophilic support and very hydrophobic amino acids sequence. The acylation rate observed with the progress of a Kaiser test was better in acetonitrile than NMP or methylene chloride. Possibly the use of DMSO at certain degree of chitosan substitution with linkers would further improve the coupling reaction rate. All three linkers allowed for synthesis produced desired tetrapeptides (Fig.5.) of a relatively good



quality. The electrospray mass spectrometry done on synthesized peptides confirmed their identity. The yield of peptide cleaved from chitosan/chitin support varied depending of the linker. In the case of Rink amide linker 250 mg of starting support yielded ~20mg of tetrapeptides. Linker H gave ~5 mg of the peptide and about the same amount generated with the vanillin linker.

Discussion. Chitosan can be derivatized easily with cleavable linkers and then used successfully as a support for peptide synthesis using Fmoc strategy. Three different linkers were used to derivatize chitosan. Rink amide linker, commonly used in commercially available supports for Fmoc peptide synthesis, is used to make *C*-terminal amide peptides. Fmoc protected form of this linker allowed to acetylate the remaining free amino groups of chitosan to exclude its interference in amino acid coupling at the following steps. We assume that coupling to hydroxyl groups in presence of readily an available amino group of chitin does not take place. The yield of synthesis from this linker is acceptable compared to other supports used in Fmoc approach.

It is possible that the yield of these syntheses would be improved by the use of higher temperatures. The peptides obtained in this procedure appeared (Fig.5.) basically in two peaks where the second one could still be the peptide complexed with linker. From theretention time of synthesized peptides, the sequence Fmoc-Cys-Aib-Aib-Met-NH_2 seems to be the most hydrophobic one, a feature expected to be important in penetration of cell membrane necessary for the inhibitory activity of those analogues. Linker H, designed for peptides with *C*-terminal acids, is very sensitive to acidic conditions, and it would allow to split peptides from their support while

Figure 5. HPLC profile of crude peptides cleaved from chitosan/chitin-linker complex. Peptides were eluted from a 15× 0.46cm Hamilton PRP3 column, using from 2 min. a linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O. The location of the profiles is vanillin, linker H and Rink linker one on another from the bottom to the top.

they are still protected on side chains. This linker gave a very pure peptide (Fig.5.) but not with a satisfactory yield. This yield failure might be the result of peptide loss due to hyperactivity of this linker or of the chitosan form used in this case as non-lyophilized acetate material. Vanillin used to form a deactivated to phenolic type of linkage also gave low yield of synthesized peptide. This would indicate that deactivation of phenolic ester by *o*-methoxy deactivation may not be sufficient. Furthermore during synthesis, some of the peptide may have got cleaved, although the product obtained this way was quite clean. The properties of chitin are similar to those of cellulose. Chitosan, unlike cellulose, does not have to be derivatized before attachment of the linker. Synthesized peptide can be readily separated from support by filtration, unlike peptide isolation procedure applied in beaded cellulose [5] case. Chitosan is readily available, cheap, and is easily coupled to a linker, then reacetylated to give linker-chitin support ready to use for peptide synthesis. This peptide synthesis procedure could be applied in standard batch synthesizers commonly used.

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Graft copolymerization of methyl methacrylate onto mercapto-chitin

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Summary
Graft copolymerization behavior of methyl methacrylate onto mercapto-chitin and some properties of the resulting hybrid materials have been examined. Mercapto-chitin was subjected to the reaction with methyl methacrylate in dimethyl sulfoxide. The grafting percentage increased with an increase in the amount of methyl methacrylate and reached 1300%. The ester groups in the side chains could be hydrolyzed with aqueous sodium hydroxide in dimethyl sulfoxide, and about 80% of the ester groups were converted into carboxylate groups under appropriate conditions. The hydrolyzed product was subsequently treated with acetic anhydride to generate carboxyl groups and to acetylate the free amino groups possibly produced during the hydrolysis process. The resulting graft copolymers showed high affinity for solvents. They were also characterized by unique properties including hygroscopic nature, biodegradability, and thermal properties.

Introduction

Chitin has three functional groups in the repeating unit and is thus expected to have high potential for diversifying the molecular structure by chemical modifications. There may be a wide variety of reactions that would chemically modify this biomass polymer, but of possible modifications, graft copolymerization is highly important. It would enable sophisticated molecular designs to afford novel types of tailored hybrid materials composed of the natural polysaccharide and synthetic polymers. The properties of the resulting graft copolymers may be widely controlled by the characteristics of the side chains including molecular structure, length, and number.

Some initiation methods for graft copolymerization of vinyl monomers onto chitin have been reported: cerium(IV) [1,2], tributylborane [3], γ-ray [4], and UV [5]. The reactions, however, have to be conducted under heterogeneous conditions owing to the insoluble nature of chitin. This sometimes causes poor reproducibility as well as structural ambiguity.

Efficient and controlled graft copolymerizations have become possible with some soluble derivatives such as iodo-chitin [6], tosyl-chitin [7], and partially deacetylated chitin [8-11]. Though insoluble, mercapto-chitin has proved to be a suitable radical initiator for styrene to introduce side chains at the labile mercapto groups [12]. Here we report the graft copolymerization behavior of methyl methacrylate onto mercapto-chitin and some physicochemical properties of the resulting hybrid materials.

Experimental

Preparation of mercapto-chitin: Mercapto-chitin was prepared from tosyl-chitin according to the procedure reported previously [13].

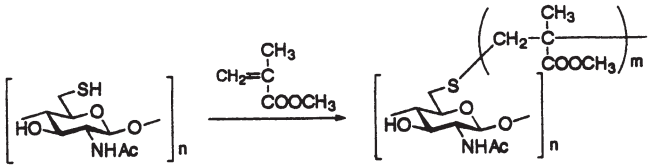
Graft copolymerization: A suspension of mercapto-chitin in dimethyl sulfoxide (DMSO) was stirred at room temperature for 3 h, and methyl methacrylate purified by distillation was added. The mixture was stirred at 80°C for 48 h and poured into methanol. The product was washed thoroughly with acetone and dried to give a graft copolymer as a white powdery material.

Hydrolysis of side-chain esters: A graft copolymer swollen in DMSO was treated with an aqueous alkali solution, and the mixture was poured into water. The resulting solution was dialyzed with water, concentrated, and freeze-dried to give a white solid.

Results and Discussion

Mercapto-chitin was prepared by tosylation of chitin followed by thioacetylation and S-deacetylation. The degree of substitution (ds) could be determined by either titration (Ellman method) or elemental analysis. The degree of substitution of mercapto-chitin used in this study was 0.65-0.70.

The SH groups of mercapto-chitin are expected to dissociate easily to generate free radicals, which will initiate polymerization of vinyl monomers by a radical mechanism. As a typical monomer, methyl methacrylate (MMA) was chosen, and the graft copolymerization onto mercapto-chitin was examined to introduce poly(methyl methacrylate) (PMMA) side chains (Scheme 1). The reaction proceeded efficiently in DMSO, and the resulting graft copolymer was isolated in methanol and acetone. Some typical results are summarized in Table 1. As evident in the table, the grafting percentage, a ratio of the weight of grafted side chains to that of main chains, increased with increasing amount of the monomer.



Scheme 1

The resulting graft copolymers, chitin-*graft*-PMMA, contained ester groups in the side chains, and thus alkaline hydrolysis was examined (Scheme 2). The ester group was quite resistive against hydrolysis, and no reaction occurred under ordinary conditions in water or aqueous methanol. However, when the graft copolymer was treated with an aqueous alkali/DMSO, the ester was transformed into the sodium carboxylate. As listed in Table 2, the extent of hydrolysis became high under appropriate conditions.

The hydrolyzed product was then treated with acetic anhydride in methanol to convert the sodium carboxylate group into the carboxyl group and to acetylate the free amino groups possibly formed by deacetylation during the alkaline hydrolysis of the side chains (Scheme 2).

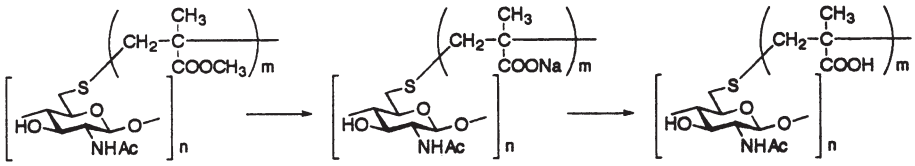
The resulting graft copolymers showed high affinity for solvents owing to the introduction of the side chains. The original chitin-*graft*-PMMA swelled highly in chloroform and DMSO. The hydrolyzed graft copolymer having carboxylate groups was readily soluble in methanol and water. On treatment of the carboxylate copolymer with acetic anhydride, the

solubility behavior changed markedly; the resulting carboxyl copolymer was soluble in polar organic solvents such as DMSO.

Table 1. Graft copolymerization of methyl methacrylate onto mercapto-chitin[a]

SH-Chitin (mg)[b]	MMA / Pyranose[c]	Yield (mg)	Grafting (%)[d]
100	50	230	250
49	70	329	990
48	100	372	1390

[a]In DMSO at 80°C for 48 h.
[b]ds 0.65-0.70.
[c]Molar ratio.
[d]Grafting (%) = (wt of branches / wt of main chains) × 100.



Scheme 2

Table 2. Hydrolysis of chitin-*graft*-poly(methyl methacrylate)[a]

Alkali/Solvent[b]	Temperature (°C)	Time (h)	Conversion (%)[c]
NaOHaq (1%)/DMSO	r. t.	24	—
NaOHaq (1%)/DMSO	50	24	61
NaOHaq (5%)/DMSO	50	24	87

[a]Grafting, 990%.
[b]Aqueous alkali solution/organic solvent = 1/9 by volume.
[c]Determined with a calibration curve based on IR spectroscopy.

Hygroscopic characteristics of the graft copolymers were then elucidated. After drying, the samples were kept under 93% relative humidity, and the weight increases were followed. The results are listed in Table 3. As anticipated, the moisture absorption ability decreased by graft copolymerization due to the hydrophobic nature of the introduced side chains. On hydrolysis, however, the hygroscopicity increased drastically. The carboxyl copolymer exhibited a hygroscopicity between those of chitin and chitin-*graft*-PMMA.

Influence of the introduction of PMMA side chains into chitin on the biodegradability was evaluated in terms of lysozyme susceptibility. Chitin is degraded with lysozyme, but chitin-*graft*-PMMA proved to be more susceptible to the enzyme. This may be reasonably interpreted by the loose arrangement of the molecules as a result of the introduction of side chains [13].

Table 3. Moisture absorption of chitin and the graft copolymers.

Sample	Grafting (%)	Weight increase[a] (%)
Chitin	0	17
Chitin-*g*-PMMA	580	8
Hydrolyzed copolymer[b]	1000	66

[a]After 8 days under 93% relative humidity.
[b]Carboxylate form. Hydrolysis conversion, 55%.

Differential scanning calorimetry measurement revealed that chitin-*graft*-PMMA showed an inflection point at around 120°C, indicating a glass transition temperature due to the PMMA chains.

Acknowledgments
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Thermal depolymerization of chitosan salts

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Summary

In this study, thermal degradation of chitosan was examined. The kinetics of bond cleavage were examined for two water soluble salts, chitosan chloride and chitosan glutamate, in order to determine if the counter ion would affect the rate of degradation. Chitosan salts were thermally degraded at 60, 80, 105 and 120 °C. The rate of degradation increased rapidly with increasing temperature and the activation energy for chitosan glutamate and chitosan chloride was found to be 99 and 112 kJ/mol, respectively. The degradation of chitosan was followed by viscometry, the plots of viscosity, η, versus time were nonlinear and in agreement with the theory of random cleavage of the glycosidic linkages. Results from SEC-MALLS showed decreasing molecular weight with increasing time and a polydispersity index close to two, also indicating that degradation of chitosan chloride and glutamate salts is a random process.

Introduction

The aim of the study was to find a reproducible process for degradation of chitosan in solid form. We wanted to investigate if a relationship could be established between the rate of degradation of chitosan salt in the solid state and the resulting viscosity of a solution made from the thermally degraded material. These data would allow us to assess the thermal stability of the glycosidic bond in chitosan. By calculating the activation energy necessary to break the glycosidic bonds, effects of the counter ion, if any, on thermal degradation would be apparent. In addition, information regarding the stability of chitosan salts at various storage temperatures would be obtained.

Degradation of polysaccharides occurs via cleavage of the glycosidic bonds. Controlled depolymerization of polysaccharides is useful in order to control properties like viscosity, solubility and biological activity.

Materials and Methods

Materials

The physico-chemical parameters of PROTASAN™ chloride and glutamate salts used in the study are given in Table 1. These chitosan salts were manufactured by Pronova Biomedical (Oslo, Norway).

Table 1: Specifications of PROTASAN™ chloride and glutamate salts used.

Parameter	PROTASAN CL	PROTASAN G
Average molecular weight ($\overline{M}_w$)	330,000 g/mol	420,000 g/mol
Apparent viscosity (1% w/w solution)	135 mPa•s	84 mPa•s
Intrinsic viscosity: salt (calculated as chitosan base)	8.0 dl/g (9.2 dl/g)	6.2 dl/g (11.3 dl/g)
Degree of Deacetylation (%DA)	84 %	83 %

Viscometry

The apparent viscosity of 1% (w/w) chitosan solutions were measured at 20 °C and 20 rpm using a Brookfield Digital Rheometer, Type RV DV-II/III. The intrinsic viscosity of chitosan solutions were determined at 20 °C using an Ubbelohde capillary viscometer (Schott Geräte AVS 360) in 0.02 M sodium acetate/ acetic acid buffer containing 0.1 M sodium chloride, pH 4.5.

Size Exclusion Chromatography -Multiple Angle Laser Light Scattering (SEC-MALLS)

The average molecular weight of the chitosan samples was determined by size exclusion chromatography using 3 TSK gel columns coupled in series (TSK PW_{XL} 6000, TSK PW_{XL} 5000 and TSK PW_{XL} 3000). The columns were eluted with 0.2 M ammonium acetate, pH = 4.5 at a flow rate of 0.8 ml/min. Polymer peaks were detected using a Waters 410 refractive index detector. Molecular weight was determined using a Wyatt DAWN multiple angle laser light scattering detector running ASTRA software.

Degradation kinetics- decrease in molecular weight and viscosity

Depolymerization is assumed to follow first order kinetics with respect to the number of cleavable bonds (n). Therefore, $-dn/dt = kn$, where k = the pseudo first order rate constant which is dependent upon the temperature and pH but not the polymer concentration. The integrated form becomes: $\ln(n_0-n) = -k(t-t_0)$. The fraction of cleaved bonds, α, equals $(n_0-n)/n_0$. The number average of residues per chain, $\overline{DP}_n$, equals $1/\alpha$. At low values of α one obtains:

$$(\alpha-\alpha_0) = k(t-t_0) \qquad (1)$$

$$1/\overline{DP}_n - 1/\overline{DP}_{n,0} = k(t-t_0) \qquad (2)$$

Since DP is proportional to the molecular weight ($M = M_0 \cdot DP$), a well-known relationship for number average molecular weight ($\overline{M}_n$) is obtained: $1/\overline{M}_n - 1/\overline{M}_{n,0} = (k/M_0)t$ and for weight average molecular weight ($\overline{M}_w$): $1/\overline{M}_w - 1/\overline{M}_{w,0} = (k/2M_0)t$. The factor 2 is a result of the fact that for a random depolymerization the polydispersity index is equal to, or approaches, 2.

The decrease in M is therefore uniquely defined by the rate constant (k). Plots of $1/\overline{M}_n$ or $1/\overline{M}_w$ versus time are linear, with slopes of k/M_0 and $k/(2M_0)$, respectively. The rate of degradation which describes the decrease in molecular weight is given by:

$$-(d\overline{M}_n/dt)=(k/M_0)(\overline{M}_n)^2 \qquad (3)$$

Because of the term $(\overline{M}_n)^2$ long polymer chains appear to be more unstable than shorter chains. However, this is simply a consequence of the fact that a fixed number of chain breaks in a population of long molecules would lead to a relative decrease in $\overline{M}_n$ which is larger than that which is obtained for the same number of breaks in a population of shorter chains [1].

The relationship between the molecular weight and the intrinsic viscosity [η] is usually given by the Mark-Houwink-Sakurada (MHS-equation)

$$[\eta] = K \cdot \overline{M}^{a} \qquad (4)$$

For a linear single stranded polymer such as chitosan, a combination of the MHS equation and equation of weight average molecular weight gives:

$$1/[\eta]^{1/a} = 1/[\eta]_0^{1/a} + k't \qquad (5)$$

where $k' = k/(M_0 K^{1/a})$; k is the rate of bond cleavage, M_0 is the monomer molecular weight, and K and a are MHS parameters. Anthonsen *et al.* [2] showed that equation (4) and (5) can be used for estimating MHS parameters (a and K) for chitosans with different degrees of deacetylation and at 0.1M ionic strength (pH= 4.5). In equations (6) and (7) the term F_A represents the fraction of groups acetylated, that is $F_A = (1-\%DA/100)$.

$$\log K_{0.1} = -0.427 - 3.821\ F_A \qquad (6)$$

$$a_{0.1} = 0.6169 + 0.7590\ F_A \qquad (7)$$

The rate constant k for degradation is obtained simply by plotting $1/[\eta]^{1/a}$ versus degradation time and evaluation of the slopes, k', of the linear curves obtained and converting to k by the equations above.

Activation energy

The activation energy for breaking chemical bonds is dependent upon the type of depolymerization and will, in general, be different for different depolymerization mechanisms.

The Arrhenius equation express that depolymerization rate (k) is dependent of temperature (T):

$$k = Ae^{-E_a/RT} \qquad (8)$$

In equation (8) R is the gas constant, T is the absolute temperature and E_a is the activation energy. Taking the natural logarithm of the equation gives:

$$\ln k = \ln A - E_a/RT \qquad (9)$$

A plot of ln k versus 1/T gives a straight line whose slope is equal to $-E_a/R$.

Color measurements

An X-Rite® 948 SpectroColorimeter was used for measuring the development of color in chitosan salt samples during exposure to various temperatures. The whiteness index, Y, relates to a value of 0 % for a black object and 100 % for a white or colorless object.

Results and Discussion

Change in viscosity/molecular weight over time

Degradation of PROTASAN™ chitosan salts was followed by viscometry. In Figure 1, the viscosity of 1% solutions of thermally degraded PROTASAN™ glutamate and chloride is plotted as a function of degradation time. PROTASAN™ glutamate and chloride salts were thermally degraded at 60, 80, 105 and 120 °C. Higher temperatures were not evaluated since decomposition of chitosan occurs at approximately 200 °C [3].

As can be seen from Figure 1, the viscosity decreases exponentially initially and then decreases at a slower rate over an extended period of time. This is simply a consequence of the fact that a fixed number of chain breaks in a population of long molecules leads to a

relative decrease in viscosity/molecular weight which is larger than what is obtained for the same number of breaks in a population of shorter chains.

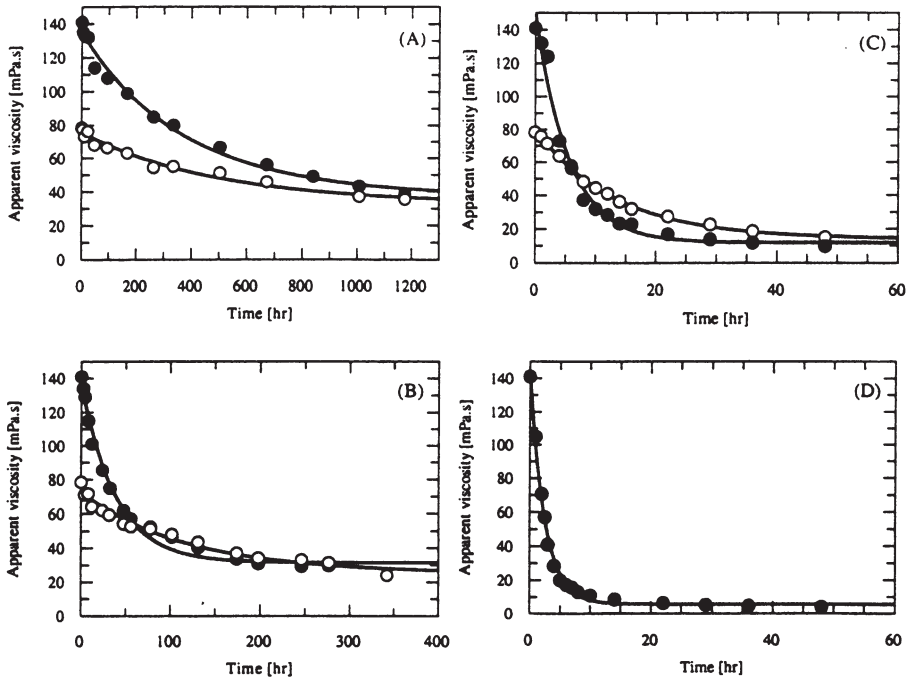

Figure 1: Thermal degradation of PROTASAN™ chloride salt (●) and PROTASAN™ glutamate salt (○) at (A) 60 °C, (B) 80 °C, (C) 105 °C and (D) 120 °C.

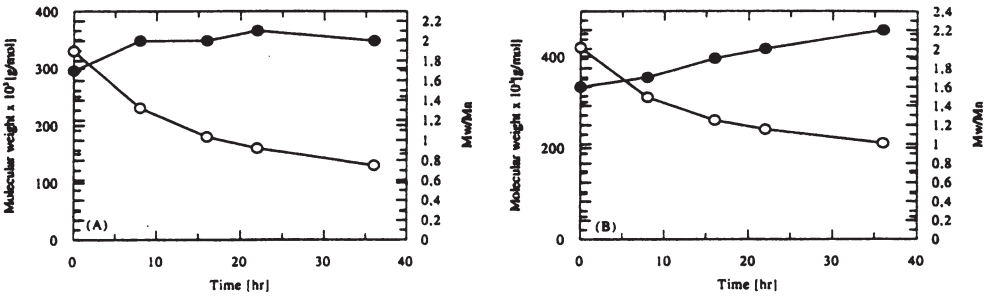

Figure 2: (○) Molecular weight and (●) polydispersity index of (A) PROTASAN CL and (B) PROTASAN G degraded at 105 °C.

Samples of PROTASAN™ chloride and glutamate salts degraded at 105 °C were analyzed by SEC-MALLS. The results indicate that molecular weight decreases with increasing degradation time and that the polydispersity index approaches to 2 (Figure 2). A value of 2 for the polydispersity index indicates that thermal degradation of PROTASAN™ salts is a random process. For the initial material the polydispersity index is lower than two - due to removal of low molecular weight material in PROTASAN™. Samples of chitosan degraded at lower temperatures were not analyzed, but a random depolymerization is expected.

We have observed that chitosan salts with high degree of deacetylation are more thermal stable than chitosan salts with low deacetylation degree. Experiments reported by Vårum and Smidsrød [4] showed that the rate of the depolymerization increases with decreasing degree of deacetylation. They concluded that the rates of acid hydrolysis of the glycosidic linkages in chitosans are of the order A-A≈A-D >> D-A≈D-D. Further experiments will be carried out to establish whether these differences also are the same in the solid state.

Activation energy

The activation energy for thermal depolymerization was obtained by plotting the natural logarithm of the depolymerization rate versus 1/T. Activation energy (E_a) is obtained from the slope, $-E_a/R$, of the straight line as shown in Figure 3. The activation energy for PROTASAN™ chloride and glutamate salts is found to be 112 ± 2 kJ/mol and 99 ± 2 kJ/mol respectively.

Color measurements

Changes in color of PROTASAN™ chloride and glutamate salts were observed during the degradation period. Before the thermal treatment of the PROTASAN™ salts the whiteness index was approximately 88 %. The change in the color was minimal when salts were degraded at 60 °C. Color changes were more rapid and greater when chitosan salts were degraded at 105 and 120 °C.

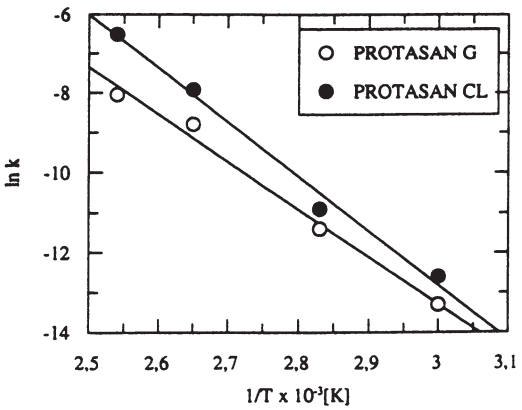

Figure 3: Activation energy for thermal depolymerization of PROTASAN CL and PROTASAN G is obtained by plotting ln k versus 1/T.

The Maillard reaction involves amino groups reacting with an aldehyde and the color change with cleavage of glycosidic bond may be caused by this reaction because of the reducing ends formed during the thermal depolymerization. Amino groups in chitosan can react with the free carbonyl species in open sugar conformation. Because of the Maillard reaction the color of PROTASAN™ salts changes from white to a more yellowish-brown color.

Degradation mechanisms

The proposed mechanisms for temperature-induced degradation of chitosan are acid catalyzed hydrolysis and oxidative reductive degradation (ORD) of the glycosidic bonds. Protonation of the glycosidic oxygen is the first step of an acid hydrolysis. The proton can be added from the protonated amino group of chitosan. Heterolysis follows which results in the formation of a cyclic carbonium-oxonium ion that most probably exists in the half-chair conformation (Figure 4). By addition of water the reducing sugar is formed [5] and the proton is regenerated. At elevated temperatures, however, water molecules will not necessarily be available. Most likely a rearrangement of the carbonium ion can occur.which would also lead to regeneration of the proton.

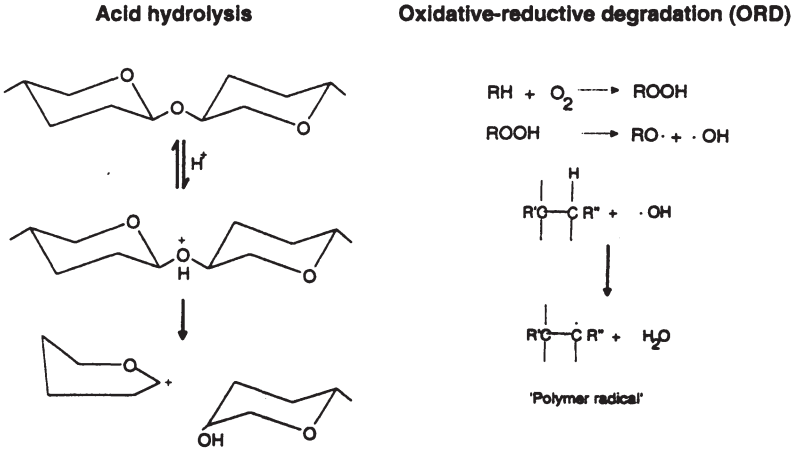

Figure 4: General mechanisms for the acid catalyzed hydrolysis and oxidative-reductive degradation (ORD) of glycosidic linkages.

Chitosan can also be degraded in the presence of molecular oxygen. ORD involves a series of free radical reactions that ultimately lead to chain scission [1]. The ORD mechanism is presented schematically in Figure 4. Reducing compounds are oxidized, yielding a peroxide. The decomposition of peroxide is catalyzed by transition metals and leads to the formation of free radicals. The free radicals will be involved in a series of chain reactions, some of which result in the attack on the polymer chain. The 'Polymer radical' is unstable, and further reactions of unknown mechanisms result in cleavage of glycosidic bonds. Optimum stability with respect to ORD is therefore obtained with minimal oxygen content, transition metals, light and autooxidable compounds. PROTASAN™ salts of ultrapure quality should offer optimum stability with regard to ORD reactions.

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Radiolysis and sonolysis of chitosan – two convenient techniques for a controlled reduction of the molecular weight

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Summary
The molecular weight of chitosan can be readily reduced to the desired level by subjecting its aqueous solutions to ionizing radiation or to ultrasound. Compared to classical degradation methods, these procedures are fast, simple and easy to control.

Introduction

Chitosan, a copolymer of β-(1-4)-D-glucosamine and *N*-acetyl-β-(1-4)-D-glucosamine, is an ionic polysaccharide derived from chitin. Due to its non-toxicity, biodegradability and many unique biomedical properties, chitosan is of particular interest for medical and pharmaceutical purposes (for reviews see *e.g.* refs.[1-3]).

Although the underlying chemical and physical effects of some of these applications are still not known in detail, considerable evidence has been gathered that in many cases the suitability of chitosan for a particular purpose and its effectiveness in exerting a specific action depends on its molecular weight [2,4-6]. The molecular weight of raw chitosan, produced by deacetylation of chitin, depends on the starting material and the conditions of treatment. Weight-average molecular weights of several hundreds to over one million Dalton are common. Therefore, there is a need for efficient and simple methods for adjusting the molecular weight of chitosan to a level suitable for a particular application.

This may be done by acid hydrolysis (*e.g.* refs.[1,7]), which, however, usually requires elevated temperatures, high acid concentrations and relatively long reaction times; in addition, side reactions may occur. Moreover, due to the many factors involved, a precise control of the final molecular weight is not easily achieved. Probably the most promising of the classical degradation methods is the reaction with nitrous acid [8], which is relatively rapid, selective and easy to control. Another widely studied degradation procedure is the enzymatic hydrolysis of chitosan [1,2,9]. Recently, degradation with ozone has been reported as well [10].

In the present work it will be shown that high-molecular-weight chitosan can be readily reduced to the desired molecular weight by subjecting the polymer in aqueous solution to ionizing radiation or to ultrasound. Only stoechiometric amounts of acid are required for dissolving the polymer. Both procedures are simple, the degradation takes typically a few minutes only, and the final average molecular weight can be reasonably well controlled.

So far, the effect of ionizing radiation on chitosan has been studied mainly with solid samples [5,11-16]. In fact, irradiation of dry chitosan is probably the simplest of all degradation methods, being a single-step operation with no need for dissolving, pH adjustment, re-precipitation, drying *etc.*, with the additional advantage of a concomitant sterilization as well as an improvement of the biocompatibility and antimicrobial activity [5,16]. However, there are also some drawbacks, *i.e.* post-irradiation changes in molecular weight continuing for weeks and coloration of the material [5,13-15]. These can be largely avoided when chitosan is irradiated in aqueous solution.

Subjection of polymers in solution to ultrasound is a well-known degradation method [17,18], yet, only very few data are available on the sonication of chitosan solutions [19], leaving still open questions concerning the mechanism of chain breakage and the influence of various parameters on the effectiveness of this treatment.

The results presented in this communication are a part of a broader project which focuses on the mechanisms of radiolysis and sonolysis of chitosan in solution.

Materials and Methods

Chitosan was prepared by deacetylation of krill (*Euphausia superba*) chitin with 50 % aqueous sodium hydroxide, at a chitosan weight fraction of 9 %, at 100 °C for 20 min followed by washing with water. This procedure was carried out three times. The degree of deacetylation was 90.5 % as determined by potentiometric titration [20], and the weight-average molecular weight was 4.0×10^5 Da. Polymer concentrations are given in mol dm^{-3} of the repeating unit (on average 165 Da).

Solutions were made up in Milli-Q-filtered water (Millipore). Perchloric acid, non-reactive towards $^{\bullet}OH$ and $H^{\bullet}$, was chosen for dissolving chitosan. Chitosan dispersed in water by stirring was dissolved by adding stoechiometric amounts of acid and filtered through a 5 μm-pore-size filter (Minisart, Sartorius).

^{60}Co-γ-Irradiations were performed at a dose rate of 0.082 Gy s^{-1}. Prior to irradiation, solutions were saturated for 1 h with N_2O purified by an Oxisorb column (Messer-Griesheim), or with a N_2O/O_2 gas mixture (4:1 v:v). Sonolysis was carried out at 20 °C in a laboratory sonicator (321 kHz, 170 W kg^{-1}) [21,22] of 0.5 dm^3 capacity. The solutions were placed in small (8 cm^3), flat-bottom, gas-tight vessels, purged with argon, inserted into the water-filled sonicator and constantly rotated during the sonication [21].

Absolute values of the weight-average molecular weight were determined by low-angle laser light-scattering (KMX-6, Chromatix, operating at $\lambda = 633$ nm), in solutions of pH 3.0 ($HClO_4$), containing 0.25 mol dm^{-3} sodium perchlorate ($dn/dc = 0.187$ cm^3 g^{-1} at 25 °C).

Results and Discussion

Radiolysis. The radical-generating system. When dilute, aqueous polymer solutions are subjected to ionizing radiation, its energy is absorbed practically only by water. In the radiolysis of water [reaction (1)], equal amounts of hydroxyl radicals, hydrated electrons and some 10 % H atoms are formed [23]. Nitrous oxide has been used to convert hydrated electrons into OH radicals [reaction (2)].

$$H_2O \xrightarrow[(1)]{\text{ionizing radiation}} {}^{\bullet}OH,\ e^-_{aq},\ H^{\bullet},\ H^+,\ H_2,\ H_2O_2$$

$$N_2O + e^-_{aq} + H_2O \xrightarrow[(2)]{} {}^{\bullet}OH + OH^- + N_2$$

The reaction of OH radicals with chitosan. Hydroxyl radicals (and H atoms) react with mono- and polysaccharides by abstracting carbon-bound hydrogens (*cf.* refs. [24,25]). As a result of this, carbon-centered (or, in the present case, also nitrogen-centered) radicals are formed at the carbohydrate polymer.

The rate constant of the reaction of OH radicals with chitosan, measured for our sample with pulse radiolysis by a competition with thymine [23], is $k = 6.4 \times 10^8$ dm^3 mol^{-1} s^{-1} (details will be published elsewhere).

Hydrogen atoms react with carbohydrates much more slowly than OH radicals. Their yield is only *ca.* 10 % under our experimental conditions and since they react also by H-abstraction, the expected products are the same.

Chain scission. Radicals formed on carbohydrate polymers undergo relatively fast reactions leading to the final, stable products. On the basis of the accumulated knowledge on the reactions of carbohydrate radicals in the absence of oxygen [23,24], one expects two main processes in the present case, elimination of a water, leading to the formation of carbonyl groups and secondary radicals, and chain breakage, the latter being of special interest in the present context.

Fragmentation of a polysaccharide chain occurs mainly by the scission of the glycosidic linkage. Radicals localized at C(1), C(4) and C(5) atoms are the main precursors of this process [23,26].

Changes in molecular weight. After decay of the radicals, the final, stable composition of the system can be evaluated. Changes in molecular weight of chitosan caused by γ-irradiation in N_2O-saturated solution are shown in Figure 1 (solid line). From the difference between the initial and final molecular weight one can calculate the effective concentration of the chain breaks (*i.e.* the concentration of chain breaks that persist after all the recombination processes are completed) [27]. They are directly proportional to the absorbed dose (see inset in Figure 1).

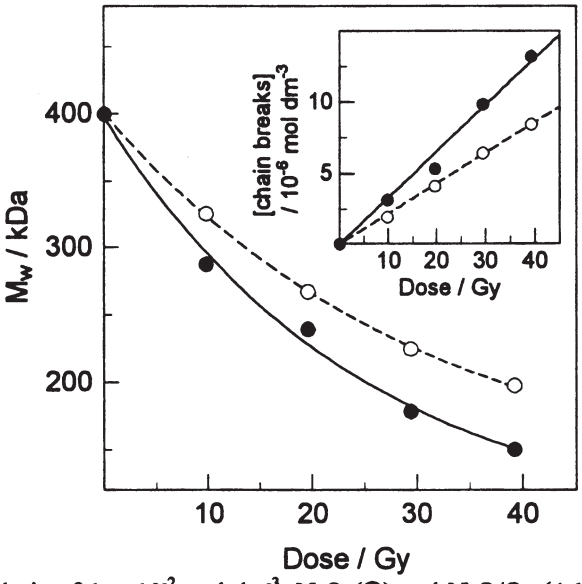

Figure 1. γ-Radiolysis of 1×10^{-2} mol dm^{-3}, N_2O (●) and N_2O/O_2 (4:1 v:v) (○) saturated chitosan in solution at pH 3.0. Weight-average molecular weight (inset: effective concentration of chain breaks) as a function of dose.

The radiation-chemical yield of scission, in terms of moles of breaks formed per unit of absorbed radiation energy, is $G_s = 3.4 \times 10^{-7}$ mol J^{-1}, *i.e.* more than 50 % of the primary radicals have caused chain scission.

In the presence of O_2 (broken line in Figure 1) the scission yield, $G_s = 2.1 \times 10^{-7}$ mol J^{-1}, is significantly lower. Such a protective effect exerted by oxygen is typical for the breakage of the glycosidic linkage [23], in contrast to most polymer systems whose free-radical-induced degradation is enhanced by O_2.

It is estimated that 100 Gy given to a 1×10^{-2} mol dm^{-3} solution only causes one chemical alteration per 300 structural units, and no post-irradiation changes are expected.

Thus, radiolysis of chitosan in aqueous solution is a simple, fast and convenient method for the reduction of its molecular weight. It runs efficiently at room temperature, requires only stoechiometric amounts of acid (for the dissolution of the sample) and can be easily controlled (irradiation can be stopped at any time and the degradation terminates at that moment). The required doses are low and can be applied in any laboratory or commercial (*e.g.* radiation sterilization) gamma or electron-beam facility within minutes. No special reactors, installations *etc.* are needed.

Sonolysis. When high-intensity ultrasound is applied to a liquid, formation and collapse of small gas bubbles takes place (cavitation) [28]. During the quasi-adiabatic compression of these bubbles, high pressures and temperatures build up for a very short period [29]. As a consequence of this, the solvent molecules contained in the gas phase are split into free radicals. In the case of water, OH radicals and H-atoms are formed [30]. They diffuse into the liquid water layer surrounding the cavitation bubble, where they either recombine or react with the substrates present in the solution, and the subsequent reactions are the same as those caused by radiolysis. Another mechanism of sonochemical degradation is the pyrolysis of substrate molecules. It may occur not only in the gas phase of the collapsing bubbles (where polymer molecules cannot enter), but also in the thin liquid layer surrounding the hot bubbles. The third mechanism, usually considered as the main one acting on macromolecules, is the rupture of the polymer chain caused by the extreme pressure differences and the related hydrodynamic forces occurring in the liquid phase in the vicinity of oscillating and collapsing gas bubbles.

When argon-saturated chitosan solutions are subjected to ultrasound, a decrease in molecular weight is observed (solid line in Figure 2). Preliminary measurements extending beyond the times shown in Figure 2 indicate that oligomers of a molecular weight < 20 kDa can be easily obtained.

It is often postulated that sonolytically-induced chain scission in polymers is mainly or solely due to the hydrodynamic force effect [17]. This may well be the case for organic solvents and low frequencies. In the present case with water as solvent and at relatively high frequency there is strong experimental evidence for a major contribution of OH radicals. As shown above, OH radicals are very efficient in initiating degradation of chitosan. In a sonolytic experiment, these radicals can be completely eliminated from the aqueous phase by adding 2×10^{-3} mol dm^{-3} of *tert*-butanol [22]. The presence of such low concentrations of *tert*-butanol does not impair the cavitation process itself. As can be seen from Figure 2 (broken line), addition of *tert*-butanol reduces, but not completely suppresses the sonolytic chain scission of chitosan. This may be taken as an indication that OH radicals contribute to chain breakage, but that they are not the only factor in the sonolytic degradation of this polymer.

From the practical point of view one can conclude that sonication, as irradiation, is an efficient and simple tool for obtaining chitosan of desired molecular weights.

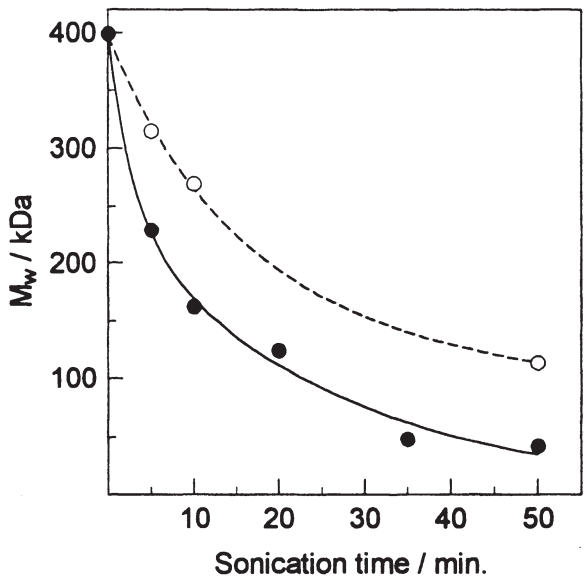

Figure 2. Sonolysis (321 kHz, 170 W, 20 °C) of 1×10^{-2} mol dm^{-3} chitosan in Ar-saturated aqueous solution at pH 3.0 ($HClO_4$) in the absence (●) and presence (○) of 2×10^{-3} mol dm^{-3} *tert*-butanol. Weight-average molecular weight as a function of sonication time.

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Thermal and UV Degradation of Chitosan

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Summary

The present study was undertaken to investigate the difference between thermo- and photodegradation of chitosan of various deacetylation degree DD from 59 to 86 %, on exposure to monochromatic UV radiation of λ=257 nm and dynamic and isothermal heating at 130 – 180 °C. Thermogravimetric, FTIR spectroscopic and dilute solutions viscosity results have shown that both thermodegradation and photodegradation processes of chitosan lead to an important change of the biopolymer structure. The decrease of viscosity for UV irradiated chitosan is interpreted in terms of both photolytic random chain scission of glicosidic linkage and a conversion of amide (chitin comonomer) into amine units. In the case of thermal degradation at conditions of higher mobility of –C-N· macroradicals (above T_g of chitosan) crosslinking is observed, leading to gel fraction. The rate of the process is faster (lower activation energy) for higher DD of chitosan.

Introduction

Chitin found in a variety of biosystems is a biopolymer, structurally similar to cellulose except for secondary hydroxy on the α carbon atom which is substituted by an acetamide group. Chitosan is prepared from chitin by deacetylation. It is a copolymer consisting of β-(1-4)-2-acetamido-D-glucose units and β-(1-4)-2-amino-D-glucose units with the latter often changing from 60 to 90. Chitosan forms clear, water insoluble films (obtained by casting from acetic solutions) which are used by themselves and as biodegradable but wet strength additive in paper for packaging applications, fibres, membranes and for other biomedical materials[1-4]. Some of these uses involve the exposure of the biopolymer to heat and light with possible degradation of the polymer chain. There are a few studies on photodegradation of chitosan [5] dealing with the wavelength sensitivity of chitosan films exposed to monochromatic UV-visible radiation. The authors propose a possible mechanism of photodegradation based on changes in FTIR spectra leading to main chain scission process and carbonyl group formation. Chain scission in chitosan is compared to a similar one in cellulose [6] via photolysis of the glycosidic linkage. In addition to chain scission process chitosan undergoes photodegradation reaction leading to an increase of amine group intensity.

The present study was undertaken to investigate the difference between photo–degradation and thermal degradation of chitosan samples of various deacetylation degrees on exposure to monochromatic UV radiation of λ=257 μm and isothermal heating at 130 - 180°C or dynamic heating up to 500 °C.

Materials and Methods

Samples

Chitosan samples of various deacetylation degrees (DD) from 59 % to 86.6 % were prepared by deacetylation of krill chitin in Sea Fishery Institute in Gdynia. Shear stress viscosity results presented previously [7,8] showed a strong dependence of the chitosan solution viscosity on DD value of the polymer at constant concentration.

Chitosan films ~ 30 μm thick were prepared using the following procedure:

1. 1% solutions of chitosan flakes in 1% acetic acid were prepared by stirring for several hours and clearing for two days.
2. These solutions were filtered using a centrifuge. It lead to removal of all nondissolved residues.
3. Chitosan acetate films were prepared by the solution casting method (on glass plates) and dried in air at room temperature for 3 days.
4. So prepared films were washed in methanol to neutralize excess acid and then dried.

Degradation processes

Thermodegradation of chitosan films was carried out in isothermal conditions in an oven at 130 to 180°C in air up to 8 hours and in dynamic conditions using thermogravimetric analyzer. Photodegradation of chitosan films occurred in UV irradiation using a low pressure mercury lamp of λ = 254 μm (90% of light) with light intensity I = 700 Lux. Time of the photo-degradation did not exceed 5 hours.

The progress of the chitosan degradation was observed by FTIR spectroscopic studies, intrinsic viscosity of dilute solutions, weight loss measurements.

FTIR studies were made on spectrophotometer Specord 75 IR Zeiss. To viscosity determinations of dilute solutions of chitosan in 1 % acetic acid an Ubbelohde viscometer was applied.

Results and Discussion

THERMODEGRADATION

1. Viscosity results.

After thermal treatment viscosity of chitosan acidic solution increases and some part of the polymer is not completely dissolved. Gel fraction increases with time of heat treatment. The solution viscosity of sol fraction increases.

2. Thermogravimetric analysis.

Weight loss kinetics can be described by the simple equation 1:

$$\frac{dC}{dt} = k \cdot (1 - C)^n \qquad 1.$$

$$k = A \cdot \exp\left(\frac{-E}{R \cdot T}\right)$$

$$C = \frac{w_0 - w}{w_0}$$

where:

w_0, w - initial and changing weight of sample,

T - temperature,
E - activation energy,
n - reaction order,
A - preexponential factor of Arrhenius equation,
t - time,
k - rate constant;

- Isothermal conditions

In isothermal conditions equation 1. can be given in the ln form:

$$\ln\left(\frac{dC}{dt}\right) = \ln A + n \cdot \ln(1-C) - \frac{E}{R \cdot T} \qquad 2.$$

The following plots 3. allow to calculate E and n:

$$1.\ \ln\left(\frac{dC}{dt}\right) = f\left(\frac{1}{T}\right) \rightarrow \mathrm{E} \qquad 3.$$

$$2.\ \ln\left(\frac{dC}{dt}\right) = f(1-C) \rightarrow \mathrm{n}$$

Chosen results of the isothermal degradation are shown in Figs. 1 and 2.:

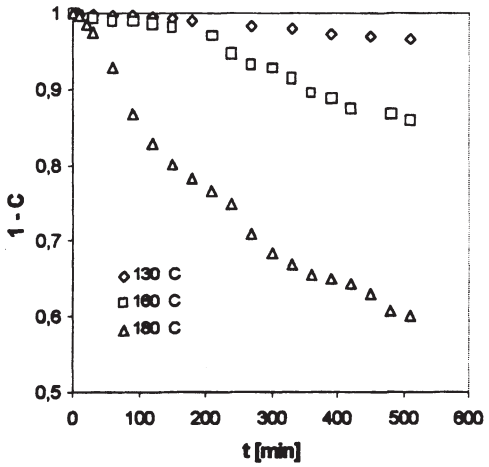

Fig. 1.
Isothermal thermogravimetric curves (1-C) = f(t) for DD=86.7 %.

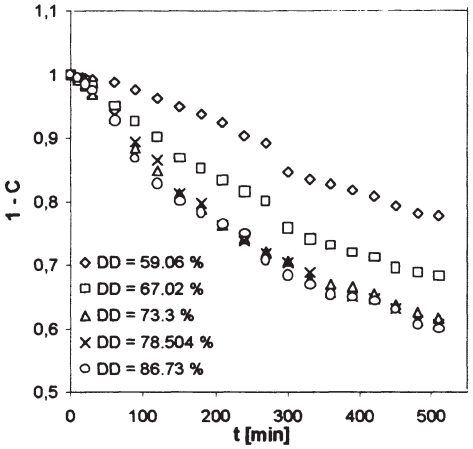

Fig. 2.
Isothermal thermogravimetric curves (1-C) = f(t) at 180 °C obtained for various deacetylation degrees (DD).

- Dynamic conditions:

Taking: $\phi = \frac{dT}{dt}$ - heating rate ≈ 7,9 °/min

equation 1. can be presented in the following form 4. in which the weight loss is dependent on temperature T:

$$\frac{dC}{(1-C)^n} = \frac{A}{\phi} \cdot \exp\left(\frac{-E}{R \cdot T}\right) dT \qquad 4.$$

Thermogravimetric curves of various chitosan samples are shown in Fig. 3. Two main stages of the thermal degradation are observed.

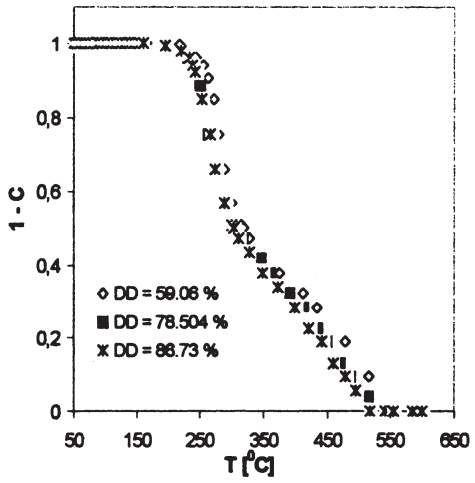

Fig. 3. Dynamic termogravimetric curves (1 – C) = f (T) for various DD.

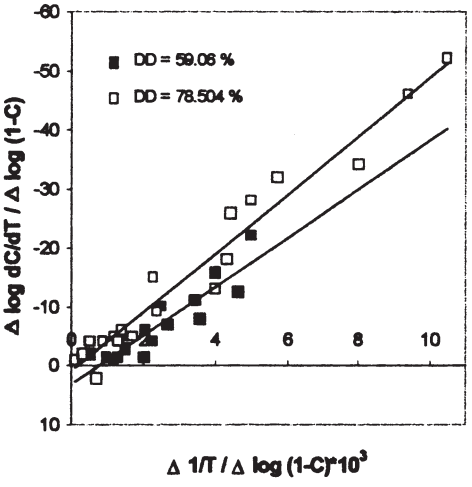

Fig. 4. Plots according to equation 6. drawn for two samples.

For thermogravimetric analysis a few known methods were applied. Two of them are presented below:

1. Friedman method [9]:

$$E = \frac{n \cdot T_m^{\,2} \cdot R \cdot \left(\frac{dC}{dt}\right)_m}{1 - C_m} \qquad 5.$$

C_m, T_m and $\left(\frac{dC}{dt}\right)_m$ values in maximal rate.

2. Freeman – Carroll method [10]:

$$\Delta \log \phi \left(\frac{dC}{dt}\right) = n \cdot \Delta \log(1-C) - \frac{E}{2{,}3 \cdot R} \cdot \Delta \cdot \left(\frac{1}{T}\right) \qquad 6.$$

Plots in Fig. 4. have shown the results analyzed by the Freeman – Carroll method.

Fig. 5. has shown the activation energy E values (and n) drawn versus DD derived from the two methods.

The micrograph in Fig. 6. has shown an example of a network structure obtained from gel fraction of chitosan after thermal degradation.

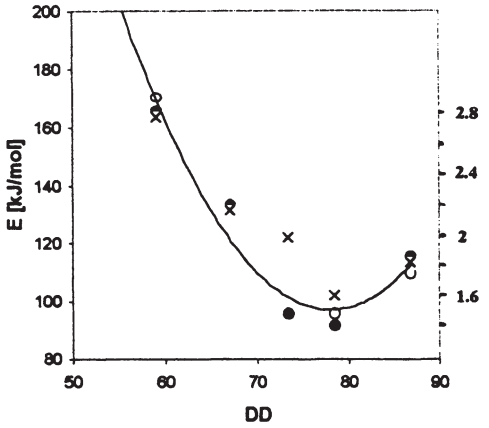

Fig. 5. Activation energy values (for 1 stage of degradation) versus DD; • - Friedman and o - Freeman–Carroll method, × - reaction order.

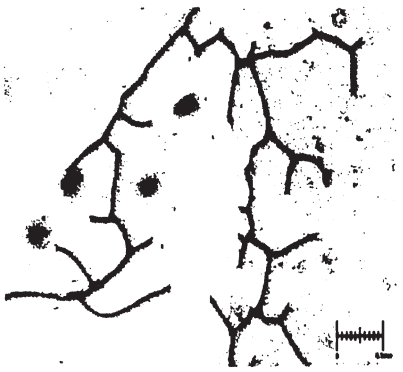

Fig. 6. Network structure in gel.

DTA curves of various chitosan samples are shown in Fig. 7.

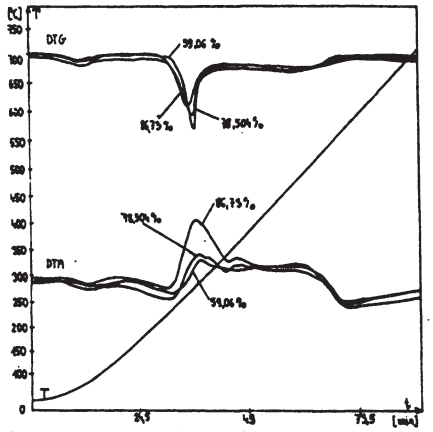

Fig. 7. Curves of differential thermal analysis for various chitosan.

One can conclude that:

1. Initial temperature of the thermal destruction decreases with increasing DD of chitosan (DD= 59, T_i = 221 °C; DD= 78.5, T_i = 201 °C; DD= 86.7, T_i = 198 °C).
2. Exothermic heat of destruction is larger for higher DD (Fig. 7).
3. Activation energy values (also n) of thermal degradation decrease with increasing DD (Fig. 5).
3. Crosslinking occurs (micrographs of chitosan network structure in gel) (Fig. 6).

PHOTODEGRADATION

The rate of photodegradation may be expressed by the rate of photolysis of a given chromophoric group which yields free radicals leading to molecular chain scission.

The number of molecular chain scissions in photolysis is described [11] by the following equation:

$$S = \frac{\Theta \cdot I_0 \cdot \varepsilon \cdot l \cdot [c_0]}{[x_0]} \cdot t = k \cdot t \qquad 7.$$

where:

- Θ - quantum yield of photolysis (chain scission)
- ε - extinction coefficient
- l - film thickness
- I_0 - intensity of incident light
- $[x_0]$ - initial concentration of polymer chain
- $[c_0]$ - concentration of chromophoric group
- k - rate constant

S values were calculated from intrinsic viscosity data $[\eta_0]$ and $[\eta_t]$, (α - viscosity coefficient was taken as 0,9) according to equation 8.:

$$S = \left(\frac{[\eta_0]}{[\eta_t]} \right)^{\frac{1}{\alpha}} - 1 \qquad 8.$$

Some results of viscosity of dilute solution (in 1 % acetic acid) are shown in Fig. 8. and the number of molecular chain scissions calculated from the viscosity data are presented in Fig. 9. The linearity of the plot S=f(t) implies that random chain scission occurs.

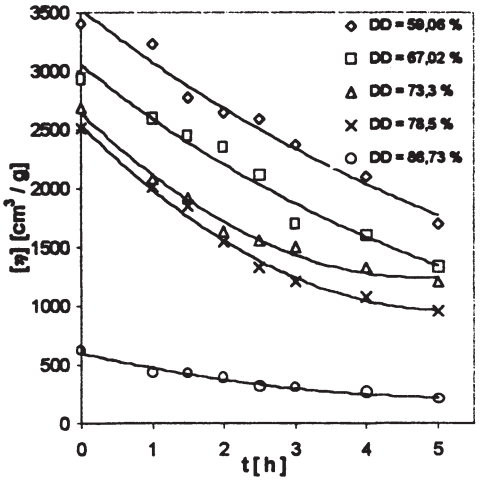

Fig. 8. Intrinsic viscosity versus time of UV exposure for various DD.

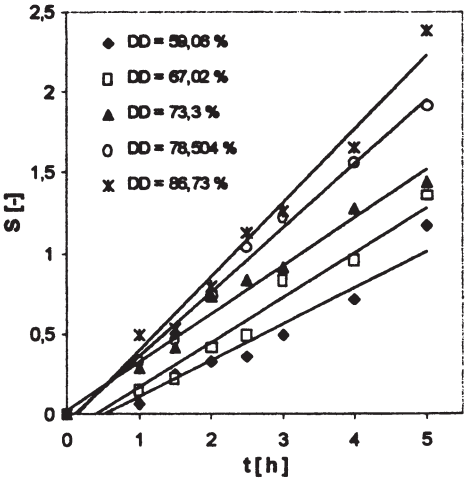

Fig. 9. Number of chain scissions versus time of UV exposure for various DD.

The rate constant k of photolysis calculated from the slope of linear dependence S= f(t) in Fig. 9 is presented in Fig. 10 versus DD of chitosan.

Huggins constants K' reflecting interactions between polymer and solvent, are calculated from viscosity data according to equation 9.:

$$\frac{\eta_{sp}}{c} = [\eta] + K' \cdot [\eta]^2 \cdot c \qquad 9.$$

They are drawn in Fig. 11 versus time of UV exposure for one chosen sample. A similar increasing tendency is observed for other samples.

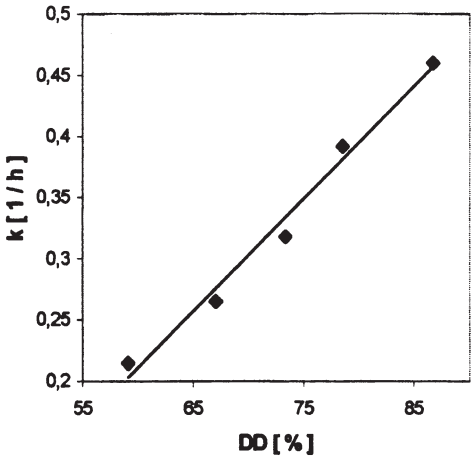

Fig. 10. Rate constant k of photolysis drawn versus DD.

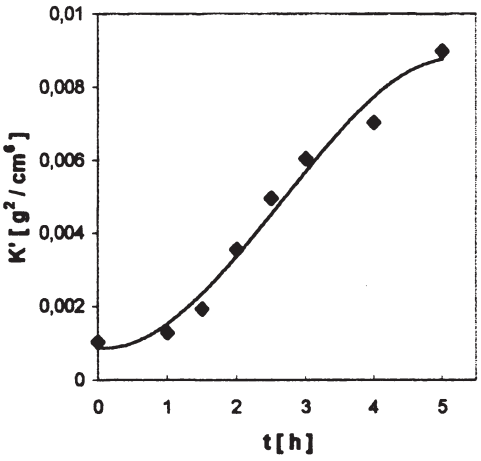

Fig. 11. Huggins constant K' (DD=78.5 %) versus time of UV exposure.

The following conclusions on chitosan photolysis resulting from viscosity data can be drawn:

1. Chitosan photolysis by UV of $\lambda = 257$ cm^{-1} is the first order reaction: $S = k \cdot t$ (Fig. 9).
2. Rate constant k of the photolysis changes proportionally to deacetylation degree DD (Fig. 10) (amine groups appear to be chromophoric groups).
3. During UV degradation the interaction between the polymer chains and the solvent changes leading to increasing solubility which is reflected in rising Huggins constant (Fig. 11).

FTIR RESULTS

Absorption at characteristic groups present in chitosan was analyzed:

2920 cm^{-1} – reference line (in UV degradation), -CH stretching vibration attributed to pyranose ring,

1469 cm^{-1} – CH_3 in amide group,

1550 cm^{-1} – vibration of amide group,
1580 – 1600 cm^{-1} – primary amine bands,
1049 cm^{-1} – C – O – C in glicosidic linkage,
1620 cm^{-1} – carbonyl groups,
1720 cm^{-1} – ester groups,
640 cm^{-1} – absorption in glicosidic ring,
1380 cm^{-1} – reference line for thermal degradation.

Fig. 12 presents an example of IR spectrum of chitosans film (DD = 86.6 %) unexposed and UV exposed.

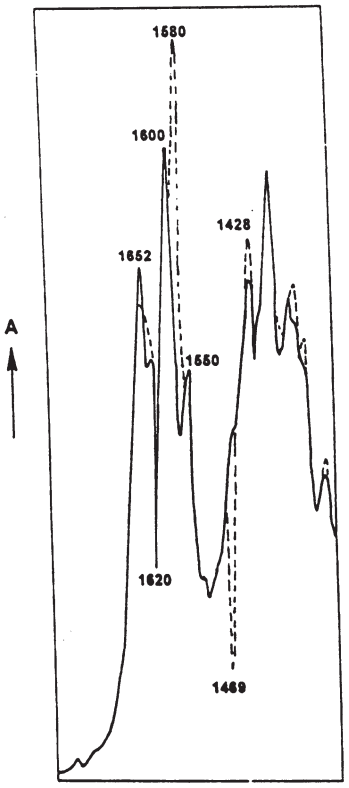

Fig. 12.
IR spectrum of chitosan (DD = 86.6 %) — unexposed and - - - exposed (t = 5 h).

The following preliminary conclusions can be drawn from FTIR studies.

From the analysis of FTIR spectrum of UV irradiated chitosan with DD = 86.6 % for time t = 5 h the following results were obtained (see Fig. 12):

1. an increase in amine groups absorption at 1580 cm^{-1} with a simultaneous decrease of $-CH_3$ group absorption at 1469 cm^{-1},
2. small decrease in C - O - C group absorption at 1049 cm^{-1},

The observed changes result from the destruction of amide groups and their conversion to amine groups and some molecular chain scission of glicosidic linkage both leading to increasing solubility and decreasing viscosity of chitosan acetic solution.

From the analysis of FTIR spectra of chitosan with DD = 78.7 % after heat treatment at T = 160 °C for time t = 3.5 h the following results were obtained:

1. a decrease in groups connected with the glicosidic ring at 640 cm^{-1} and 2920 cm^{-1};
2. a decrease in amine groups absorption at 1590 cm^{-1};
3. an increase of absorption of carbonyl groups at 1670 cm^{-1} or some increase at 1040 cm^{-1} 1160 cm^{-1}.

The observed changes result from thermal destruction of the glicosidic ring, possible thermooxidation of polymer and crosslinking of macroradicals of various chains (increasing gel fractions).

FTIR investigation will be continued.

Conclusion

Thermogravimetric, FTIR and viscosity results have shown that both thermodegradation and UV degradation of chitosan lead to an important change of the biopolymer structure. However, the mechanism of the processes is completely different. It will be discussed later.

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Heat-induced physicochemical changes in highly deacetylated chitosan

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Summary
The objective of this study was to determine the heat-induced chemical changes in highly deacetylated chitosan. Heat may be employed to facilitate the processing of chitosan and to confer sterility on chitosan-based products. However, dry heat at 160°C and saturated steam at 125°C induced coloration and lowered the aqueous solubility of the chitosan samples. These changes were not accompanied by apparent chemical modifications, as there were only minor differences observed in the ^{13}C CP-MAS NMR and FTIR spectra. The heated samples exhibited sharper IR absorption bands, which, coupled with X-ray diffraction results, indicate that the physical changes may be attributed to heat-induced alignment of chitosan molecular chains.

Introduction
Chitosan, a polycationic electrolyte, has potential applications in diverse fields such as medicine, food, agriculture and waste disposal. Heat is often employed to facilitate the processing of chitosan, and may be used to sterilize chitosan-based pharmaceuticals or medical products. Sterilization of pharmaceuticals and medical products is commonly effected by exposure to dry heat at 160°C or to saturated steam at 125°C.

Several studies [1-4] have shown that heat can modify the properties of chitosan. This has implications on the performance of heat-treated chitosan products. We have earlier reported that dry heat at 160°C and saturated steam at 125°C can cause intensive coloration of highly deacetylated chitosan, rendering the polymer poorly soluble even in aqueous acids [1]. The chemical basis for these changes has not been established, although Toffey *et al* (1996) have proffered, through NMR and thermal analyses, that the water-soluble chitosan acetate may be converted to water insoluble chitin upon heating [2].

The objective of this study was to determine the chemical reactions, if any, which may be brought about by heating highly deacetylated chitosan molecules, and to correlate the results to the heat-induced physicochemical changes obtained in our earlier study.

Materials and Methods
Chitosan (Tokyo Kasei Kogyo Co. Ltd., Japan) was further deacetylated by a 2-stage solid state hydrolysis process, using 40% w/v sodium hydroxide and 1% $NaBH_4$, intermitted with distilled water washing. The polymer was dissolved in 3% v/v acetic acid, regenerated with 1N sodium hydroxide, washed till neutral and freeze-dried (Dynavac Freeze Dryer). Molecular weight of the chitosan was determined by solution viscometry (Cannon Ubbelohde,

30°C, 0.2M CH_3COOH/0.1M CH_3COONa as solvent) to be 8.73 (± 0.55) x 10^5 (K = 6.6 x 10^{-3}, a = 0.88)[5]. The degree of deacetylation, measured with the first derivative UV spectophotometric method[6], was ≥ 95%. All other chemicals and reagents were of analytical grade.

Triplicate samples (100mg) of the purified chitosan on glass petri dishes were exposed to dry heat (Memmert oven with air vent opened) at 120°C or 160°C, or to saturated steam (Hirayama autoclave) at 125°C. Samples were also heated in the oven at 120°C under negative pressure of –100kPa. The heated samples were removed from the heat source after 1 h and stored in desiccators at ambient temperature for at least 24 h prior to analysis. Solid-state NMR experiments were carried out on a Bruker Avance 400 instrument at a resonance frequency of 100 MHz for ^{13}C nuclei. The proton spin-lock field strength was 13kHz. To obtain the IR spectra, pellets were prepared with 2.0mg chitosan and 180mg KBr, and scanned from 4000cm^{-1} to 400cm^{-1} (Jasco FTIR). X-ray diffraction pattern in the 2θ range of 5 to 30° was acquired using Ni-filtered CuKα radiation generated at 30 kV and 20 mA (Philips PW 1280) and a scan rate of 3° (2θ)/min.

Results and Discussion

The control chitosan sample was white. Exposure of the samples for 1 h to dry heat at 160°C or to saturated steam at 125°C caused the sample to turn yellowish brown and dark brown respectively. The intense coloration was accompanied by reduced solubility in aqueous acetic acid, the autoclaved chitosan sample becoming insoluble and forming a swollen gel in the acid medium. Dry heat at 120°C did not change the physical appearance of the sample, although it improved sample interaction with the aqueous acetic acid.[1] Heating *in vacuo* at 120°C also did not cause significant changes to the physical properties of the polymer.

X-ray diffraction patterns show the control chitosan sample to be largely amorphous (Figure 1). Exposure to dry heat at 120°C did not change the X-ray diffraction pattern, but

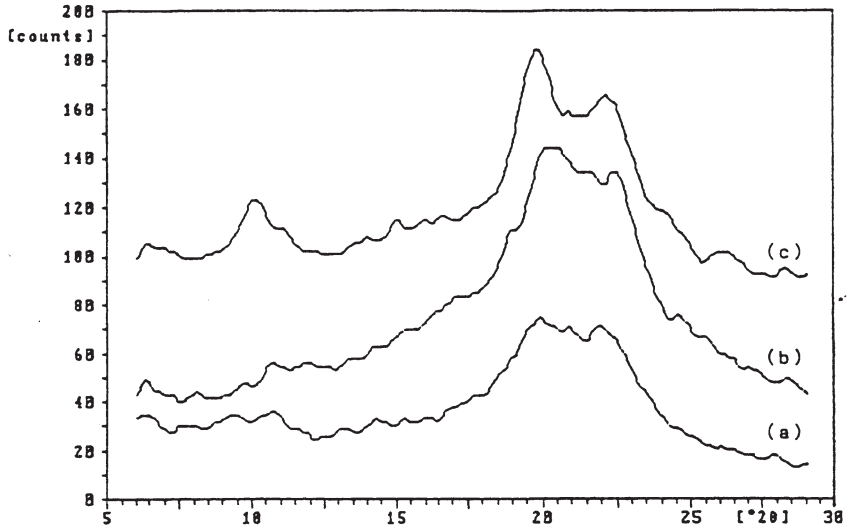

Figure 1. Effects of heat on the X-ray diffraction pattern of highly deacetylated chitosan samples (a) control (b) dry heat at 160°C (c) saturated steam at 125°C.

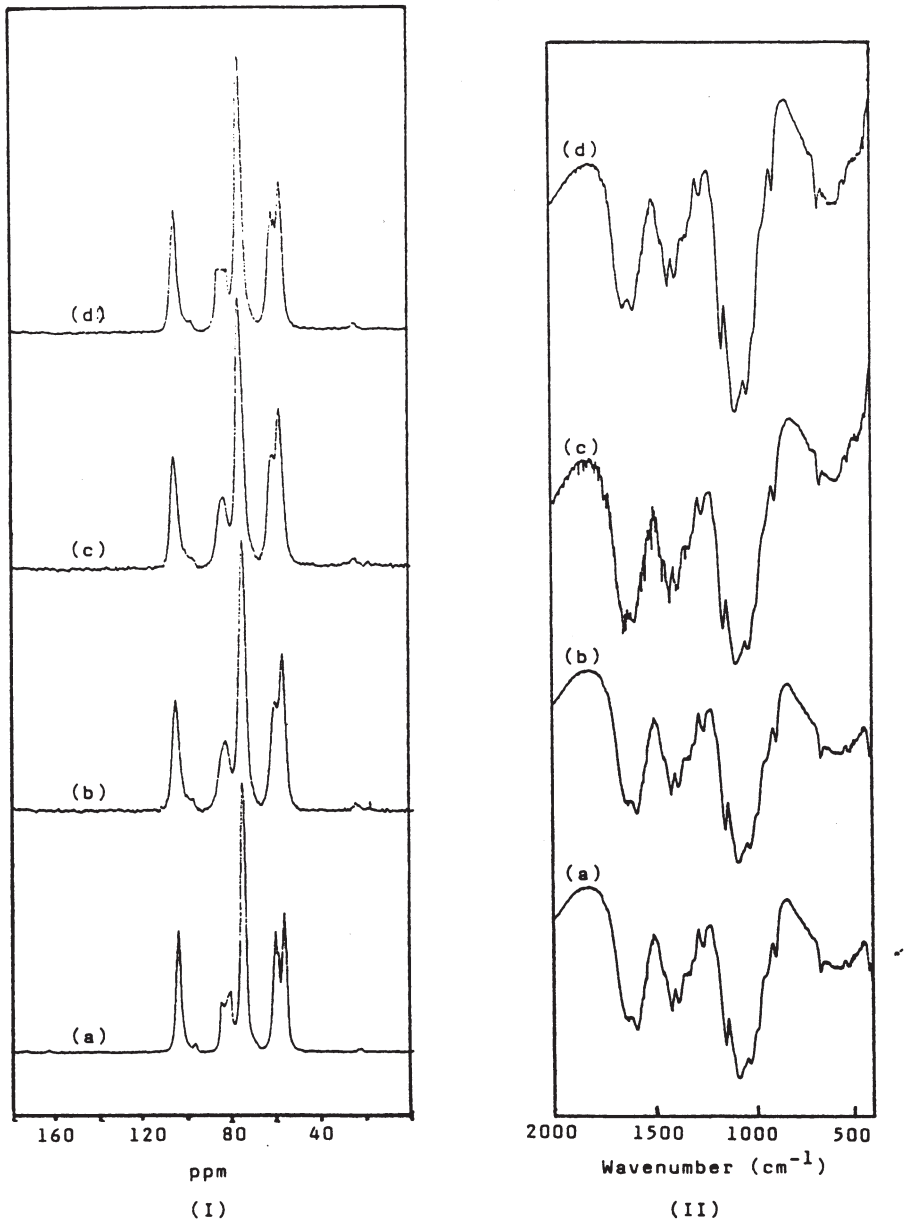

Figure 2. Solid-state ^{13}C CP-MAS NMR (I) and FTIR (II) spectra of selected heat-treated chitosan samples. (a) Control sample (b) Sample exposed to dry heat at 120°C (c) Sample exposed to dry heat at 160°C (d) Sample exposed to saturated steam at 125°C.

exposure to dry heat at higher temperature or to saturated steam increased the ordering of molecular chains, causing a sharpening and an increase in the number of X-ray diffraction peaks. The changes were more marked for the autoclaved sample, which exhibited sharp peaks at 10° (2θ), which may be attributed to the presence of hydrated crystals of chitosan[4], and at 20° (2θ).

Figure 2 shows the solid-state ^{13}C CP-MAS NMR and FTIR spectra of selected heat-treated chitosan samples. Notwithstanding the intense coloration produced by dry heat at 160°C and saturated steam at 125°C, there were no apparent new signals in the ^{13}C CP-MAS NMR spectra after heat treatment of the chitosan. The untreated sample raised six ^{13}C signals at δ 57, 61, 76, 82, 86 and 105 ppm. Based on the electronic effects exerted by the neighbouring chemical groups, these signals may be assigned to C_2, C_3, C_6, C_4, C_5 and C_1, respectively, of the glucosamine repeating unit. Exposure to dry heat or saturated steam reduced the relative intensity of the ^{13}C signal at δ 61 ppm, and caused the merging of signals at 82 and 86 ppm to give only one signal at 84 ppm. These minor changes, present also in the NMR spectrum of sample exposed to dry heat at 120°C, imply that no significant chemical changes were produced by the heat treatment.

IR spectra are also presented in Figure 2 using the same ordinate scale for transmittance. Characteristic bands in the 2000 to 400 cm^{-1} region included the N-H stretch at 1600 cm^{-1}, the oxygen bridge at 1152 cm^{-1} and the C-O stretch at 1090 cm^{-1}. The spectrum for the control sample confirmed that it was highly deacetylated; the amide I band at 1650 cm^{-1} was small compared to the N-H peak at 1600 cm^{-1}. As was observed for the ^{13}C NMR spectrum, heat treatment did not significantly change the IR spectrum of the chitosan sample. However, samples that had been exposed to dry heat at 160°C, and particularly those exposed to saturated steam at 125°C, gave sharper IR absorption peaks compared to the control. This was not seen for samples exposed to dry heat at 120°C. The sharpening of IR absorption peaks for chitosan has been associated with enhanced crystallization in the sample.[7]

The intense brown coloration and reduced aqueous solubility of the heated chitosan samples has drawn comparisons with chitosan samples cross-linked with glutaraldehyde.[1] This study affirms the lack of evidence for heat-induced chemical modifications and effectively rules out the possibilities of cross-link formation in heated chitosan samples. Rather, the sharpening of the IR absorption bands and the X-ray diffraction peaks suggests that the heat-induced coloration and changes in physical properties are due to an enhanced degree of chain ordering in the polymer.

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Chitosan fibre and its chemical *N*- modification at the fibre state for use as functional materials

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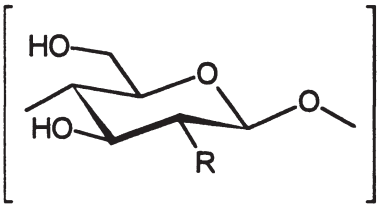
Summary
A solution of chitosan in aq. 2% acetic acid-methanol (2:1, v/v) was spun through a viscose-type spinneret at room temperature into a novel coagulating solution aq. 5-10% ammonia solutions saturated with ammonium sulfate (ca. 43%). The extruded chitosan fibre was chemically *N*-acylated (d.s. 0.8-1.0) at a solid state by treatment with several carboxylic acid anhydrides in methanol at room temperature. Chitin fibre and chitin cellulose blended fibre were treated at a solid state with 40% NaOH at 90-100°C for 4 h to afford the corresponding *N*-deacetylated fibre. These data indicata that chitosan fibre and chitin fibre are *N*-modifiable at the solid state. Some characteristics of these fibres for use as functional materials were also describes.

Introduction
Chitosan fibre and chitin (*N*-acetylchitosan) fibre have been reported by several investigators [1,2], and some *N*-arylidene- and *N*-alkylidene-chitosan fibres have been prepared in our laboratory [3]. However, little is known about the other *N*-modified chitosan fibres because of the difficulty of their direct spinning. The present paper aims to prepare some novel *N*-acyl (fatty acyl, benzoyl and succinyl) chitosan fibres by the chemical *N*-modification of the chitosan fibre at a solid state for use as biofunctional materials (Scheme 1).



Scheme 1. R=-NHC(=O)CH_3 (1); -NHC(=O)CH_2CH_3 (2); -NHC(=O)$(CH_2)_2CH_3$ (3); -NHC(=O)$(CH_2)_4CH_3$ (4); -NHC(=O)$(CH_2)_6CH_3$ (5); -NHC(=O)C_6H_5 (6); -NHC(=O)C_2H_4COOH (7)

Materials and Methods

The sample of crab shell chitosan had d.s. 0.12 for NAc and MW 24 x 10^4. Chitin fibre [4,5] and chitin-cellulose blended fibre [6] were used for *N*-deacetylation in the present study. The degree of substitution (d. S.) for *N*--substitution was determined by the elemental analyses, and other methods were described in our previous paper.

Results and discussion

Chitosan fibre: Chitosan was dissolved at a 3% concentration on aqueous 2% acetic acid-methanol (2:1, v/v), and the solution was spun through a viscose-type spinneret (12.5x18x0.3 mm in size) having 300 holes of each 0.1 mm in exit diameter at a rate of 8 m/min into aq. 5-10% ammonia solutions saturated with ammonium sulfate (ca. 43%) at room temperature. After stetching to 1.2 - 1.4-fold in a solution of ethyleneglycol-aq. 10% NaOH (9.1, v/v) at room temperature, the extruded fibre was washed with water and then with aq. 30% methanol, and dipped into aq. 50% methanol overnight. The chitosan fibre was collected by filtration and air-dried with mechanical stretching (80-95% yields). As shown in Table 1, the mechanical prperties were similar to those of the chitosan filament (D) extruded in aq. 10% NaOH solution containing 30% sodium acetate [3].

Table 1. Some mechanical properties of chitosan flament

Filament	Titre (denier)	Tenacity (g/denier)	Elongation (%)
A	14.5	1.23	12.1
B	14.8	1.10	9.9
C	4.0	1.11	13.4
D	3.74-9.02	0.78-1.24	13.0-17.3

A, not stretched during the spinning step, but stretched during the air-drying step; B, Not stretched during both the spinning and air drying steps; C, Stretched during both the spinning and air-drying steps; D, A 5% chitosan solution in aq. 2% acetic acid the filament was stretched during the spinning step, but not stretched during the air-drying step (3).

Table 2. Some mechanical properties of *N*-acylchitosan filaments

Filament	Titre (denier)	Tenacity (g/denier)	Elongation (%))
1	4.86	1.33	10.6
2	4.78	1.61	12.6
3	5.05	1.75	11.4
4	4.45	1.26	12.0
5	4.65	1.46	13.0
6	6.18	1.21	12.5
7	5.54	1.38	13.0

1, *N*-acetylchitosan (chitin); 2, *N*-propionylchitosan; 3, *N*-butyrylchitosan; 4, *N*-hexanoyl-chitosan; 5,*N*-octanoylchitosan; 6, *N*-benzoylchitosan; 7, *N*-succinylchitosan.

N-*Acylchitosan fibres*: The treatment of chitosan fibre with carboxylic anhydrides gave rise to novel *N*-butyryl (d.s. 1.0), *N*-hexanoyl (d.s. 1.0), *N*-octanoyl (d.s. 1.0), *N*-benzoyl (d.s. 0.97), *N*-succinyl (d.s. 0.81) chitosan fibres, respectively.

These filaments (Table 2) showed 1.21-1.75 g/denier for the tenacity and 10.6-13.0% for the elongation, which were similar to or slightly higher than those of the original chitosan filament. The *N*-succinylchitosan fibre was soluble in water but the other fibres were insoluble.

These mechanical properties of *N*-acylchitosan filaments were essentially similar in the mechanical properties to those of *N*-alkylidene- and *N*-arylidene-chitosan filaments (titre 4.37-5.72; tenacity 1.23-1.83 g/denier; elongation 12.9-17.2%) [3].

Table 3. Some mechanical properties of *N*-deacetylated filaments

Filament	Titre (denier)	Tenacity (g/denier)	Elongation (%)
8	2.96	0.79	18.9
9	3.27	0.95	21.4

8, Chitosan prepared from the chitin filament by treatment once with aq. 40% NaOH at 95-100 °C for 9 h; 9, chitosan-cellulose blended filament prepared from the chitin cellulose blended filament by treatment once with aq. 40% NaOH at 95-100 °C for 9 h.

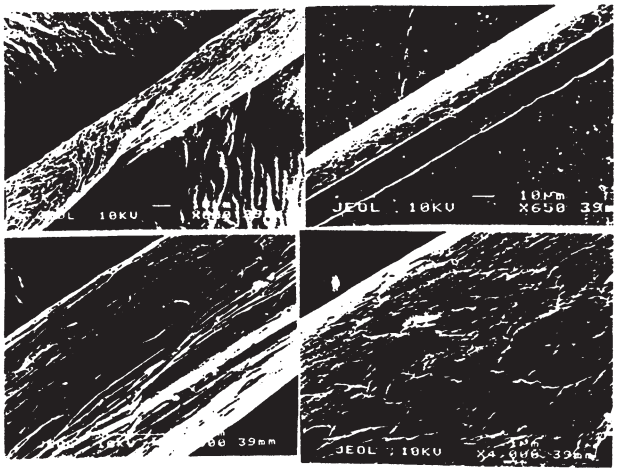


Figure 1. SEM photographs of chitosan filament (right) and *N*-octanoylchitosan filament (left).

N-*Deacetylation of chitin fibre and its cellulose blended fibre:* Each of chitin fibre [4,5] and cellulose-chitin blended fibre [6] was treated with aq. 40% NaOH at 90-100 °C for 4 h. The alkaline treatment was repeated once more. The obtained chitosan fibre (d.s. 0.1 for *N*-acetyl) was soluble in aq. 2% acetic acid, and the obtained novel cellulose-chitosan blended fibrer was only swelled in aq. 2% acetic acid solution. Their tenacity values slightly decreased after this treatment (Table 3).

Fibre characteristics: A scaly pattern appeared on the surface of both the chitosan and *N*-acylchitosan filaments as examined by SEM analysis (Figure 1). The pattern is considered to produce during the neutralization and dehydration processes of chitosan filament. The molecular crysallinity in the filament is unknown. Relative hydrolysis rate of the fibre by egg-white lysozyme was in the rate of 1 : 0.3 : 0.0 for *N*-acetyl-, *N*-propionyl- and *N*-butyryl-

chitosan fibres. *N*-Hexanoyl- and *N*-octanoyl-chitosan fibres were blood compatible better than *N*-acetylchitosan (chitin) fibre.

Conclusions

1) The chemical *N*-acylation (d.s. >0.8) of chitosan fibre was performed at the solid state by the post-treatment.
2) The post-*N*-modification effected little on the mechanical properties of chitosan fibre.
3) Chitosan fibre and ist cellulose blended fibre were prepared by chemical *N*-deacetylation from chitin fibre and its cellulose fibre, respectivily.
4) The waste coagulating solution of ammonium sulfate may be usable as an agricultural fertilizer.
5) The present new products may be usable as biofunctional materials including antithrombogenic, hemostatic (embolus), and cell-eliciting, controlled digestible fibres.

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Preparation of a fiber-reactive chitosan derivative with enhanced antimicrobial activity

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Summary

A chitosan derivative with enhanced antimicrobial activity and functional groups that can form covalent bonds with cotton fabrics was prepared for antimicrobial textile finishing application. To increase antimicrobial activity and give water-solubility on chitosan, quaternary ammonium salt groups were introduced on the *O*-positions (mainly on C-6) of chitosan based on a Schiff base intermediate (salicylidene chitosan). To impart fiber-reactivity on chitosan, dichlorotriazinyl groups were introduced on the amino groups of chitosan. The final product had poor water-solubility and may require organic co-solvents for use as a textile finishing agent.

Introduction

Chitosan, β-1,4-linked glucosamine, is the principal derivative of chitin that is the second most abundant polysaccharide found on earth next to cellulose. Chitosan, as a natural renewable resource, has a number of unique properties such as antimicrobial activity, nontoxicity, and biodegradability, which enable chitosan to attract scientific and industrial interest in the field of biochemistry, medicine, food science, and textile [1].

The antimicrobial activity of chitosan against a variety of bacteria is well known. Chitosan, however, shows its antimicrobial activity only in acidic condition due to its poor solubility above pH 6.5. Therefore, water-soluble chitosan derivatives soluble in the entire range of pH would be good candidates as antimicrobial reagents [2]. Recently, a number of chemical modifications have been applied to synthesize water-soluble chitosan derivatives having enhanced antimicrobial activity [2, 3, 4].

In recent years, consumer demand for textile products with antimicrobial property and the safety of them toward humans and the environment is increasing. As a biodegradable natural polymer, chitosan attracts considerable attention on this respect [5]. As a result, chitosan itself and some chitosan derivatives with enhanced antimicrobial activity were applied to textile fabrics [5, 6]. However, there were few studies on the covalent linkage of chitosan and its derivatives onto fabrics to increase their durability against repeated washings.

The ultimate object of this work is to synthesize a novel chitosan derivative expected to have enhanced antimicrobial activity as well as functional groups that can be covalently linked onto cotton fabric. Two functional groups were introduced on chitosan: quaternary ammonium salt group to impart enhanced antimicrobial activity as well as water-solubility, and dichlorotriazinyl group to impart fiber-reactivity on chitosan. For this purpose, site-selective chemical modification (Figure 1) of chitosan was studied, i.e., introduction of

quaternary ammonium salt group on the *O*-position of chitosan and dichlorotriazinyl group on the *N*-position. Schiff base derivative (2) of chitosan was employed as an intermediate to protect amino groups of chitosan.

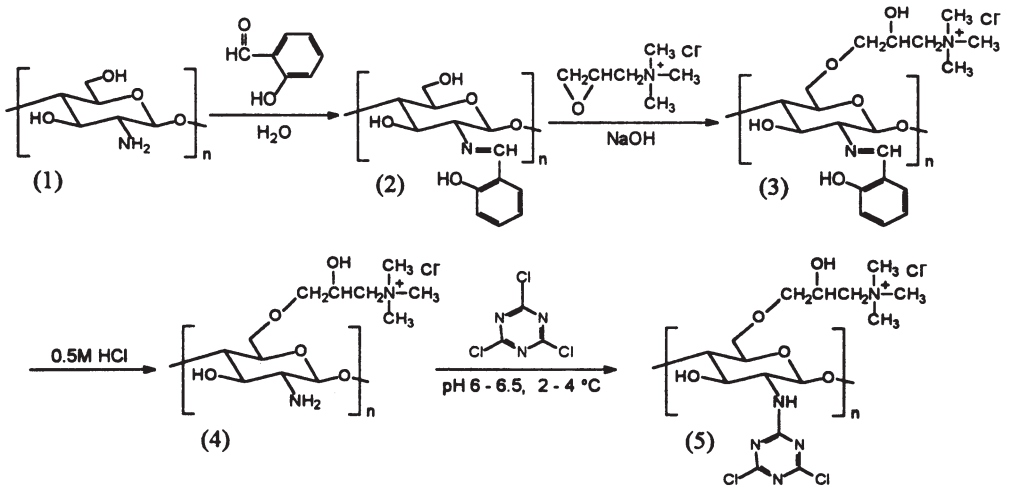

Figure 1. Synthesis scheme of a fiber-reactive chitosan derivative. ((1) deacetylated chitosan, (2) salicylidene chitosan, (3) *O*-substituted GTMAC salicylidene chitosan, (4) *O*-substituted GTMAC chitosan derivative, (5) *N*-dichlorotriazinyl-*O*-substituted GTMAC chitosan derivative).

Materials and Methods

General procedures: All solvents and reagents used were commercially available and used without further purification. The degree of deacetylation (DD) of chitosan and the degree of substitution (DS) of each reaction product were determined either by elemental analysis or conductometric titration. For the elemental analysis, the reaction products were sent to Atlantic Microlab, Inc. (Georgia, USA). Conductometric titration was performed with Orion Benchtop Conductivity meter (Model 162). All IR spectra were obtained by using a Nicolet 510P FT-IR spectrophotometer. The data collection parameters employed were 4.0 cm^{-1} resolution and 32 scans. The samples were prepared as KBr pellets and were scanned against a blank KBr pellet background.

Preparation of highly deacetylated chitosan (1): Finely ground commercial chitosan (60 g, Natural Polymer, Inc.) with 83.5% DD was treated with 50% (w/w) aqueous NaOH solution (1200 g) at 120°C for 2 hours. The reaction mixture was washed with distilled water until neutral, finally with methanol, and air-dried. This deacetylation procedure was repeated one more time. The DD obtained from conductometric titration was 0.95. The viscosity molecular weight was determined to be 6.10×10^5 g/mol by the method of Wang et al. [7].

Salicylidene chitosan (2): Well-dried chitosan (18.5 g, 95% DD) was reacted with 61 mL of salicylaldehyde (ca. 10 mole excess) in 300 mL of distilled water at room temperature. After 8 hours stirring, the yellowish reaction product was filtered over a glass filter, washed with ethanol and diethyl ether several times, and air-dried. It was extracted with diethyl ether in a Soxhlet extractor overnight to remove unreacted salicylaldehyde and dried overnight at 60°C in vacuum. The DS of the salicylidene chitosan was 0.90 from elemental analysis. To

increase the DS up to 1.00, the reaction was repeated for a prolonged time (20 hours) and the product was purified by the same method. From elemental analysis, it was confirmed that the primary amino groups were completely substituted with Schiff base groups (DS 1.00).

O-substituted GTMAC chitosan derivative (4): Salicylidene chitosan (4 g) was well dispersed in 30 mL of 1:2 mixture of water and iso-propanol at 50°C in the presence of NaOH (0.3 g, ca. 0.5 mole portion to salicylidene chitosan) as a base catalyst. To the mixture, glycidyltrimethylammonium chloride (GTMAC, 30 mL, ca. 10 mole excess) was added and stirred at 50°C for 24 hours under nitrogen purge. Once the reaction was complete, the reaction product was poured in ethanol (300 mL) and stirred for 30 minutes. It was filtered over a glass filter, washed with ethanol until neutral, and finally with acetone, and air-dried. The dried pale yellowish product was dissolved in 0.5M HCl solution (200 mL) at room temperature for 40 hours to completely remove any remaining Schiff base groups. The solution was precipitated in 1 L of 1:1 mixture of acetone and ethanol. The precipitate was filtered over a glass filter and washed well with ethanol until neutral and finally with acetone, and air-dried. This dried product was dissolved in water (150 mL) and 50% (w/w) NaOH solution was added drop by drop to pH 10 to regenerate the free amino groups of chitosan. This solution was precipitated in 1L of acetone and the precipitate was Soxhlet extracted with ethanol for two days. The extracted product (4) was dried in vacuum at room temperature overnight. It was dissolved in deionized water (200 mL) and the solution was allowed to flow down through an ion exchange column (Amberlite IRA-402 (Cl^- form)). It was collected and dried at 50°C. After evaporation of all water, the product was collected and dried at 60°C in vacuum overnight. The DS of the water-soluble chitosan derivative determined from conductometric titration was 1.09.

N-dichlorotriazinyl-O-substituted GTMAC chitosan derivative (5): The *O*-substituted GTMAC chitosan derivative was dissolved in distilled water (30 mL) and stirred in an ice bath at 2-4°C. A known amount of cyanuric chloride (0.1010 g, 0.6 mole portion to the chitosan derivative) was dissolved in 10 mL of cold acetone and this solution was added slowly to the chitosan derivative solution which was being stirred. During the reaction, the pH was kept at 6.0-6.5 by adding 0.3M Na_2CO_3 aqueous solution drop-wise. After 2 hours, the final pH was adjusted to 7.5 and the reaction was stopped. The reaction solution was precipitated in acetone (150 mL), filtered over a glass filter, and washed thoroughly with acetone to remove unreacted cyanuric chloride. It was further washed with water and finally with acetone. It was dried at room temperature in vacuum for 24 hours. The DS calculated from elemental analysis was 0.19.

Conductometric titration: Deacetylated chitosan and *O*-substituted GTMAC chitosan derivative were dissolved in 90 mL of distilled water with 10 mL of 0.1M HCl and 100 mL of deionized water, respectively, and then titrated with 0.1N NaOH and 0.017N $AgNO_3$, respectively.

Results and Discussion

Degree of deacetylation of deacetylated chitosan: As shown in Figure 2, the titration curve showed two deflection points. The first deflection point corresponds to the neutralization of excess H^+ ions of HCl by OH^- ions of NaOH added. The range between the first and the second deflection points corresponds to the neutralization of the protonated amino groups of chitosan. After the second deflection point, the conductivity goes up with a higher value of slope, which is due to the excess of NaOH added. The number of moles of NaOH used between the first and the second deflection point equals the number of moles of amino groups

of the chitosan sample, which was divided by that of completely deacetylated chitosan to give a value of DD 0.95.

FTIR analysis: The absorption peaks of FTIR spectra (Figure 3) of chitosan and its derivatives were assigned as following: (a) 1595 cm^{-1} (NH_2), (b) 3060 cm^{-1} (aromatic C-H) and 1630 cm^{-1} (C=N of Schiff base), (c) 1480 cm^{-1} (C-H of (2-hydroxy-3-trimethylammonium)propyl group), and (d) 1560 cm^{-1} (C=N of dichlorotriazinyl group) and 845 cm^{-1} (C-Cl of dichlorotriazinyl group).

Synthesis of O-substituted GTMAC chitosan derivative: To introduce quaternary ammonium salt groups on the *O*-positions (mainly C-6) of chitosan, NaOH was used as a base catalyst. It was found that the salicylidene chitosan decomposed gradually and unexpectedly in alkaline condition although the literature [8] suggests that the salicylidene chitosan was stable in alkaline condition. Due to the decomposition of salicylidene chitosan during the reaction, it was supposed that some ammonium salt groups were introduced on the *N*-positions of chitosan. Although the salicylidene chitosan did not completely protect the amino groups of chitosan, it showed higher reactivity than that of chitosan itself with GTMAC in the preliminary experiment.

The DS obtained for *O*-substituted GTMAC chitosan derivative was 1.09 (the sum of DS on both *O*- and *N*-positions). From the titration method, the DS on the *O*- and *N*-positions of chitosan could not be differentiated. Maresch et al. [9] investigated hydroxypropylation of chitosan by reacting chitosan with propylene oxide. From a series of reactions in a different concentration of NaOH as a catalyst, they found that the DS on the *O*-position was at least *two times higher than that on the N-position of chitosan. Based on their result, it could be* thought that the *O*-substituted GTMAC chitosan derivative (DS 1.09) has at least 60% free amino groups and this amount of free amines was thought to be enough to be used as reactive sites for the next step of synthesis with cyanuric chloride.

Solubility of N-dichlorotriazinyl-O-substituted GTMAC chitosan derivative: In the solubility test of the final product in water, it did not dissolve, but it was swollen to a gel-like form. The water-insolubility of the product could be due to either hydrophobicity of the

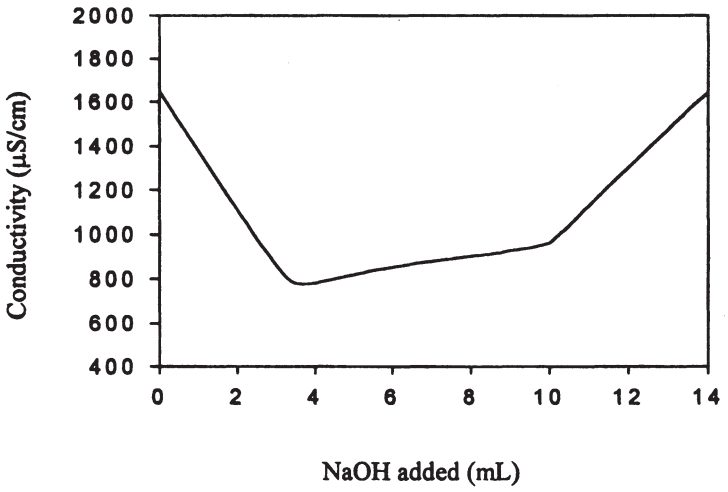

Figure 2. Conductometric titration curve of deacetylated chitosan.

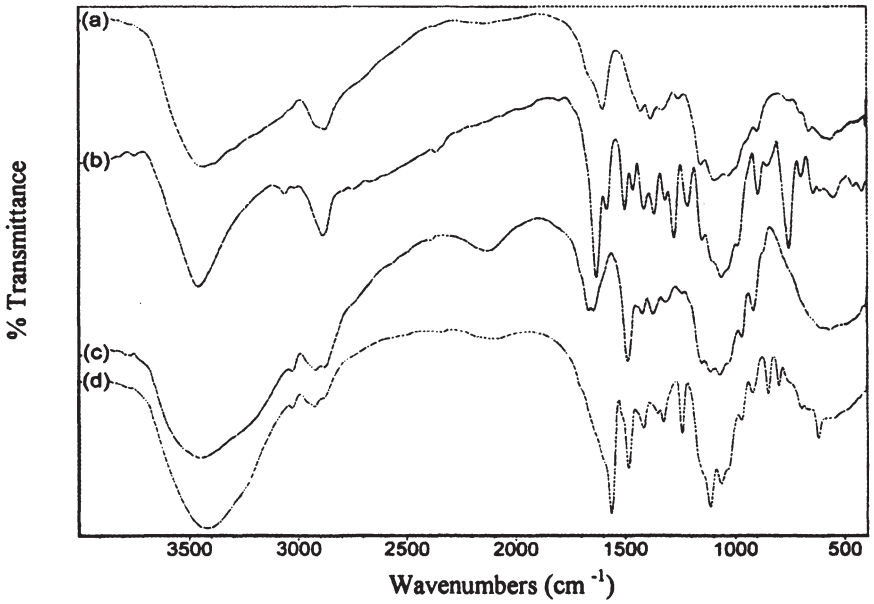

Figure 3. FTIR spectra of (a) deacetylated chitosan, (b) salicylidene chitosan, (c) *O*-substituted GTMAC chitosan derivative, (d) *N*-dichlorotriazinyl-*O*-substituted GTMAC chitosan derivative.

introduced dichlorotriazinyl groups or the crosslinking of chitosan that is caused by the reaction of the second chloride atom of dichlorotriazinyl group with unreacted free amino groups of chitosan.

In terms of hydrophobicity, as the reaction proceeded the reaction solution became clear, which means that dichlorotriazinyl groups introduced were not so hydrophobic to induce precipitation of the chitosan derivative in the aqueous medium. It was found that after precipitating the reaction solution in acetone and immediately draining the acetone, the precipitate dissolved well in water. From this, it could be thought that some crosslinking between chitosan chains were occurred during precipitation and filtering at room temperature. Even though it is known that the second chloride atom of dichlotriazinyl group reacts at 30-50°C, a few of them may react at room temperature and this could make the water-soluble chitosan derivative insoluble. This was also confirmed by reacting *O*-substituted GTMAC chitosan derivative with a very small portion of cyanuric chloride (0.1 mole portion), which would give much less hydrophobicity than 0.6 mole portion of cyanuric chloride. The reaction product also did not dissolved in water, but swollen. From the result, it was concluded that it might require organic co-solvents for use as a textile finishing agent or other fiber-reactive groups that are stable at room temperature should be investigated to prepare a water-soluble chitosan derivative.

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Chromatographic separation of rare earths with complexane types of chemically modified chitosan

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Summary
Adsorptive separation among rare eaerths from dilute sulfuric acid solution on chemically modified chitosan prepared by immobilizing the ligand of ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) or diethylenetriamine-*N,N,N',N'',N''*-pentaacetic acid (DTPA) onto polymer matrices of chitosan was investigated in order to develop novel mutual separation technology of rare earths at cheap cost. On the basis of the equilibrium data, chromatographic mutual separation was exemplidied by using the column packed with these chemically modified chitosan for the pair of samarium and yttrium as well as for the group of light rare earths, lanthanum, cerium, praseodymium and neodymium, to verify their ability of recognition or separation for rare earths. It was found that satisfactorily clear separation between yttrium and samarium as well as that among these 4 kinds of light rare earths can be successfully achieved with these chemically modified chitosan.

Introduction
In recent years, demands for highly purified rare earths are increasing to produce many kinds of novel advanced materials in high-tech industries. Ion exchange is one of the technologies for separating and purifying various metals including rare earths. According to conventional process by means of ion exchange for the separation and purification of rare earths, after all of rare earths are collectively adsorbed on cation exchange resin packed in an adsorption column, aqueous solution of chelating reagents such as EDTA or DTPA which form metal-chelates with each of rare earths with different stabilities corresponding to each of them is passed through the column. During passing through the column, the chelating reagents desorb the rare earth adsorbed on the resin according to the order of the stability of these metal-chelates, that is, that with highest stability with chelating reagents as such is eluted at first and that with weakest stability at the last. That is, this separation process is dependent on the recognition or separation ability of water soluble chelating reagent, not on the ion exchange resin. In this process, majority of expensive chelating reagents are wasted without being resused, which increases the production cost of purified rare earths. If some chelating resins with high recognition and separation ability for rare earths were available, each of rare earth would be mutually separated from each other at high purity and at cheap cost by using cheap mineral acids such as hydrochloric, nitric or sulfuric acids as elutes. From the point of view as such, some novel chelating resins with the ability for mutual separation among rare earths have been developed to date [1,2]. However, the synthetic routes of these resins are

complicated and, therefore, the production costs are considered to be very expensive, unacceptably by industry.

In the previous papers [3-6], we prepared two kinds of complexing agent types of chemically modified chitosan by immobilizing the ligands of EDTA or DTPA onto polymer matrices of chitosan, abbreviated as EDTA- and DTPA-chitosan, respectively, hereafter, and demonstrated that the adsorption of metal ions is much enhanced compared with original chitosan and that the order of selectivity for some base metal ions in the adsorption on these chemically modified chitosan is nearly the same with that of the stability of these metal chelates with these complexing agents.

In the present paper, the adsorption behaviors of these chemically modified chitosan for rare earths were investigated by batch experiments and compared with those of polyallylamine, a synthetic polymer, chemically modified with the same functional groups. Further, an experimental work for mutual separation of some pair or group of rare earths were carried out by using a column packed with them to exemplify their mutual separation among rare earths; that is, mutual separation between samarium and yttrium and among lanthanum, cerium, praseodymium and neodymium were carried out by using the adsorption column packed with EDTA-chitosan and DTPA-chitosan, respectively, as typical examples for the cases the mutual separation of which is very difficult according to the conventional method.

Materials and Methods

EDTA- and DTPA-chitosan were prepared according to the method described in the previous papers [3-6] by interacting chitosan with anhydride of EDTA or DTPA. Polyallylamine chemically modified with EDTA or DTPA, abbreviated as EDTA- and DTPA-polyallylamine, respectively, hereafter, which were used to compare the adsorption behavior for rare earths with the chemically modified chitosan were prepared according to the method also described in the previous paper [6]. The extent of the introduction of functional groups on polymer matrices measured by means of neutralization titration was as follows: EDTA-chitosan; 100 %, DTPA-chitosan; 35 %, EDTA-polyallylamine; 87 %, DTPA-polyallylamine; 52 %.

Aqueous solutions were prepared by dissolving reagent grade rare erth chlorides in dilute sulfuric acid solution. The concentration of rare earths in the solution was measured by using a Shimadzu model ICPS-1000 III ICP-AES spectrometer.

Vacuum dried adsorption gels were used as they are for batch experiments to examine the adsorption behaviors for rare earths, i.e. the relationship between their distribution ratio and equilibrium pH, in the same manner described elsewhere [3-6]. In the column experiments, after sieved so as to uniform the particle size of the adsorption gels, they were packed in an adsorption column made of glass and coated with water jacket to keep the temperature constant at 303 K as shown in the previous paper [5]. The adsorption gel sufficiently swollen with ion-exchanged water in advance was packed between layers of glass beads in the column. Metal ions were loaded on the gel by letting aqueous solution containing 100 g/m3 each of rare earths in question the pH of which was adjusted by adding small amount of sulfuric acid run through the column. After washing the column with ion-exchanged water, each of the loaded rare earths were eluted from the column by letting dilute sulfuric acid solution the pH of which was adjusted corresponding each rare earths in question run through the column. The sample solutions from the outlet were collected using a fraction collector, and the rare earth concentrations were measured by using the above-mentioned ICP-AES spectrometer.

Results and Discussion

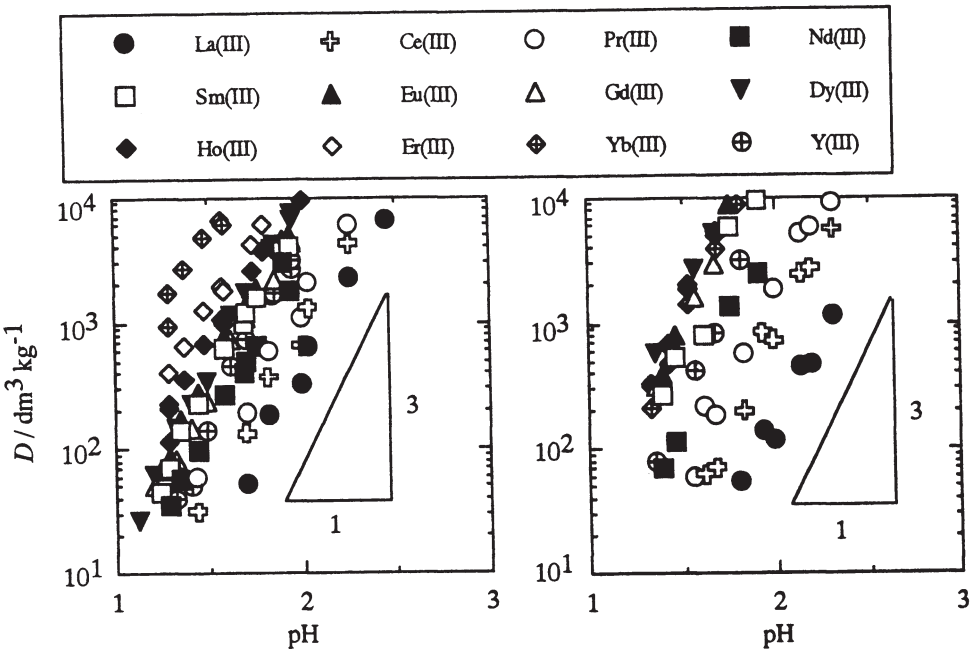

Figure 1. pH Dependency of the distribution ratio of some rare earths in the adsorption from dilute sulfuric acid solution on EDTA-chitosan.

Figure 2. pH Dependency of the distribution ratio of some rare earths in the adsorption from dilute sulfuric acid solution on DTPA-chitosan.

Figures 1 and 2 show logarithmic plots of distribution ratio of some rare earths against equilibrium pH in the equilibrium adsorption on EDTA- and DTPA-chitosan, respectively. As seen from these figures, the plots in both figures appear to lie on straight lines with the slope of 3, suggesting that 3 hydrogen ions are released for the adsorption of unit rare earth ion to form stable metal chelate with a chelating functional group according to cation exchange mechanism. From the intercepts of these straight lines with the ordinate, apparent adsorption equilibrium constants were evaluated for each rare earth and for both adsorbents. Figure 3 shows the plots of the thus evaluated apparent adsorption equilibrium constants for both adsorbents against atomic number of rare earths. The relationships between the adsorption equilibrium constants on EDTA- and DTPA-chitosan and atomic number shown in this figure resemble those between the stability constants of rare earth chelates with EDTA and DTPA, respectively, and atomic number. This result suggests that the chelating characteristics of EDTA and DTPA are maintained still after the immobilization of these ligands on polymer matrices of chitosan also in this case similar to those of base metal ions [6]. In Fig. 3, the diferences in the adsorption equilibrium constants on DTPA-chitosan between adjacent elements in light rare earths, lanthanum, cerium, praseodymium and neodymium, are large enough for their mutual separation while those in other rare earths are small, suggesting difficult mutual separation among them. On the other hand, those on EDTA-chitosan are also large for all elements except for some middle and heavy rare earths, yttrium, samarium, europium, gadolinium, dysproseum and holmium. From this result, mutual separation

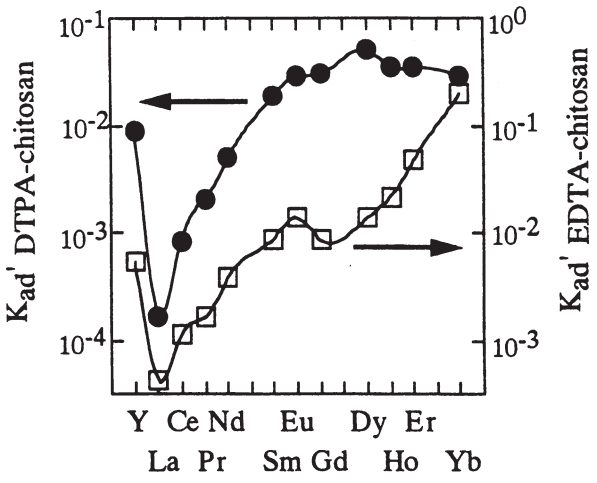

Figure 3. Apparent adsorption equilibrium constants of rare earths on EDTA- and DTPA-

between yttrium and samarium as well as that among lanthanum, cerium, praseodymium and neodymium was carried out by using the column packed with adsorption gels of EDTA- and DTPA-chitosan, respectively, to test their actual separation abilities as will be mentioned later.

Figures 4 and 5 show the logarithmic plot of distribution ratio of some rare earths against equilibrium pH in the adsorption on EDTA-polyallylamine and DTPA-polyallylamine, respectively, under the same condition with EDTA-chitosan and DTPA-chitosan. Different from the cases of EDTA- and DTPA-chitosan, nearly all plots except for lanthanum appear to cluster on a single curve, suggesting very difficult mutual separation among these rare earths with this adsorbent. From this result, it is inferred that the excellent separation ability exhibited by EDTA- and DTPA-chitosan is not attributable to the chelating functional groups of EDTA or DTPA but to the "synergistic effect" of these functional groups in combination of polymer matrices of chitosan.

Figure 6 shows chromatogram for the separation of samarium and yttrium by using the column packed with EDTA-chitosan together with the change in pH in the eluent. As seen from this figure, even these two metal ions the mutual separation of which appears very difficult from the result of batch experiment as mentioned earlier can be clearly and easily separated under the condition described in the figure caption by using the column packed with EDTA-chitosan.

Figure 7 shows chromatogram for the separation of 4 kinds of light rare earths, i.e. lanthanum, cerium, praseodymium and neodymium, by using the column packed with DTPA-chitosan together with the change in pH in the eluent. As seen from this figure, the peaks of chromatogram corresponding to these 4 kinds of light rare earths are satisfactorily apart from each other. Among them, it should be especially noteworthy that even the pair of neodymium and praseodymium the separation of which is the most difficult among rare earths can be satisfactorily mutually separated by this column using dilute sulfuric acid solution as eluent.

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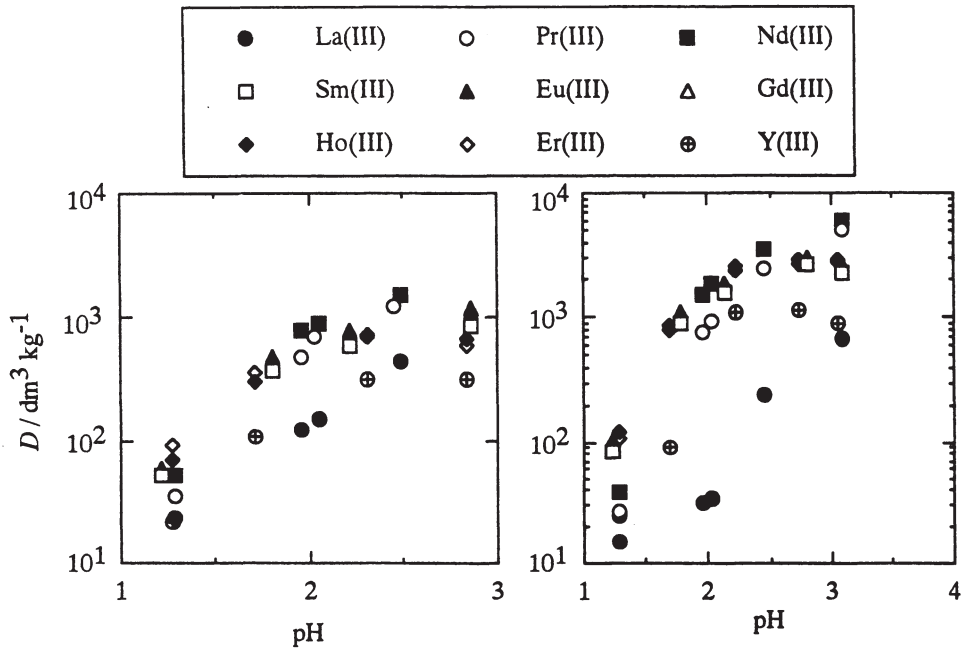
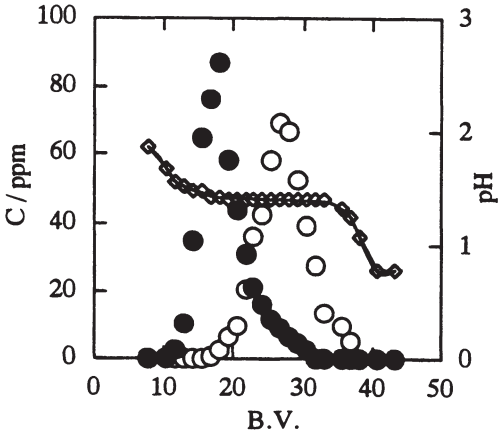


Figure 4. pH dependency of the distribution ratio of some rare earths in the adsorption from dilute sulfuric acid solution on EDTA-polyallylamine

Figure 5. pH dependency of the distribution ratio of some rare earths in the adsorption from dilute sulfuric acid solution on DTPA-polyallylamine



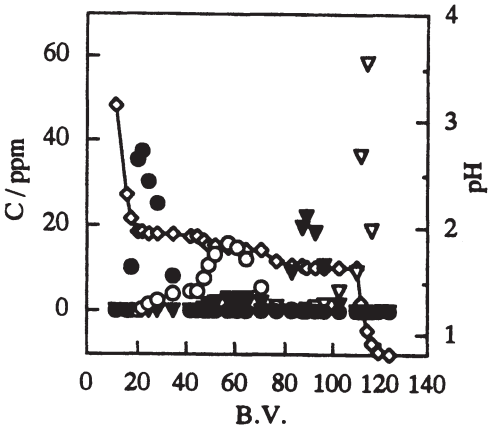


Figure 6. Chromatogram for separation of samarium and yttrium by using the column packed with EDTA-chitosan. Dry weight of the packed adsorbent: 0.30 g. Flow rate: 6.0 ml/h. ●: Y, ○ : Sm, ◊ : pH

Figure 7. Chromatogram for separation of 4 kinds of light rare earths by using the column packed with DTPA-chitosan. Dry weight of the packed adsorbent: 0.30 g. Flow rate: 6.0 ml/h. ●: La, ○ : Ce, ▼ : Pr, ▽: Nd, ◊ : pH

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The effects of detergents on chitosan

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Summary
The effects of a number of washing agents and of sodium perborate, the most common bleaching agent used in household detergents, has been studied on both particulate chitosan and chitosan-coated cotton. The results show that with perborate-containing detergents there is a considerable decrease in the chain length of the chitosan and a steady loss of chitosan itself from the coated fabric. However the chitosan is resistant to washing agents that do not contain sodium perborate or, presumably, other oxidative bleaches.

Introduction

There is considerable interest in utilising chitosan in the textile area. Applications that have been examined include the production of fibres, both alone and as a component of blend fibres [1], and its use as a finishing agent to impart shrink resistance to woollen fabrics [2] and to improve the dyeing behaviour of fibres [3,4]. The topic has recently been reviewed in detail by Hudson [5].

If chitosan is to be used in the field of consumer textiles, as distinct from that of technical textiles or medical textiles, then it is almost certainly going to undergo regular laundering during its lifetime, and must be able to withstand such treatments. Detergents are normally complex mixtures and may contain, in addition to the surfactant: bleaching agents, fluorescent brightening agents, enzymes, and inorganic salts such as phosphates and silicates. In such mixtures it is the bleaching agent that is most likely to attack chitosan.

Chitosan is known to be attacked by a number of bleaching agents such as sodium hypochlorite, hydrogen peroxide and sodium perborate [6], the latter being one of the most common bleaching agents incorporated in household detergents. It was therefore of interest to investigate the effect of a range of commercial detergents on chitosan.

Materials and Methods

Materials

The chitosan used for both particle treatment and fabric coating was a high viscosity sample of Protan Pro-floc P, (Lot No. 047-342-07G).

The cotton was a plain weave fabric (123 g/m^2; warp: 94 ends/inch; weft 74 picks/inch) and was prepared for dyeing and printing by conventional scouring and bleaching treatments. Coating was carried out by padding with a 10 g/L chitosan solution in aqueous acetic acid, neutralising and crosslinking with glutaraldehyde.

The dye (C.I. Acid Orange 7 [Orange II]) was a commercial sample that had been purified by recrystallisation from aqueous ethanol.

The washing agents were selected to give a representative cross-section of the various permutations found with commercially available products (A, F, O), together with 'ECE reference detergent for testing for colour fastness to domestic and commercial laundering to ISO 105: CO6 : 1981' (D), natural soap flakes (X) and sodium perborate (P). The important components of each are given in Table 1.

Table 1. Major constituents of the washing agents used.

Washing agent	Type	Major constituents		
		Oxygen-based bleach	Enzyme addition	Fluorescent brightening agent
A	Synthetic detergent	–	✓	✓
D	Synthetic detergent	–	–	–
F	Synthetic detergent	✓	✓	✓
O	Synthetic detergent	✓	–	✓
P	Sodium perborate only	✓	–	–
X	Natural soap	–	–	–

Methods

The % deacetylation and amine group concentration values were determined by dye adsorption [7].

The % solubility was determined by dissolving 2 g of chitosan in 200 mL 1% (v/v) acetic acid, stirring for 24 hours, then filtering the solution and determining the dry weight of insoluble material retained by the filter.

Solution viscosities were determined on 1% (wt/v) solutions in 1% (v/v) acetic acid using a Brookfield Rotating Viscometer.

The CIE Whiteness Index [8] and the Colour Strength measurements were carried out using a Spectraflash SF600 reflecting spectrophotometer (Datacolour International Ltd).

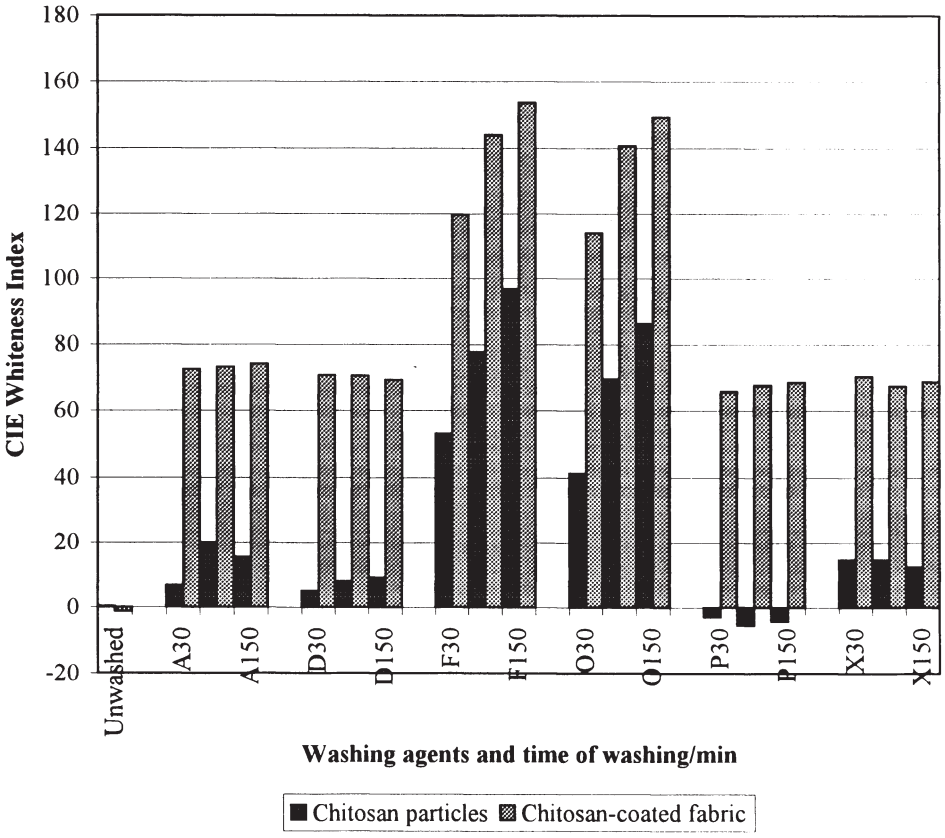
All wash tests were carried out at 60°C to simulate British Standard Method BS 1006 : 1990 (CO6 : Colour fastness to domestic and commercial laundering) [9]. Samples were given from 1 to 5 washes, each of 30 minutes duration. For example, A30, A90 and A150 refer to samples washed for totals of 30, 90 and 150 minutes respectively using washing agent A. Fresh solutions containing 5 g/L of washing agents or 0.5 g/L sodium perborate were made up for each 30 minute wash.

Results and Discussion

Chitosan in particle form was given up to five 30-minute washes at 60°C in each of the washing agents, and the samples analysed for whiteness, amine group concentration and viscosity. The effect of the washing treatments on the whiteness of chitosan was assessed instrumentally, and the results are shown in Figure 1. The large increase in whiteness obtained with detergents A, F and O is due to the presence of fluorescent brightening agents (FBAs), with varying affinities for chitosan, in these detergents. The decrease in whiteness relative to

the unwashed reference sample that occurs on treatment with perborate alone suggests that there is some oxidative degradation of the chitosan occurring, leading to discolouration of the samples. This discolouration is masked by the much greater brightening effect of the FBAs in detergents F and O which also contain sodium perborate.

Figure 1. The effect of washing on the CIE Whiteness Index values for chitosan particles and chitosan-coated fabric

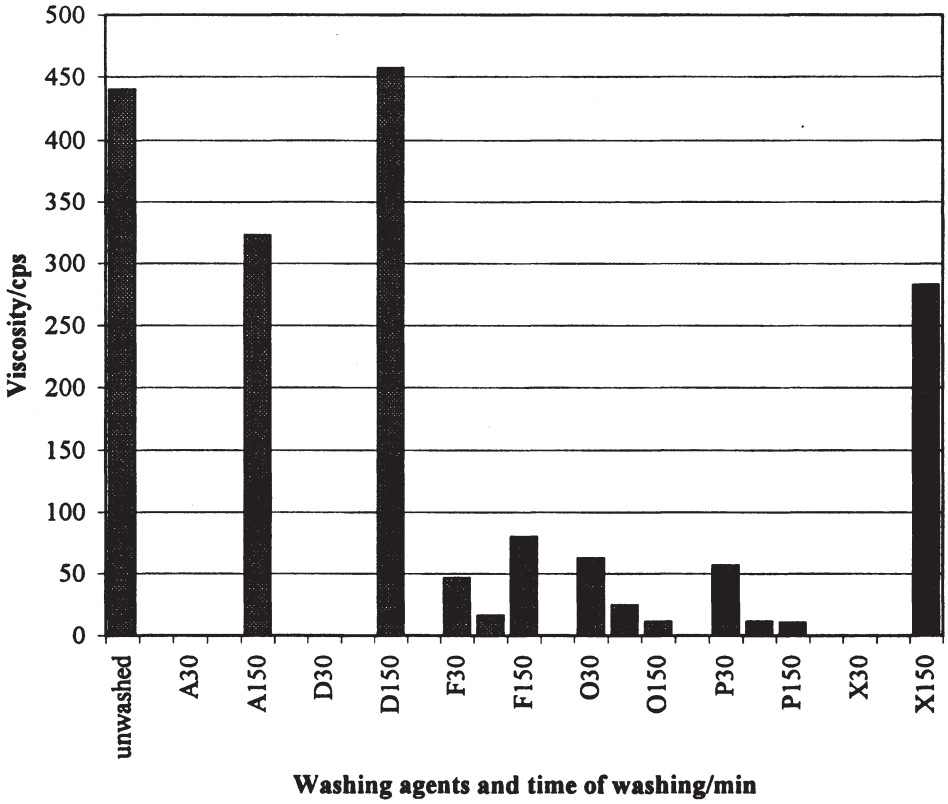


The results of the amine group concentration analysis are shown in Figure 2. In all cases, except for F150, there is very little change in the amine group concentration, which ranges from 4.18 mols kg^{-1} – 3.98 mols kg^{-1}. Even in the case of sample F150 the amine group concentration had only dropped to 3.81 mols kg^{-1}. These results suggest that only a small fraction of the amine groups have been attacked during the washing process.

Figure 2. The effect of washing on the amine group concentration of chitosan particles

However the results of the viscosity measurements show that sodium perborate, and the detergents containing perborates, cause considerable chitosan chain degradation under conventional washing conditions. The effect is very apparent even after only one wash cycle, causing a drop of approximately 90% in the concentrated solution viscosity (Figure 3).

Figure 3. Solution viscosity of chitosan particles after treatment with washing agents

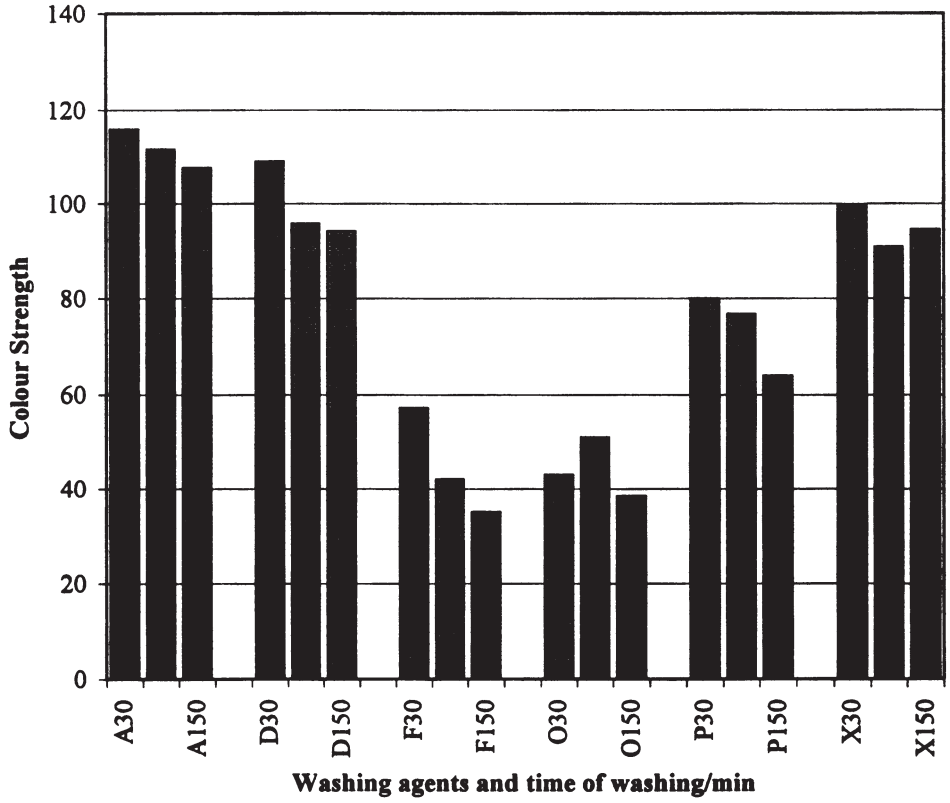


The effects of sodium perborate and the other washing agents on chitosan in the form of a crosslinked coating on cotton fabric was then examined by dye adsorption. The basis of the technique, which involves dyeing under acid conditions after the washing treatments, is the assumption that there is a 1:1 stoichiometry between the monosulphonated dye used, C.I. Acid Orange 7 (Orange II) and protonated amine groups of the chitosan coating. This dye has no affinity for the cotton fabric substrate so the extent of dye adsorption is a measure of the amine group content of the coating. It is measured by reflectance spectroscopy and the Colour Strength determined. This is the ratio, expressed as a percentage, of the intensity of colour of a given sample relative to that of the unwashed, dyed standard.

The results are given in Figure 4. These show that the perborate-containing detergents F and O cause a considerable reduction in the Colour Strength, more so even than the perborate alone. Taking these results, together with the amine group concentration and viscosity results (Figures 2 and 3) suggest a model for the degradation process due to perborate. The attack occurs at the amine groups, breaking the chains and reducing the solution viscosity. Where two breaks occur close together the relatively short chain segment

produced may be washed away. This will not affect the overall amine group concentration to any significant extent (Figure 2) but will reduce the total amount of chitosan present and hence reduce the amine groups available to act as dye sites (Figure 4). The presence of detergent will aid the removal of short chain segments, and hence the greater decrease in the Colour Strength due to perborate-containing detergents compared to that due to sodium perborate itself. It is reasonable to assume that laundering of already dyed chitosan-based textile materials would give considerable loss in Colour Strength if perborate-containing detergents were used.

Figure 4. Colour Strength values for chitosan-coated fabric after treatment with various washing agents



Conclusions

Chitosan-based textile materials may be safely laundered provided that a perborate-free detergent is used. In general these are marketed as suitable for coloured materials. However, use of a perborate-containing detergent will lead to rapid breakdown and loss of the chitosan. It is likely that sodium percarbonate, which is another bleaching agent commonly used in detergents, would have a similar degradative effect on chitosan.

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Chitosan-alginate PEC films prepared from chitosan of different molecular weights

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Summary

In a previous study, we have successfully prepared homogeneous chitosan-alginate polyelectrolyte complex (PEC) films by casting suspensions containing chitosan-alginate coacervates. The objective of this study was to evaluate the characteristics of chitosan-alginate PEC films prepared with chitosan of different molecular weights. The shorter chain chitosan produced finer coacervates with the sodium alginate, resulting in more homogeneous PEC films. Film homogeneity was confirmed with scanning electronic microscopy and atomic force microscopy. Further differentiation in film characteristics was seen in the capacity for water uptake in different aqueous media.

Introduction

Chitosan is derived from chitin, the second most abundant natural polymer after cellulose. Chitosan is comprised mainly of 2-amino-2-deoxy-D-glucose units, and it can be dissolved in inorganic and organic acids following the protonation of the primary amino groups [1]. When dissolved in acids, chitosan forms a linear cationic polyelectrolyte. Alginate is also a natural linear polymer. Extracted from brown algae, it is made up of two uronic acid monosaccharides: D-mannuronic (M) and L-guluronic (G) acids. Though alginic acid itself is insoluble in water, some salts of alginates, e.g. potassium and sodium alginate, dissolve in water to give polyanionic polymers. Both chitosan and alginate are biodegradable and biocompatible, and have potential applications in fields such as food, agriculture and medicine.

The polycationic chitosan is capable of reacting with a variety of oppositely charged polymers such as carrageenan [2], alginate [3], and even DNA molecules [4]. Several studies have reported on the properties and applications of the chitosan-alginate polyelectrolyte complex. In almost all of the studies, the complex was prepared in the form of beads, spheres and capsules [5]-[8]. Such systems are advanced when used to formulate tablets or injectables. But when applied as wound dressings, tablet coatings or membrane-controlled drug release systems, the presence of too much interface and unreacted components can be disadvantageous. For these applications, a homogeneous chitosan-alginate PEC material is preferred.

We have developed a method to prepare films from the chitosan-alginate coacervates [9]. The homogeneity and other properties of the films were dependent on the processing parameters. Since the molecular weight of chitosan is known to affect its properties and applications [10], the objective of this study was to investigate the effect of chitosan molecular weight on the characteristics of the chitosan-alginate PEC films.

Materials and Methods

Chitosan (Aldrich Chemical Company, Inc., Milwaukee, USA) was further deacetylated by refluxing twice with 40% w/v sodium hydroxide in the presence of 1% $NaBH_4$, intermitted by washing with plenty of distilled water. The polymer was then dissolved in 3% v/v acetic acid, regenerated with 1N sodium hydroxide, washed with distilled water and freeze-dried. Three batches of chitosan were used: high molecular weight (HM) (Lot No. 11403DS), medium molecular weight (MM) (Lot No. 06126MN) and low molecular weight (LM) (Lot No. 07421MR) samples. The M_v of the purified chitosans was measured by solution viscometry (Cannon Ubbelohde, 30°C, 0.2M CH_3COOH/0.1M CH_3COONa as solvent, $K = 6.6 \times 10^{-3}$, $a = 0.88$) [11] while their degrees of deacetylation were determined by the first derivative UV spectrophotometric method [12]. Sodium alginate (Lot #2445310L, BDH Ltd., Poole, England) was used as received. It has an intrinsic viscosity of 3.23, which may be approximated to a M_v of 1.04×10^5 (Cannon Ubbelohde, 25°C, 0.1M NaCl solvent, $K = 6.9 \times 10^{-6}$, $a = 1.13$) [13]. All other chemicals and reagents were of analytical grade.

Chitosan solutions (0.25% w/v) were prepared by adding equal volumes of acetone to filtered solutions of the polymer (0.5% w/v) in 2% v/v aqueous acetic acid. Solutions containing 0.25% w/v alginate were prepared by dissolving the sodium alginate in distilled water. Coacervation was effected by adding dropwise 25ml of the chitosan solution to 25ml of alginate solution under vigorous manual agitation. The resultant suspension was stirred for 20 min, cast into a petri dish (Sterilin, UK, internal ∅ 85mm) and dried overnight at ambient temperature (25°C). After drying, the film was immersed in 50ml distilled water for 1h, washed thrice with distilled water and dried at 30°C under vacuum.

The PEC films were measured for weight (Mettler-Toledo) and thickness (Mitutoyo thickness gauge), and stored in desiccators at ambient temperature until use. Surface morphology of the films, coated with gold-platinum (Ion sputter JFC-1100, 5 min), was observed under the scanning electron microscope (SEM) (JEOL JSM-T220A, 15kV and 200x magnification, Mamiya camera). Surface images of the films were also acquired using the atomic force microscope (AFM) (Digital Instrument NanoScopeIII, scan rate: 0.5-0.8Hz, number of acquisition: 256, image data: height).

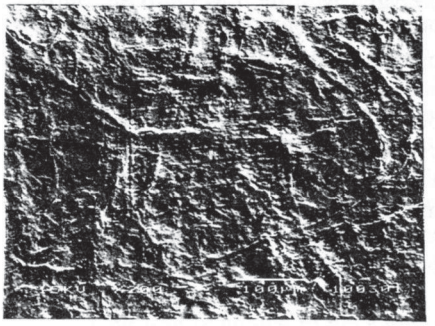
The capacity of the PEC films for water uptake in distilled water, 0.1N HCl and pH 7.4 phosphate buffer solutions were determined. Triplicate samples of films (∅ 30mm) were stored in desiccators until constant weight (W_o) before immersion in 100ml of test media at 37.0 ± 0.1°C. The extent of water sorption is expressed as the change in film weight, measured at periodic intervals by retrieving the films from the media, and weighing the films after blotting with filter paper (W_t).

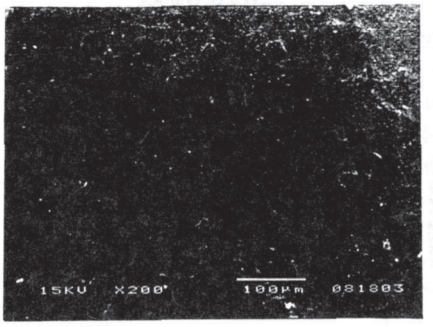
Results and Discussion

The M_v values for the chitosan samples were $1.30 (\pm 0.06) \times 10^5$, $5.33 (\pm 0.21) \times 10^5$ and $10.0 (\pm 0.11) \times 10^5$ for the LM, MM and HM chitosans respectively. All three batches were highly deacetylated, possessing the respective degrees of deacetylation of 95.14 ± 0.21 %, 93.43 ± 0.03 % and 94.50 ± 0.03 %.

Under the conditions employed, the HM chitosan gave gel-like, clumpy coacervates while the LM chitosan gave finer, more fibrous coacervates with the same alginate sample. The suspension of LM chitosan-alginate coacervates was milky and more homogeneous compared to that of HM chitosan-alginate coacervates. Coacervates of MM chitosan and alginate were intermediate in character.

SEM photographs of the PEC films prepared from suspensions of the coacervates are given in Figure 1. The air-surface of PEC films prepared with HM chitosan showed striations





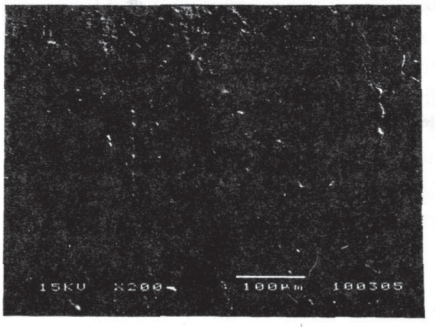
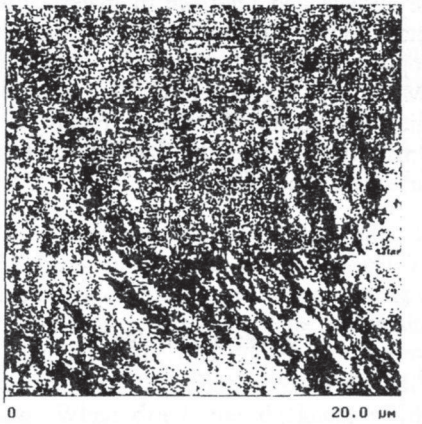


Figure 1 SEM of HM(top), MM(middle) and LM(bottom) PEC films



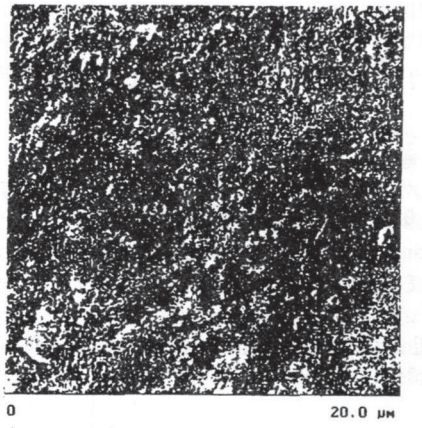


Figure 2. Phase Imaging of HM(top) and LM-PEC(bottom) films by AFM.

and greater degree of undulation compared to films prepared with the LM chitosan. Surface roughness was of the order: LM-PEC < MM-PEC < HM-PEC, while the reverse order was seen for surface homogeneity. The atomic force microscope (AFM), a powerful tool to study the surface and interface of materials at the nanoscale range, was able to provide greater details of the film surface. The topography of all 3 types of PEC films, as observed under the tapping mode, did not indicate significant variations. Phase imaging, however, showed the LM chitosan-alginate PEC films to have a more homogenous image than the MM- and HM chitosan-alginate PEC films (Figure 2). This confirms that the LM chitosan-alginate PEC films, prepared with the finer coacervates, were more homogeneous in both composition and surface morphology.

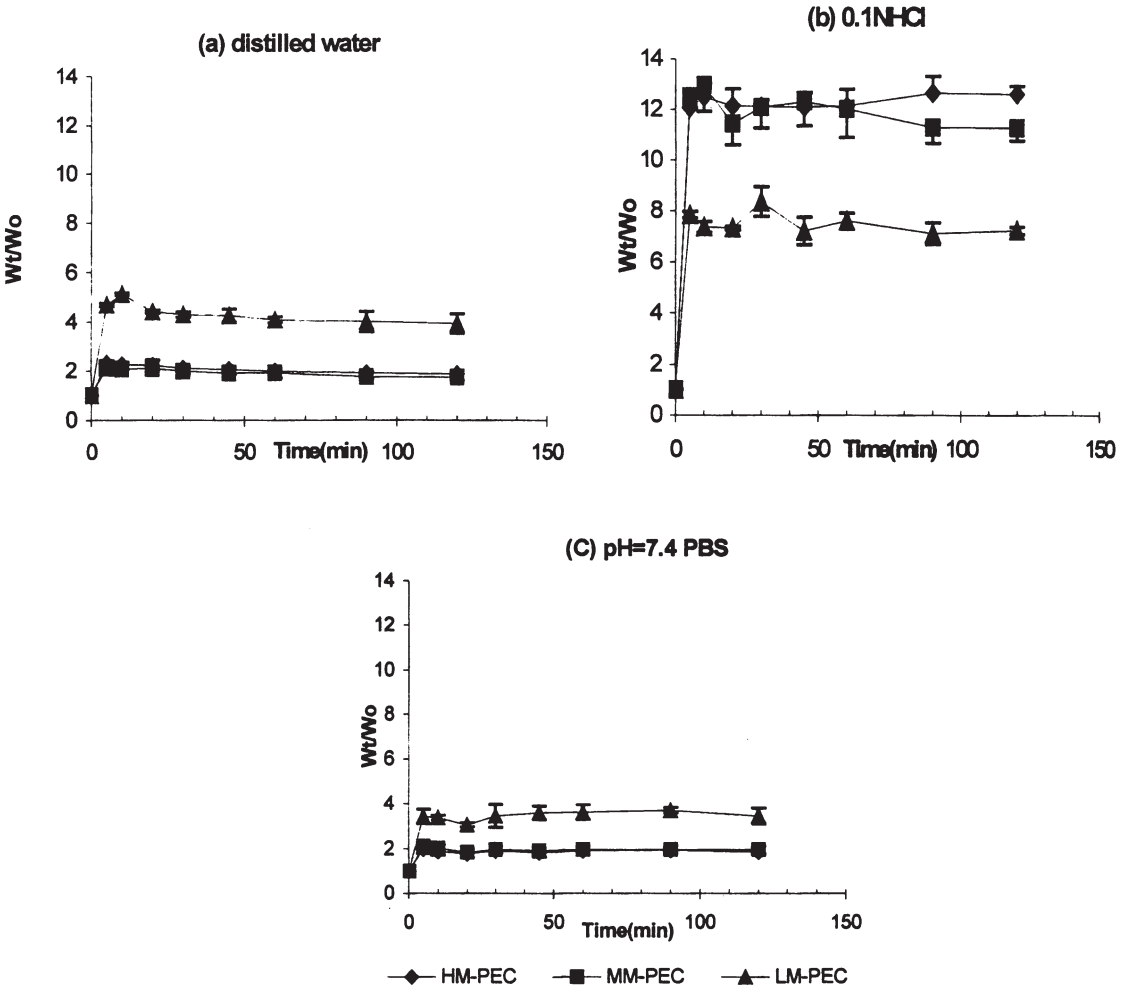


Figure 3. Water Uptake capacity of different molecular weights chitosan-alginate PEC films in different aqueous media.

Figure 3 shows the water uptake capacity, expressed as the ratio of W_t/W_o, of the PEC films as a function of immersion time in aqueous media. Compared to the MM- and HM-PEC films, the LM-PEC films had higher water uptake in distilled water and pH 7.4 phosphate buffer, but lower water uptake in 0.1N HCl. On the other hand, water uptake by the MM- and HM-PEC films were similar in the 3 aqueous media. It is not surprising that the amount of water imbibed by all 3 types of films was highest in 0.1N HCl. The acid medium could protonate unreacted amino groups in the films, thereby promoting film expansion and hydration. The differential results obtained in this medium implied that the number of unreacted amino groups increased in the order of LM-PEC < MM-PEC ≈ HM-PEC. The LM chitosan is likely to have a more extended conformation in acetic acid, thereby allowing it to react more completely with the alginate molecules.

Chitosan is known to have poorer water uptake in neutral or basic aqueous media. To explain the difference in water uptake amongst the films in distilled water and pH 7.4 phosphate buffer solution, it would be necessary to include a discussion on the processing of the chitosan-alginate PEC films by distilled water during preparation. The immersion followed by washing of the dried PEC films with distilled water was intended to remove excess acetic acid from the films. It was noted that the LM-PEC films expanded with a significant increase in surface area upon processing. When dried, the thickness of the processed films was 24.9 ± 3.81μm. Film expansion was not noted for similarly processed MM- and HM-PEC films, which had the respective thickness of 33.3 ± 4.60 μm and 41.5 ± 6.65 μm. It could be implied that the MM- and HM-PEC films were more condensed and therefore less amenable to the penetration of water molecules in distilled water than the LM-PEC films. Upon processing, the LM-PEC films became even more porous, accounting for their greater ability to imbibe water compared to the other two types of films at near neutral pH range.

Conclusion

The molecular weight of chitosan can affect the properties of PEC films prepared from chitosan-alginate coacervates. Chitosan samples with M_v values of 1.30 (± 0.06) x 10^5 reacted more completely with the alginate to give finer coacervates, leading to the formation of more homogeneous films compared to PEC films prepared with chitosans of higher M_v *viz.* 5.33 (± 0.21) x 10^5 and 10.0 (± 0.11) x 10^5. The latter films were also less permeable to water at neutral pH range. Films prepared with the two higher molecular weight chitosans had similar properties, indicating that a threshold molecular weight value must be reached before differences in film properties are observed.

Acknowledgements

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Enzymes of chitin metabolism for the design of antifungals

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Summary
The premises are stated upon which the design of antifungals rests, and the three basic strategies followed to that aim are outlined: (1) the conventional, (2) the biochemical target-based and (3) the structure-based, 'rational' approaches. The status is pinpointed of inhibitors of specifically fungal enzymes at the lead discovery level of these strategies. The reasons are detailed for considering the triple enzyme system chitin synthase(CS)-chitinase-ß-*N*-acetylhexosaminidase (HexNAc'ase) a 'supertarget' for future antifungal drug research. The present paper provides comprehensive lists of the best inhibitors of these enzymes (only compounds are mentioned displaying K_i/IC_{50} values ≤ 100 µM for CS, $\leq \sim 50$ µM for chitinase, and ≤ 1 µM for HexNAc'ase). Whereas CS-inhibitors represent a gamut of structural motifs, those of chitinase are exclusively allosamidin-based, and those of HexNAc'ase are glycon-based substrate analogues, intermediates or close transition-state mimics. The evaluation of these lists identify the following compounds as the presently best leads for further investigations into the design of novel antifungals targeting chitin metabolism (numbers in bold print concern the compound no. in the corresponding table; numbers in square brackets are K_i's or EC_{50}' [µM]): CS inhibitors – Calcofluor White (**6**[6]), a dibenzazetine (**11**[6]), a tetrahydropyrane (**14**[4]), oleanoic acid (**7**[2]), amphotericin B (**2**[1]), pentachloronitrobenzene (**3**[1]) and nikkomycin $MeOB_x$ (**1**[0.4]); chitinase inhibitors – allosamidin (**1**[~ 0.02]) and the dibiotinylated derivative of **1** (**13**[0.007]); HexNAc'ase inhibitors – de-branched nagstatin (**15**[0.006]), 2-acetamido-2-deoxynojirimycin (**5**[~ 0.005]) and a derivative of *N*-acetylglucosaminono-1,5-lactone-*O*-(phenylcarbamoyl)oxime (**11**[0.004]).

Introduction

The rationale and strategies of antifungal drug design

The need for novel specific agents in the treatment of fungal diseases in agriculture and human medicine is well documented; this necessity concerns not only the number but, even more importantly, the short-comings of present-day fungicides and antimycotics (ref's in [1,2]). Setting apart strategies for the therapy of fungal diseases that are directed towards enhancing host defense mechanisms, as well as disregarding sheer serendipity and the fact that a prepared mind is prone to meet chance, the actual quest for new antifungals relies heavily on the design of antifungals. This is based on two premisses: (1) an inhibitor of an enzyme that is specifically fungal as well as essential for growth, development or infectivity is a potential antifungal (provided it can reach the site of its action as an intact entity and in a sufficiently high concentration), and (2) specificity at the site of action *in vivo* of an inhibitor of an enzyme that,

per se, is not specific for fungi can arise from differences between the pathogen and the host in the cellular location of the target and the biochemical context in which the agent acts. Currently, mainly three approaches are followed for the discovery of new antifungals:

(1) The conventional design: the systematic chemical modification of established antifungals (found previously by random screening campaigns) towards enhancing their efficacy and reducing adverse side effects according to quantitative structure-activity relationships (QSARs).

(2) The biochemical target-based design: the search for sites of action not explored yet in antifungal therapy (see [3]), followed by the selection of leads detected by screening, particularly automated high-throughput screening (HTS) of suitably arranged libraries of low-molecular-weight compounds (MW $\leq \sim 10^3$ Da; natural products and chemosynthetics [4,5]), using the activity of the target enzyme as the discriminator, followed by optimization of leads through QSAR studies.

(3) The structure-based or 'rational' design: the biochemical and physicochemical elucidation of target enzyme-ligand interactions [6,7], ideally with a 3D model [8,9], and the contrived synthesis of high-affinity binding compounds, i.e. of structures that mimick the shape and the charge of the substrate at the transition state of the reaction [10].

The main body of this article presents examples of inhibitors of chitin metabolism discovered by these strategies.

Enzymes of chitin metabolism – targets for the design of antifungals

On economic considerations, only broad-spectrum antifungal products are likely to be developed by major companies [11]. Thus, at the level of drug discovery, the search for novel targets/agents turns out to be largely identical in pharmaceutical and agrochemical industries, and the source of the enzyme serving as the 'screen' for HTS or as the model for structure-based design becomes of minor importance. The decision to develop a new antifungal compound into a product for application in medicine or in agriculture will then be taken at a later stage. Allowing for the tenet that a particular antifungal agent should not be used for both purposes, the final conclusion reached will depend on other considerations, among which its host toxicity and pharmacological characteristics, the possibility of conferring or increasing pathogen specificity through a proper formulation of the agent and the influence of this on nontarget organisms present in the host's environment, as well as on economic factors.

Critical to success with approaches (2) and (3) to the discovery of specific antifungals, of course, is the proper identification and selection of target enzymes (for some concepts of target evaluation and validation, see [1,3]). For the following reasons, the metabolism of chitin is generally considered a 'supertarget' in the search of novel antifungals (as pointed to earlier by others [12,13]:

(1) In antagonistic interactions of fungi with plants and vertebrates, chitin is restricted to the pathogen – the prospects are good, therefore, to specifically control the parasite through inhibition of its chitin-synthesizing ability without disturbing host metabolism.

(2) Chitin represents the fibrillar skeleton of the fungal surface structure, maintains the shape of the cell and offers protection against adverse environmental conditions – interference with the synthesis of the polymer must thus affect ist physiological function.

(3) *In situ,* chitin becomes covalently bound to other, equally essential compounds, among which a β(1-3)-β(1-6)-glucan heteropolymer, the cross-links thus generated contributing significantly to the mechanical strength of the wall fabric [14-16] – a situation that

intrinsically potentiates any damage resulting for the cell from an impairment of its ability to synthesize chitin.

(4) Fungi dispose of a complex chitinolytic system, which is constitutively expressed during growth , differs from that involved in nutrition or gross autolysis ([12,17]; for further ref's, see [18]) and which can provide for a dynamic re-modelling of the tight chitin network, as required in hyphal tip elongation and branching as well as in budding of yeasts [12,19,20] – thus, exogenous agents that interact with the chitinolytic system of growing fungi will alter the status of chitin in the wall and, thence, diminish its performance.

(5) *In vivo*, the lysis and the synthesis of chitin are linked in a co-ordinately regulated manner, to prevent excess softening or rigidification of the wall at sites of growth (for a biochemical model of the concerted action of the enzymes concerned, see Fig. 3) – interference with the proper function of either, lysis or synthesis, will inevitably affect also the other.

(6) The search for inhibitors of chitin metabolism as potential agents acting against fungi can benefit parallel efforts to develop new products for the control of animal organisms synthesizing chitin that feed on crops (particularly insects) or cause diseases in humans, among which amebiasis [21,22], giardiasis [23], trichomoniasis [24], *Pneumocystis carinii* pneumonia [25], and filariasis [26], or produce chitinolytic enzymes to infect a chitinous structure of the transmitting vector [27,28]. – In view of the structural similarity between chitin and peptidoglycan and the role the murein hydrolysing system plays during growth of bacteria [29], inhibitors of chitin catabolizing enzymes might, finally, be of interest also for investigations into new antibacterials.

A portrait of the key enzymes of chitin metabolism – chitin synthase (CS), chitinase and β-*N*-acetylhexosaminidase (HexNAc'ase)

Theoretically, all enzymes of the chitin biosynthetic and catabolic pathways as well as of effector-producing reactions could serve as targets in antifungal drug design (e.g., glucosamine-6-phosphate synthase [30-32]). *Prima vista* at least, the chances for success appear to be considerably better if the choice of an enzyme as a target is based on additional considerations, among which are (1) the feasability of minimizing interference of prospective inhibitors with metabolic pathways of the host, (2) the possibility of providing for a sufficiently high concentration of the agent at its site of action, (3) the availability of blueprints for the chemical synthesis of transition state mimics, the design of which requires a fair knowledge of the reaction mechanism of the enzyme concerned, and (4) the amount of information on the 3D structure of the target. Accounting for these points and the role chitin plays in fungal live (see second chapter of the Introduction), CS, chitinase and HexNAc'ase are, as a whole, held to be particularly well suited to act as targets, even though the latter enzyme is probably always present in the host as well (humans, animals, plants; see list in [1]).

Reactions catalyzed and structural features

Chitin synthase (CS). UDP-*N*-acetylglucosamine (UDPGlcNAc) represents the obligate substrate of CS; the absolute requirement of the enzyme for Mg^{2+}, Mn^{2+} or Co^{2+} as a cofactor is probably due to the formation during catalysis of a six-membered pyrophosphate-metal complex, as is believed to be the case with glycosyltransferases in general [33]. Chain assembly occurs without the formation of free chito-oligomers [34], and the experimental evidence implies that CS acts on a covalently bound primer, which, according to the definition [see caption to Fig. 1(1)] is chitin (i.e. R_1 = CS). For reasons detailed elsewhere [35], the existence

has been postulated of a priming protein as a fundamental property of the CS system (analogous to the situation with the synthesis of glycogen and some other polysaccharides; ref's in [36-38]). The as yet hypothetical enzyme, for which the name 'chitogenin' (CG) has been coined [Fig. 1(1), R_1 = CG], would generate covalently-bound chito-oligosaccharide as a primer for CS *sensu stricto* (= R_2). The question remains open whether the synthesis of chitin proceeds through the formation of a lipid-linked chito-oligomer bound to a protein associated with the CS complex [Fig. 1(1); R_1 = a protein; ≠ CS ≠ CG]. – Besides non-allosteric activation (or priming) as well as latency, homotropic-heterotropic regulation with GlcNAc as the positive allosteric effector is the main and mechanistically well studied regulatory feature of CS (see [34] for the stereochemical requirements of the allosteric site of CS for ligand binding).

Knowledge of the structural features of CS is quite limited and mainly concerns amino acid sequences deduced from cloned chitin synthesis genes (see [35] for a 3D model of the catalytic site of CS that has been based on these sequence data as well as some additional information from other β-glycosyltransferases). Most previous reports of the isolation of purified CS (ref's in [35,39,40]) are rather inconclusive because the evidence for a particular constituent protein of the enzyme preparation to represent CS *sensu stricto* [Fig.(1): = R_2] remained somewhat circumstantial. The use of a new procedure for the purification of the enzyme has now allowed the positive identification of a 60-kDa protein as a genuine CS polypeptide embodying the catalytic site responsible for chain elongation (Fig. 2; [39,40]). Applying a combination of molecular biology methods (particularly PCR protocols) as well as protein and enzyme technology, the prospects appear good, therefore, of thus gaining bulk quantities of CS for HTS of libraries, or establishing a refined 3D odel of the active site(s) of the enzyme suitable for a truly 'rational' design of such ligands [according to strategies (2) and (3) of Introduction].

Chitinase. As depicted in Fig. 1(2), the enzyme catalyzes the hydrolysis of its substrates according to an *endo*-mechanism, with $(GlcNAc)_2$ and (traces of) the trimer as the end product(s). The occasionally used term "*exo*-chitinase" is misleading, inasmuch as it implies cleavage of chitin or soluble fragments thereof ($n \leq \sim 10$) by a processive mechanism of hydrolysis, starting at the non-reducing end of the substrate and successively releasing GlcNAc or chitobiose (see also [41]). However, the existence of the latter type of chitinase has yet to be demonstrated conclusively, and enzymes displaying *exo*-activity with hydrolytic products of chitin by releasing monomers should, more appropriately, eventually be referred to as HexNAc'ases [Fig. 1(3)] with substrate chain-length specificity extending to chito-oligomers with $n > 2$. As yet, neither of these (hypothetic) chitinases has been listed in Enzyme Nomenclature. Apart from terminological nonchalance, three major causes [(1)-(3)] appear to be responsible for the wide-spread confusion existing in the terminology of *exo*- vs *endo*-chitinase. (1) Most chitinase preparations used for mechanistic studies have not represented pure entities and in all probability have been contaminated with HexNAc'ase (in fact, the isolation of homogeneous chitinase from other sources than culture media is quite elaborate; for examples, see [42]). Even if present in a very small amount only, HexNAc'ase will hydrolyze the end product of chitinase action and, thus, mimick an *exo*-action of this. (2) Chitinase as well as HexNAc'ase have transglycosylating activity; the product pattern obtained with 'semi-purified' chitinase can be faulty, therefore, and is not suitable, in any case, for assessing the *regio* specificity of the attack. (3) Chitinase is usually assayed chromogenically or fluorogenically. Although methods of choice for the rapid and sensitive estimation of chitinase activity, these assays cannot, however, be used reliably to investigate the mode of action of the

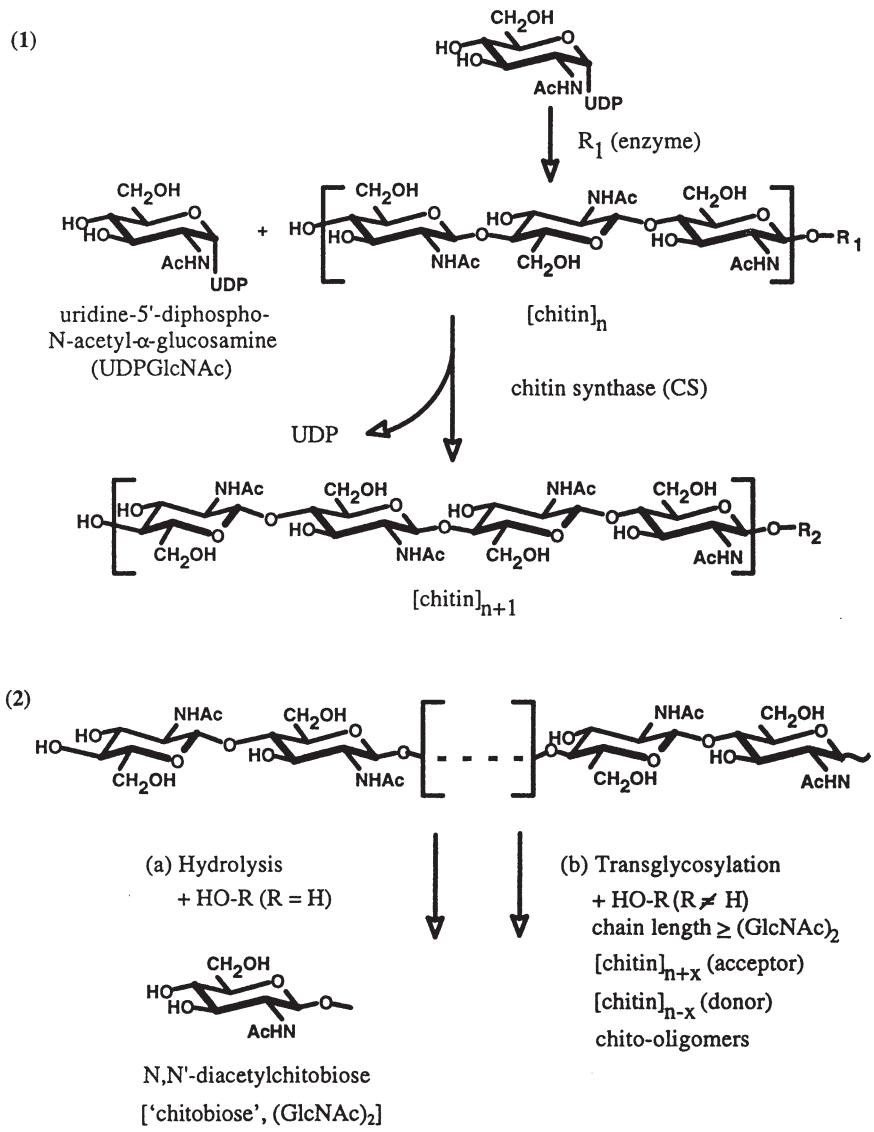
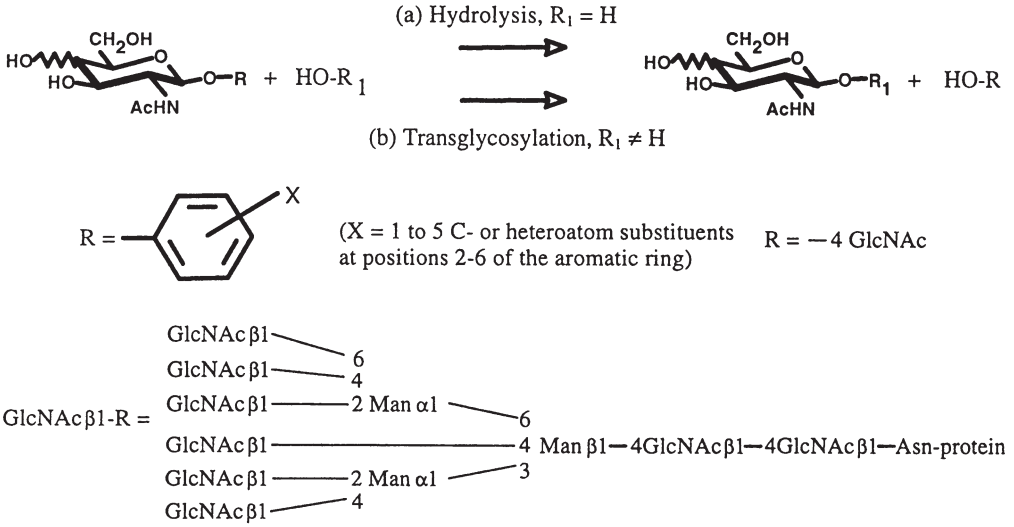


Figure 1. Schemes of the reactions catalyzed by chitin synthase (CS), chitinase and β-*N*-acetylhexosaminidase (HexNAc'ase) [(1)-(3)]. (1) CS: UDP-*N*-acetyl-D-glucosamin:chitin β-*N*-acetylglucosaminyl-transferase (EC 2.4.1.16); R_1 = H, CS (= R_2), priming UDPGlcNAc-transferase ('chitogenin', CG; ≠ CS) or another protein (≠ CS, ≠ CG) (refer to [35] for details and ref's]). (2) Chitinase (EC 3.2.1.14): catalyzes random hydrolysis of the polymer and of chito-oligomers (substrate chain length ≥ 4) with the dimer as the end product and has intrinsic transglycosylating activity (x, chain length of the chitin or chito-oligomer residue transferred). (3) HexNAc'ase (EC 3.2.1.52): catalyzes the hydrolysis of terminal nonreducing HexNAc residues in *N*-acetyl-ß-D-hexosaminides; also functions as a HexNAc-transferase (scheme from [1]).

Fig. 1(3)



enzyme, since, with the conjugated substrates, a cleaving pattern is produced that differs from that obtained with the free chito-oligomers [42-45]. Failure to correctly identify a chitinolytic enzyme with respect to its *endo-* or *exo*-mechanism of hydrolysis might, finally, also explain why family 18 of Henrissat's classification of glycosyl transferases, based on similarities in amino acid sequences [46,47], has remained heterogeneous, inasmuch as it combines typical chitinases (EC 3.2.1.14), unspecified "endo-chitinase" and "exo-chitinase", "chitotriosidase", a representative of EC 3.2.1.52, endo-GlcNAc'ase H (EC 3.2.1.96) and other "chitinases" not hydrolyzing chitin. This situation, of course, reduces the predictive value of this classification for the structure of the active site of chitinase proper.

The mechanism of chitinase action involves protonation, by the catalytic acid, of the substrate on the way to assuming the transition state geometry (see [48] for hypotheses) and the formation of an oxazoline intermediate stabilized by the neighbouring C2' acetamido group [49]. As appears to hold for family 18 chitinases in general [50-53], the only two fungal chitinases studied with respect to mechanistic aspects act with retention of the configuration [42] and, in accordance with this, display also transferase activity [42,45]. Therefore, both are to be considered family 18 chitinases. – The crystallographic models of chitinases described in [52,54,55] could be used in refined inhibitor design [according to strategy (3)], using novel ideas brought forward in a recent article on retaining glycosidases [48].

HexNAc'ase. This enzyme is by definition an *exo*-glycosidase [EC 3.2.1.52; see caption to Fig. 1(3)] and is, thus, clearly distinct from *endo*-GlcNAc'ase (EC 3.2.1.96). The HexNAc glycon can be *gluco-* or *galacto*-configurated (see [1] for GlcNAc'ase/GalNAc'ase activity ratios). Whether this seemingly rather low glycon-specificity includes the *allo*-analogue has apparently not been studied systematically. The same holds, partly, also for the influence of substitution at C-6 of *N*-acetylhexosaminides. The tolerance of HexNAc'ase for the aglycon moiety R [Fig. 1(3)] is generally high with carbohydrate chains of glycoproteins, but it is even higher with non-carbohydrate residues, as represented, e.g., by phenols (see [1]), alkaloids [56] and steroids [57].

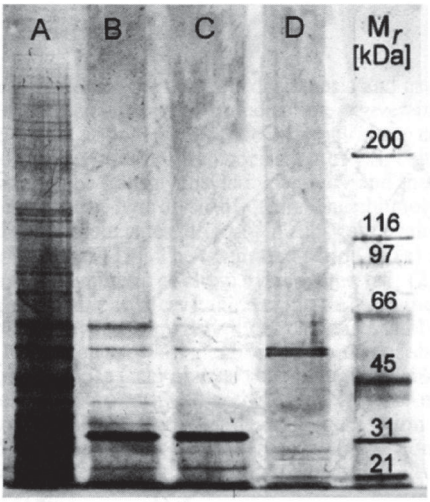


Figure 2. Identification of a 60-kDa CS polypeptide: SDS patterns (silver staining) of three different preparations of active CS obtained by subjecting chitosomal CS to affinity chromatography (AC) procedures. A Gradient-purified chitosomes (peak fraction; isolated according to [58]; D, as A, purified by heparin AC (as described in [39]); B, as A, obtained upon removal of contaminating proteins by conventional ConA-AC and desorption of the CS that had remained tightly bound to the column packing by a mixture of methyl-α-mannopyranoside and NaCl (each 0.5 M) applied under batch conditions (for experimental details, refer to [40,59]; C, as B, but desorption effected with NaCl only (0.5 M). B and C: the protein bands at 32 kDa and below are due to ConA leakage from the AC column.

The reaction carried out by HexNAc'ase is formally a nucleophilic substitution at the anomeric carbon. It proceeds with overall retention of configuration and, thus, can also transfer the glycosyl residue to alcohols. The polar acetamido group is involved in catalysis by stabilizing the reaction intermediate through formation of an oxazoline and, thence, accelerating aglycon departure (see [1] for a detailed account of the reaction mechanism of HexNAc'ase). – There exist 3D models of HexNAc'ase [1,60].

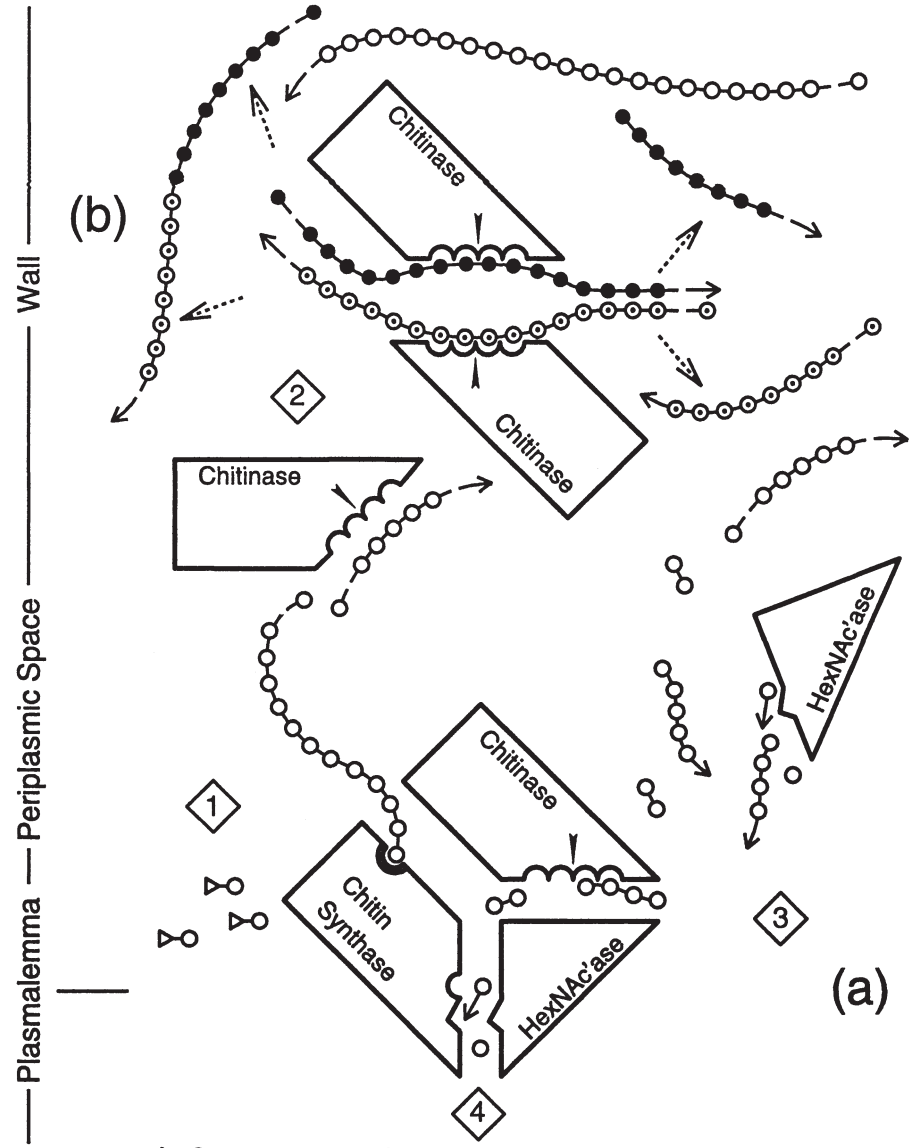
CS, chitinase and HexNAc'ase are glycoconjugated (see [35,39,40,59,61] for CS, [62,63] for chitinase, and [1,42] for HexNAc'ase), which concurs with the existence of surface-located isoforms of any of these enzymes [1].

The triple enzyme system chitin synthase-chitinase-ß-N-acetylhexosaminidase

Although the regulation of CS activity by allosteric and non-allosteric activation is sufficient to allow some control of the enzyme's action *in vitro*, additional mechanisms must exist that provide for a tight regulation of chitin synthesis *in vivo*, among which is the concerted action of CS with components of the chitinolytic system. Fig. 3 depicts a speculative scheme for the controlled synthesis and lysis of chitin in growing fungi. The hypothesis is based mainly on the facts (1) - (7); for details and ref's, refer to [1,19,35]:

(1) The *locus operandi* of CS is the cell surface, encompassing the plasma membrane, the periplasmic space as well as the wall fabric itself.

(2) In log-phase fungal cells, CS, chitinase and HexNAc'ase co-occur, and all have genuinely wall-associated forms.



O , GlcNAc; ▷O , UDPGlcNAc; O-O , chitobiose; the sign — —O marks the non-reducing end and — → points to the reducing end of the polymer; reducing GlcNAc units generated through the action of chitinase and HexNAc'ase are shown as O→ . The active sites of the enzymes are marked as (chitin synthase), (chitinase), (HexNAc'ase), , allosteric site of ChS for GlcNAc; , site of hydrolytic attack of chitinase. To better illustrate transglycosylation, the GlcNAc residues of two chains between which the event is depicted to take place are tagged differently (-●-●-●- and -⊙-⊙-⊙-).

Figure 3. For legend, see next page.

Figure 3. A biochemical model for the controlled synthesis and catabolism of chitin *in situ* as occurring during fungal growth. An indication is given in the graph of the approximate spatial arrangement of the enzymes at the cell surface. Part (a) depicts an integrated triple enzyme system consisting of CS, chitinase and HexNAc'ase. Part (b) illustrates the transglycosylating activity and the tandem action of chitinase and HexNAc'ase in the hydrolysis of chitin. The chitinolytic enzyme species of parts (a) and (b) are isozymes differing in substrate chain length preference and some other properties [19]. The integrated action of (a) and (b) encompasses four steps: (1) *de novo* synthesis of chitin by activated CS; (2) remodelling of nascent as well as of preformed, partly crystalline chitin through the combined transglycosylating/hydrolysing activity of chitinase and HexNAc'ase; (3) progressive lysis of chitin, with increasing amounts of shorter chito-oligomers and *N,N'*-diacetylchitobiose ('chitobiose') becoming available for chitinase and HexNAc'ase associated with CS; and (4) HexNAc'ase-mediated cleavage of the glycosidic bond of chitobiose resulting in the generation of the CS-activator GlcNAc and, simultaneously, potentially providing a donor for transglycosylation reactions. (Reproduced from *Chitin and Chitinases* [64]; by permission of *Birkhäuser Verlag*).

(3) GlcNAc is the authentic allosteric activator of CS [34].
(4) Some CS-species contain a chito-oligomer residue that could conceivably serve as a region of complexation for the non-catalytic high-affinity binding domain of chitinase.
(5) The chitinolytic system of growing fungi consists of several HexNAc'ases and two types of chitinases differing in pH-optimum, affinity to polysaccharide gels, ability to degrade preformed chitin and response to treatment with protease.
(6) The binary system chitinase/HexNAc'ase performs degradation of chitin to GlcNAc by action of the two enzymes in tandem.
(7) Genuinely, probably covalently wall-associated chitinase and HexNAc'ase effect transglycosylation.

The caption to Fig. 3 presents a short description of the events that allow a quasi-simultaneous degradation and dynamic remodelling of the chitin skeleton in a subtle manner as well as a concerted action of the binary system with the triple enzyme system CS-chitinase-HexNAc'ase, thus linking lysis with the synthesis of chitin through the intermediacy of HexNAc'ase.

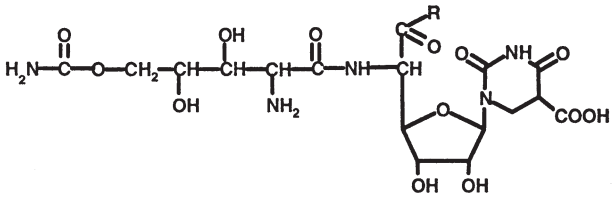
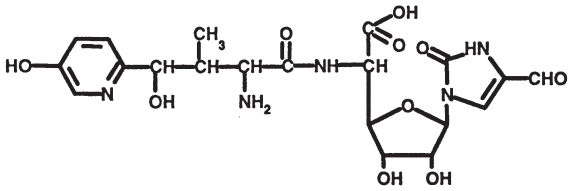
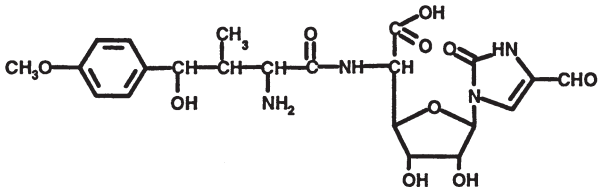
Inhibitors of chitin synthase, chitinase and ß-*N*-acetylhexosaminidase – old and new ones

***Inhibitors of CS*:** For obvious reasons, none of the CS- inhibitors known to date (listed in Table 1) has been discovered using strategy (3); and the majority of them were found by a combination of strategies (1) and (2). Thus, compounds **1 - 5** were detected as antifungals prior to knowledge of CS as the (or a) site of their action. For the compounds shown under number **6**, an interaction with CS as the cause for their antifungal activity is implicit in several studies (for ref's, see [35]). This similarly holds for triterpenoid saponins and related compounds (**7** and **8**), displaying structural features that have long been known to be associated with antifungal activity [65]. The CS-inhibitory activity of compounds **9 - 10** and **11 - 14** has been detected, respectively, by small-scale natural products screening and HTS of a large library of chemosynthetics.

Nikkomycins and polyoxins (**1**) are the best CS-inhibitors known to date, closely followed by some strongly amphiphilic natural products (**2**, **7**) as well as PCNB and some other chemosynthetics (**3**, **6**, **11**, **14**). Because of the complexity of the CS-system, the elaborate procedure to obtain sufficiently-purified enzyme preparations suitable for selective screening,

and the quite limited knowledge of the structure of the enzyme complex (see chapter *Reactions catalyzed* ...), a detailed account of the mode of action of these inhibitors can be meaningful

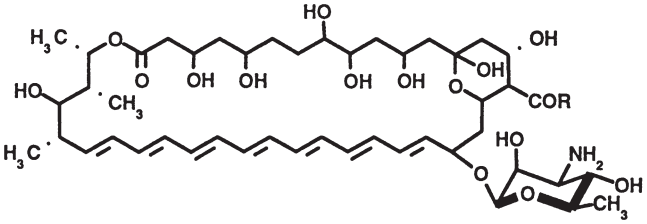
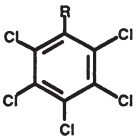
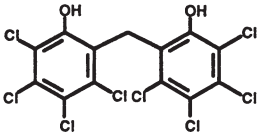
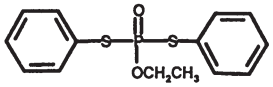
Table 1. A list of chitin synthase (CS) inhibitors: a gamut of motifs.

Compound class [1]	Source of fungal test enzyme	CS-inhibitory activity K_i IC_{50} [μM]	References
1 Nucleoside peptides			
			
R = OH, polyoxin D	*Mucor rouxii*	2	[66]
	Candida albicans	0.6	[67]
		0.2	[68]
R =octanoyl-phenylalanyl-polyoxin D	*C. albicans*	7	[67] [2]
			
nikkomycin X	*M. rouxii*	0.5	[66,69] [2]
	Agaricus bisporus	2	[70]
	Coprinus cinereus	1	[71] [2]
			
nikkomycin $MeOB_x$	*C. cinereus*	0.4	[71]

(continued)

[1] Only compounds with K_i or IC_{50} values ≤ 100 μM are listed. [2] These papers provide K_i 's of various other polyoxin and nikkomycin analogues as well as references to earlier publications; for some recent papers, see [35].

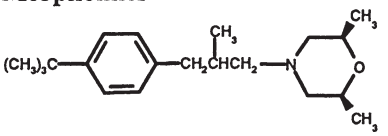
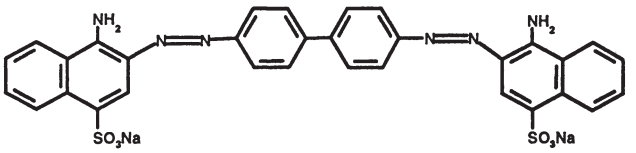
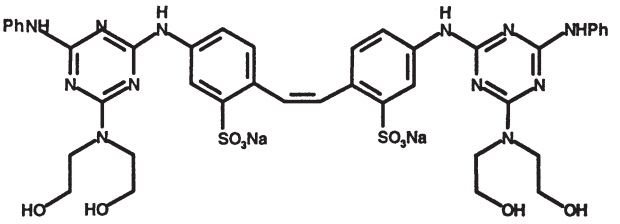
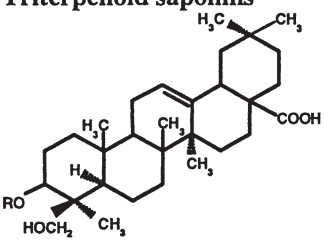
Table 1 (contd.)

Compound class [1]	Source of fungal test enzyme	CS-inhibitory activity K_i [μM]	CS-inhibitory activity IC_{50} [μM]	References
2 Polyene macrolides				
				
R = OH, amphotericin B (AmB)	*M. rouxii*		10 [3]	[72]
	A. bisporus		1 [3]	[73]
R = OCH_3, AmB methyl ester	*M. rouxii*		85 [4]	[74]
			90 [4,5]	[75]
3 Chlorinated benzenes				
				
R = NO_2, pentachloronitro-benzene (PCNB)	*A. bisporus*	1 [6]	2	[73,76]
R = H		25		
R = OH		75		
				
hexachlorophene	*C. cinereus*	9		[77]
4 Phenylphosphorodithioates				
				
'ediphenphos'	*Fusarium graminearum*	50	~100	[78]

(continued)

[3] With chitosomes (100 S-CS), in the presence of an organic solvent. [4] With 100 S-CS, no organic solvent. [5] With digitonin-solubilized 16 S-CS ex 100 S-CS. [6] Details will be published elsewhere.

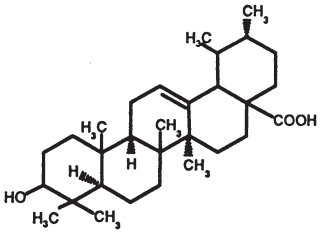
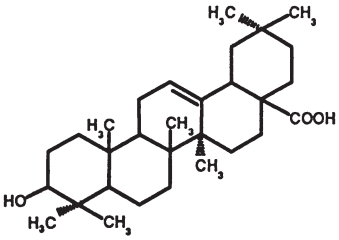
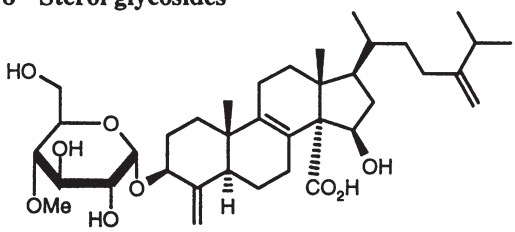
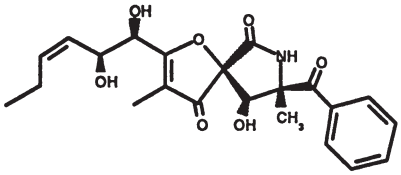
Table 1 (contd.)

Compound class [1]	Source of fungal test enzyme	CS-inhibitory activity K_i IC_{50} [μM]	References
5 Morpholines			
			
'fenpropimorph'	*A. bisporus*	50	[73]
6 Polyaromatics			
			
Congo Red	*M. rouxii*	50	[79]
			
Calcofluor White	*M. rouxii*	75	[79]
	A. bisporus	28 [7]	[73]
		8 [7]	
		6 [7]	
7 Triterpenoid saponins			
			
R = rhamnose (1→2), arabinose (1→)			
α-hederin	*S. cerevisiae*	84	[80]

(continued)

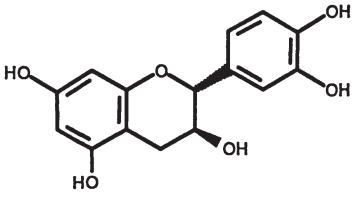
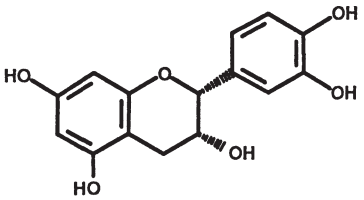
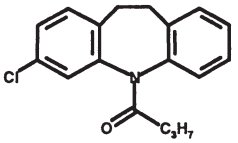
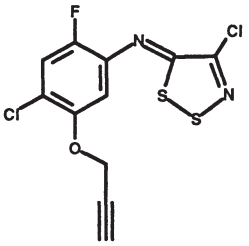
[7] Depending on type of enzyme preparation; details will be published elsewhere.

Table 1 (contd.)

Compound class [1]	Source of fungal test enzyme	CS-inhibitory activity K_i [μM]	CS-inhibitory activity IC_{50} [μM]	References
				
ursolic acid	*S. cerevisiae*		12	[80]
				
oleanoic acid	*S. cerevisiae*		2	[80]
8 Sterol glycosides				
				
ascosteroside	*S. cerevisiae*		11	[81]
9 Pseurotins				
				
pseurotin A	*C. cinereus*	93		[82]

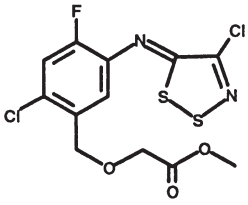
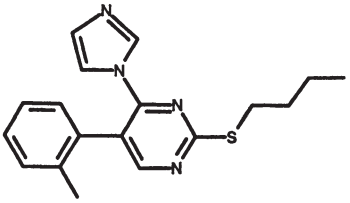
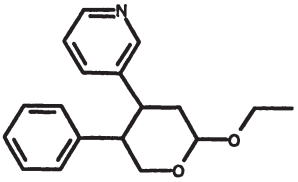
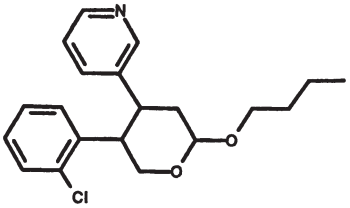
(continued)

Table 1 (contd.)

Compound class [1]	Source of fungal test enzyme	CS-inhibitory activity K_i IC_{50} [μM]	References
10 Flavonoids			
			
(±)- catechin	*S. cerevisae*	52	[83]
			
(±)- epicatechin	*S. cerevisae*	100	[83]
11 Dibenzazepines			
	*C. cinereus*	6	[84]
12 Aryliminodithiazoles			
			
3-propargyloxyphenylimino-dithiazole	*M. rouxii*	25	[85]

(continued)

Table 1 (contd.)

Compound class [1]	Source of fungal test enzyme	CS-inhibitory activity K_i IC_{50} [μM]	References
			
3-alkyloxymethylenphenyl-iminodithiazole	*M. rouxii*	16	[85]
13 Pyrimidines			
			
4-imidazoyl-5-phenyl-pyridine	*B. cinerea*	14	[85]
14 Tetrahydropyranes			
			
2-ethoxytetrahydropyrane	*B. cinerea*	39	[85]
			
2-butoxytetrahydropyrane	*B. cinerea*	4	[85]

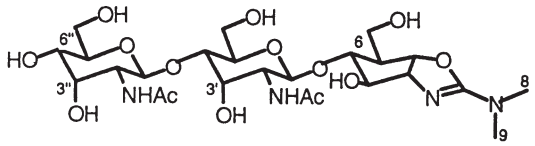
only in the case of the nucleoside peptides (for some suggestions relating to **2**, **3** and **7**, see the papers cited in the Table). Nikkomycins and polyoxins probably represent analogues of the substrate UDPGlcNAc approaching its transition state, the inhibitor's side chain acting as a mimic of the pyrophosphate-Mn^{2+}-complex formed during catalysis [33]. With the more easily accessible, highly purified CS preparation recently described ([40]; see also Fig. 2) it should be possible to reliably test further potential transition-state analogues for their interaction with the enzyme(s) concerned [see caption to Fig. 1(1)], e.g. the coupling product of acetamidoazaglucose and chitotriose [86].

Inhibitors of chitinase: Allosamidin (Table 2, **1**) and some of its congeners [87] have been discovered, some 20 years ago, by natural products screening for chitinase-inhibitory activity, using as the test systems crude extracts from insects (see footnote 1 to Table 2) and from fungi. Although having served as a lead for the synthesis of numerous analogues, the inhibitory potency of **1** has been surpassed by none of its derivatives – with two exceptions (see below). Disregarding the notoriously high IC_{50}-values with enzyme preparations from *S. cerevisiae* as well as some erratic data with test systems from other fungi, **1** can be considered to generally have IC_{50}-values in the 1 μM-region (Table 2). This falls well within the range of K_i's displayed by transition-state analogues of other glycosyl hydrolases ([88]; for HexNAc'ase, see [1] and Table 3). Indeed, the allosamizoline group binds in the center of the active site of chitinase [52].

There are two modifications [(1), (2)] of the basic allosamidin structure that did afford analogues with increased inhibitory potency. (1) Demethylation at position 9: this results in an about 10-100fold increase in inhibitory activity (compare values of **2** with those of **1**); a similar situation holds for the 9-demethyl derivative of 6''-*O*-methylallosamidin (compare values of 7 with those of **6**). In both cases, however, such an increase of activity was not observed with the test system from *Trichoderma* sp (see below for an explanation). (2) Derivatization at the non-reducing end of **1**, using a diol function at C-3'' and C-4'': although the allosamidindiol **12** is *ca* five times less inhibitory than the reference **1** (0.080 *vs* 0.016 μM), the corresponding dibiotinylated derivative is twice as active than **1** [89]. Thus, compound **13** represents the best chitinase inhibitor available do date.

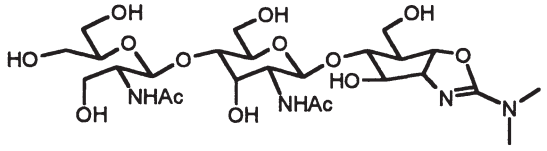
It is an intrinsic requirement of transition state mimics theory that these should follow the same pathway towards the transition state as the substrate. The situation is puzzling, therefore, that analogues of allosamidins having reversed configuration at the C-3',3''-positions are less inhibitory than the parent compounds (compare values of **8** and **9** with those of **1** and **2**), particularly with *Trichoderma* as the enzyme source. The same strong reduction in inhibitory activity does not occur, however, with analogues differing from the corresponding allosamidins only with respect to the configuration at C-3' (compare **10** and **11** with **6** and **7**). For the following arguments, (1) - (4), the suggestion is made that the seemingly poor performance of the *gluco*-analogues is not a consequence of configuration selectivity at the non-reducing end of pseudochitotriose, but due to the simultaneous occurrence of HexNAc'ase in the test systems used: (1) HexNAc'ase is a regular component of crude chitinase preparations serving in bioactivity testing (the isolation of HexNAc'ase-free chitinase is quite elaborate and, therefore, is not normally done for that purpose; for examples of pure fungal chitinase, see [42]); (2) there occurs some degradation of chitobiosyl-allosamizoline with the *Trichoderma* enzyme used in the studies concerned [90]; (3) some representatives of this genus dispose of a particularly effective chitinolytic system (see [91]), whereas yeasts are not high-producers;

Table 2. A List of chitinase inhibitors: allosamidin-based motifs.

Compound	Source of fungal test enzyme [1]	Inhibitory potency (IC_{50} [μM])	Ref's
1 Allosamidin			
			
	Saccharomyces cerevisiae	67	[92]
		54	[93]
		34	[94]
		34	[90]
		~18	[95]
		12	[92]
	Candida albicans	10	[93]
		0.300	[96]
		~0.020	[97]
	Mucor rouxii	6	[98]
	Neurospora crassa	2	[99]
	Trichoderma sp.	1	[93]
		0.482	[94]
		0.482	[90]
		0.016	[89]
	Pisolithus tinctorius	0.480	[100]
	Paxillus involutus	0.290	
	Boletinus cavipes	0.045	
	Suillus variegatus	0.010	
2 9-Demethylallosamidin			
	C. albicans	1	[93]
	cerevisiae	~0.8	[95]
		0.493	[93]
		0.493	[101]
		0.328	[94]
	Trichoderma sp.	1	[93]
		0.821	[94]
		0.821	[101]
3 8,9-Didemethylallosamidin			
	S. cerevisiae	13	[94]
	Trichoderma sp.	5	

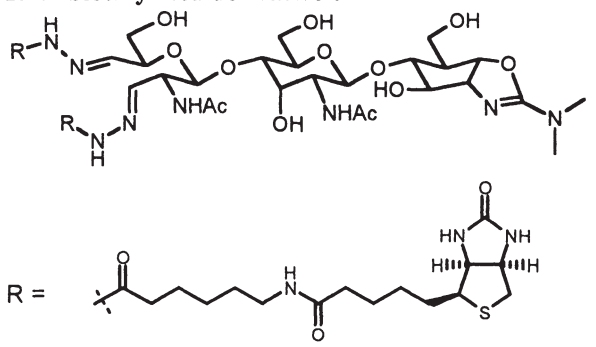
(continued)

Table 2 (contd.)

Compound	Source of fungal test enzyme [1]	Inhibitory potency (IC_{50} [μM])	Ref's
4 8-Ethyl-9-demethylallosamidin [2]			
	S. cerevisiae	3	[94]
	Trichoderma sp.	5	
5 6"-*O*-Phenylacetyl-allosamidin			
	S. cerevisiae	38	[94]
	Trichoderma sp.	0.890	
6 6"-*O*-Methyl-analogue of 1			
	cerevisiae	58	[93]
	C. albicans	14	
	Trichoderma sp.	2	
7 9-Demethyl-analogue of 6			
	S. cerevisiae	0.641	[93]
	C. albicans	0.962	
	Trichoderma sp.	2	
8 3',3"-*gluco*-analogue of 1			
	S. cerevisiae	77	[90]
	Trichoderma sp.	144	
9 3',3"-*gluco*-analogue of 2			
	S. cerevisiae	0.821	[101]
	Trichoderma sp.	94	
10 3'-*gluco*-analogue of 6 (glucosallosamidin A)			
	S. cerevisiae	49	[93]
	C. albicans	5	
	Trichoderma sp.	1	
11 3'-*gluco*-analogue of 7 (glucosallosamidin B)			
	S. cerevisiae	0.802	[93]
	C. albicans	1	
	Trichoderma sp.	3	
12 C-3",C-4"-Allosamidindiol 			
	Trichoderma sp.	0.080	[89]

(continued)

Table 2 (contd.)

Compound	Source of fungal test enzyme [1]	Inhibitory potency (IC_{50} [μM])	Ref's
13 Dibiotinylated derivative of 12 [3] 	*T.* sp.	0.007	[89]

[1] IC_{50}'s [μM] observed with enzyme preparations from representatives of other types of organisms, Bacteria, Arthropoda and Protozoa, range, respectively, from 4 - 0.083 [102,103], 1 - 0.048 [43,90,94,104-107] and 0.120 - 0.008 [108-110]. For chitinase of the nematode *Oncocerca gibsoni* a K_i value of 0.0002 μM was reported [111]. [2] For the chitinase-inhibitory activity of further *N*-monoalkyl derivatives of **1**, see [94]. [3] The IC_{50}'s of the corresponding monobiotinylated analogues amount to 0.040 [μM].

(4) in the presence of chitinase and HexNAc'ase, there occurs not only hydrolysis of the 3''-*gluco*-analogue of allosamidin to chitobiose and allosamizoline, but this is probably also the donor for a transglycosylation reaction yielding chitobiose [112] – a situation that casts doubt, indeed, on the reliability of IC_{50}' thus obtained, even disregarding the likelihood that higher oligomers of the hydrolysis products are generated under these conditions [113]. Contamination by HexNAc'ase of test systems used to assess the chitinase-inhibitory action of chitobionolactone oxime [19] and of histidine chitobiosylamide [105] might, likewise, have been one of the reasons for their poor performance (with K_i's 175 and 500 μM, respectively).

Considering the present knowledge of the reaction mechanism of chitinase, two approaches appear to be promising to obtain new inhibitors: the hydroximolactam- and the lactone (phenylcarbamoyl)oxime-based design of short-chain chito-oligomer derivatives ([114; this paper]; see also chapter *Inhibition of HexNAc'ase*). Should such exercises not only yield further insight into the enzyme's mode of action but, beyond this, also afford novel antifungals, either concept will have to account for the observations discussed in the above paragraph and for the fact that there is an intimate association between chitinase and HexNAc'ase *in vivo* as well, both topologically and functionally (see chapter *The triple enzyme system ...*). An obvious way to prevent such compounds to act as substrates for co-occurring HexNAc'ase, and to thus render them inactive, is a combination of modification at the non-reducing end and selection of an appropriate side chain structure at the reducing end as, for example, inherent in structures **13** and **2** (Table 2) and **11** (Table 3).

Inhibitors of HexNAc'ase: All compounds listed in Table 5 are glycon-based analogues of the substrate and – with the exception of nagstatin (**14**; discovered by natural products screening) – are all chemosynthetics (of course, apart from the parent sugar used in synthesis). They inhibit the enzyme competitively. Based on their high inhibitory potency (K_i's in the 1 - 5 nM range), compounds **5**, **15** and **11** are presently the best leads available for further HexNAc'ase inhibitor design.

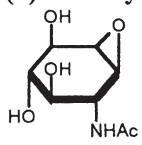
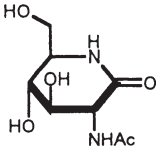
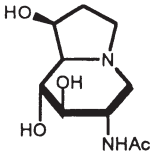
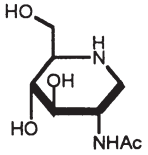
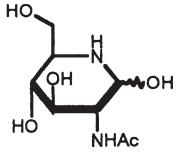
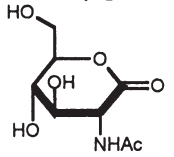
Mechanistically, these inhibitors can be arranged roughly into two groups: (1) analogues **1** - **5**, reflecting the occurrence of charge-charge interactions between the substrate and a catalytic amino acid according to the general acid-base mechanism of Sinnot [115], and (2) lactone oxime-based analogues **6** - **11**, likewise being charge mimics, but more closely approximating the transition-state stereochemistry than group (1) structures (see [48]). Compounds **13** -**16** may be considered to be but a cyclic extension of the lactone 'concept', and **12** is an individual case. The substituted phenyl ring of **11** probably represents the best mimic presently available for the role the aglycon part of β-*N*-acetylhexosaminides plays in catalysis. In fact, this question seems to have been somewhat neglected hitherto by inhibitors designers.

Compound **11** appears to be particularly interesting also for the following reasons: (1) it has a 10fold higher inhibitory activity than the mother substance **10** , and this, on its part, is already about 350 and 600 times more efficient than the underivatized GlcNAc-lactone (**6**) and -oxime (**8**), respectively – both widely cited as transition state analogues; (2) with a K_i/K_m-ratio of 6300 [116], the new inhibitor (also a new compound) seems to approximate the transition-state of the substrate more closely than any of the others and, therefore, is an excellent candidate for future use in mechanistic studies with a 3D model of HexNAc'ase (see [1]); (3) considering the fact that the 1,5-lactone derivative of tetra-*N*-acetylchitotetraose is a very good inhibitor of lysozyme [117], the introduction of this new side chain structure into the field of chitinase inhibitor design, likewise, would appear to be a promising approach towards novel agents with a higher potency than that displayed by inhibitors based on the allosamidin motif (for details, refer to chapter *Inhibitors of chitinase*).

Epilogue

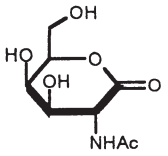
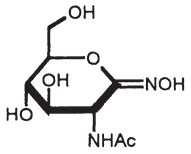
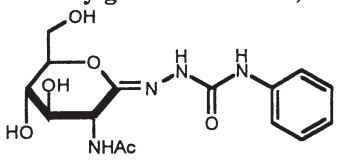
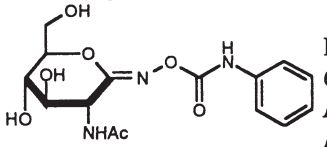
As attested by the 'history' of the discovery of specific inhibitors of chitin metabolism, good structures can be found by following any of the strategies outlined in the Introduction. It is not really relevant, therefore, whether the process of discovering an antifungal agent with the desired target specificity relies on HTS of compound libraries and modification of inhibitors found according to QSARs, or whether it involves the design of ligands upon a 3D model of the enzyme. For "She will have to sail between the Scylla of neglecting too many degrees of freedom in a simple model that gives a quick and cheap answer which is, however, useless due to its lack of precision, and the Charybdis of using a too detailed and complex molecular model that does not give a timely answer at reasonable costs"[8]. In either case, there follows the difficult task of converting specific enzyme inhibitors into speficic antifungals. Work at this stage of the discovery process can be characterized quite appropriately by the famous adage "For though many are called, few are chosen". If we are going to reach the final goal of finding novel antifungal agents speedily, an interdisciplinary approach is required, therefore.

Table 3. A List of HexNAc'ase inhibitors: glycon-based motifs.

Compound [1]	Origin of enzyme preparation	K_i [μM]	IC_{50} [μM]	References
1 (±) *N*-Acetylconduramine B trans-epoxide 	Bovine kidney	0.39		[118]
	Canavalia ensiformis	0.83		
2 *N*-Acetylglucosaminono-1,5-lactam 	Bovine kidney	0.67		[119]
3 6-Acetamido-6-deoxy-castanospermine 	Porcine placenta		0.4	[120]
	Human placenta		0.5	
	Bovine epididymis		0.7	
4 1,5-Dideoxy-1,5-imino-*N*-acetylglucosaminitol (2-acetamido-1,2-dideoxynojirimycin) 	*C. ensiformis*	0.36		[121]
		0.14		[122]
	Bovine kidney	0.37		[119]
	Zea mays	0.77		[123]
	Human liver	1.0		[122]
5 1,5-Dideoxy-1,5-imino-*N*-acetylglucosamine (2-acetamido-2-deoxynojirimycin) 	*C. ensiformis*	0.001		[122]
	Bovine kidney	0.002		[119]
	Helix pomatia	0.5		[122]
6 N-Acetylglucosaminono-1,5-lactone 	Bovine kidney	0.16		[124]
		0.036		[119]
	Rat epididymis	0.09		[125]
	Human liver	0.1		[126]
	C. ensiformis	0.8		[127]
		0.31		[124]

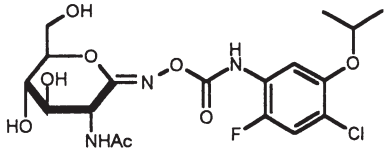
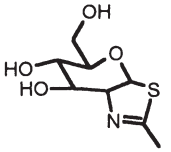
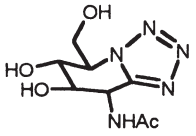
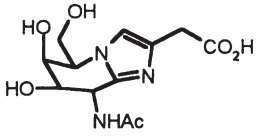
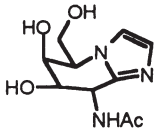
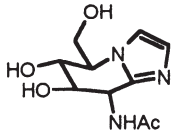
(continued)

Table 3 (contd.)

Compound [1]	Origin of enzyme preparation	K_i [μM]	IC_{50} [μM]	References
7 N-Acetylgalactosaminono-1,5-lactone				
	Porcine epididymis		0.26	[128]
	Rat epididymis	0.41		[125]
8 N-Acetylglucosaminono-1,5-lactone oxime				
	Bovine kidney	0.45		[124]
	C. ensiformis	0.62		
9 N-Acetylglucosaminono-1,5-lactone-4-phenylsemicarbazone				
	Bovine kidney	0.13		[129]
	Artemia salina	0.71		
10 *N*-Acetylglucosaminono-1,5-lactone *O*-(phenylcarbamoyl)oxime				
	Bovine kidney	0.11		[124]
	C. ensiformis	0.10		
	Phallusia mammillata	0.047		[130]
	Penicillium chrysogenum	0.068		[131]
	Suillus variegatus		0.61	[100]
	Armillaria ostoyae		0.50	
	Phytophthora cinnamomi		0.24	
	Pisolithus tinctorius		0.08	
	Aspergillus niger	0.55		[132]
	Botrytis cinerea	0.55		[116]
	Pleospora bjoerlingii	0.25		
	Pyricularia oryzae	0.06		
	Mucor rouxii	0.04		[124]
		0.03		[1,116,132]

(continued)

Table 3 (contd.)

Compound [1]	Origin of enzyme preparation	K_i [μM]	IC_{50} [μM]	References
11 ***N*-Acetylglucosaminono-1,5-lactone *O*-(2-fluoro-4-chloro-5-isopropoxyphenyl-carbamoyl)oxime** 	*P. bjoerlingii* *P. oryzae* *M. rouxii*	0.022 0.010 0.004		[116]
12 NAG-Thiazoline 	*C. ensiformis*	0.280		[133]
13 NAG-Tetrazole 	Bovine kidney	0.20		[134]
14 Nagstatin 	Porcine kidney Bovine kidney	0.017	 0.013	[135] [136]
15 Nagstatin; de-branched 	Bovine kidney		0.006	[136]
16 Nagstatin; de-branched, *gluco*-configuration 	Bovine kidney		0.007	[136]

[1] Only compounds with K_i or IC_{50} values ≤ 1 μM are listed. For further inhibitors, with K_i values ≥ 1 μM, but ≤ 1 mM, see Table in ref. [1], and [121, 137-144].

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Enzymatic degradation of chitin by microorganisms

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Summary
The degree of deacetylation of chitin substrates supplied for enrichment cultures increased during the incubation period up to 15%. Enzymatic release of acetate from chitin by crude extracts and cell debris was highest in those enrichment cultures which exhibited the highest increase in degree of deacetylation of the chitin substrate. These results indicate that part of the bacteria in enrichment cultures degrade chitin via the chitosan pathway. However, none of 25 isolated pure strains showed chitin deacetylase activity under the conditions tested until now.

Introduction

Two different catabolic pathways for chitin degradation may exist in nature. These pathways have been proposed by Davis and Eveleigh in 1984 [1]. The two pathways are shown in Fig. 1.

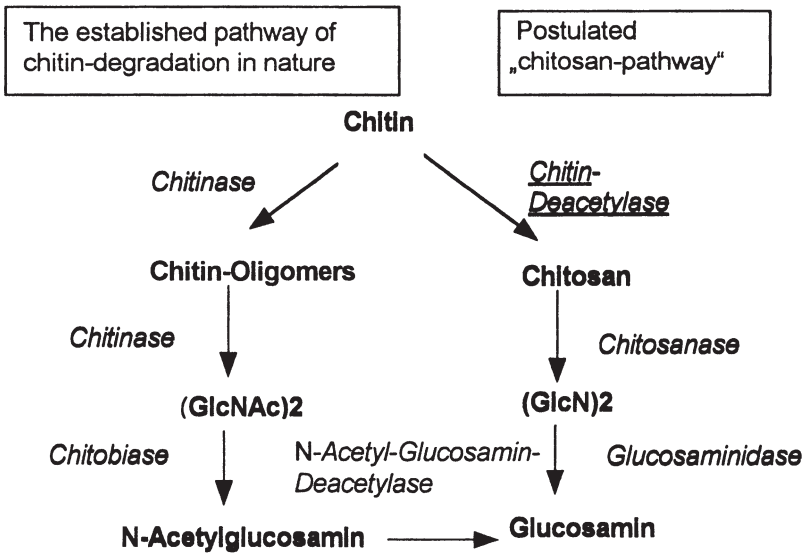

Figure 1. Two different pathways of chitin degradation in nature?

The degradation of chitin by chitinases to smaller subunits and further enzymatic steps yielding the monomer N-acetylglucosamin is well established and documented by a large number of publications of microorganisms isolated in pure cultures, containing all enzymes to metabolise chitin as sole source of energy, carbon and nitrogen. The other pathway, the so called "chitosan pathway", has been postulated by analogy to other degradation pathways of natural polymers. The key enzyme of this postulated pathway is the chitin deacetylase which converts chitin to chitosan releasing acetate. Chitin deacetylases have been found in several microorganisms, especially in fungi, but not in organisms, which grow on chitin, with one exception [2]. Nevertheless there are strong indications that in marine environments chitin is degraded via chitosan, this has been shown by Gooday and co-workers [3]. They found that the degree of deacetylation of chitin exposed to the water body or the sediment of the North Sea increased significantly especially under aerobic conditions. Simultaneously they demonstrated high activity of chitin deacetylase in situ. So far, however, no microorganisms, neither marine nor terrestrial, are known to degrade chitin via the "chitosan pathway". Therefore the aim of the experiments presented is to prove the existence of the "chitosan pathway" by isolating microorganisms in pure culture associated with this pathway.
In further studies chitin deacetylase of these microorganisms shall be examined, an enzyme which is of considerable interest for the production of chitosan from chitin.

Material and Methods

Enrichment cultures. The medium for enrichment cultures consisted of natural sea water supplemented with ammonium and iron, containing 0.5% chitin or chitosan preparations as the substrate. For inoculation, partly decomposed marine chitinous materials (crab shells) have been collected from sediments of the beach of Juist, the beach of Knock (near Emden) and the East Frisian mud-flats next to Juist. Samples of chitin and chitosan from shrimp shells and squid pen with known and differing degree of deacetylation served as substrates for enrichments. The percentage of deacetylation varied from 12.4 % to 78.0 %. Some of them were coarse ground, others finely ground. Most of the substrates were produced in our lab, others were bought from "Fish Contract" in Bremerhaven, Germany. The enrichment cultures were incubated for seven days at 30°C and transferred several times.

Isolation of chitinolytic microorganisms. Isolation from the enrichment cultures was carried out on agar plates containing 0.2% acetate as the sole carbon source. The obtained isolates were plated on chitin agar prepared as overlay media, with the upper layer containing 0.5% colloidal chitin as the sole carbon source. Plates were incubated for 3-14 days at 30°C.

Determination of the degree of deacetylation of chitinous materials. The degree of deacetylation of chitinous materials used as the substrate for enrichment cultures was determined via IR spectroscopy. Subsequent to a seven day enrichment at 30°C, the utilized chitinous materials were washed cell free, dried, and the degrees of deacetylation of the utilized chitin samples were examined again.

Determination of chitin deacetylase activity. The release of acetate from chitin due to enzymatic activity of the enrichment cultures was measured using an enzymatic test for acetate commercially available from Boehringer, Mannheim, Germany. Chitin deacetylase derived from *Mucor rouxii* served as the reference following a procedure described by Dunkel [4]. For enrichment cultures the test conditions were slightly modified [5].

Cell free extracts of the bacteria enriched. After several transfers (growth conditions see above) the bacteria were thoroughly separated from the chitin/chitosan pellet by sieving and resuspending the bacteria in 200 mM Tris-HCl buffer, pH 7.6. The obtained bacteria were centrifuged at 15000 rpm for 10 min, resuspended in buffer and ruptured by means of glass

beads with a laboratory whirl mixer. The centrifuged cell debris and the supernatant crude cell extract were incubated (2 ml each) with 10 mg of colloidal, purified chitin (Sigma, Deisenhofen, Germany) at 25°C both for 2 h and overnight. The reaction was stopped by adding 1 ml of 0.2% hydrochloric acid and boiling for 10 min. Following centrifugation at 10000 rpm for 5 min the supernatant was tested for acetate.

Results and Discussion

Ten different enrichment cultures with seawater and different kinds of chitin/chitosan substrates were inoculated with different environmental samples as described in Material and Method. After five to ten days appropriate bacterial growth occured in all cultures as have been measured by registration of the oxygen consumption by the cultures. The cultures were harvested by centrifugation. The degree of deacetylation of the remaining chitin substrate was determined and the enzymatic activity releasing acetate from colloidal chitin was measured in cellfree extracts and in cell debris. The results are shown in table 1.

An increase of deacetylation was observed in all cultures. The only negative value is within the range of the mistake of the infrared method used. The differences of degree of deacetylations ranged from 0.8 % to 15.6 %. The highest values in table 1 are marked.

Table 1. Increase of the degree of deacetylation of chitin and release of acetate from chitin in enrichment cultures.

enrich-ment	source of chitin employed	degree of deacetylation of the chitin			release of acetate from chitin by crude extracts and cell debris of bacteria from enrichment cultures in mg/l after 120 min	
		before cultivation	after cultivation	difference	crude extract	cell debris
3.2	shrimp	48,0%	47,2%	-0,8%	1,9	2,4
3.3	shrimp	31,3%	36,6%	5,3%	3,0	4,7
3.4a	squid	12,4%	28,0%	15,6%	3,2	4,2
3.4b	shrimp	24,1%	38,1%	14,0%	-	-
3.4c	shrimp	31,0%	38,1%	7,1%	-	-
3.5a	squid	12,4%	24,1%	11,1%	3,7	3,0
3,5b	shrimp	31,0%	33,1%	2,1%	-	-
3.11	shrimp	31,0%	31,8%	0,8%	2,2	2,9
4.1	shrimp	31,0%	40,1%	9,1%	3,9	3,5
5a.1	shrimp	-*	-	-	2,7	3,3
5a.2	shrimp	-	-	-	2,7	2,8
5b.1	shrimp	-	-	-	3,1	2,1
5b.2	shrimp	-	-	-	2,9	3,5

*not determined

The release of acetate from colloidal chitin by mechanically destroyed cells generally was higher in cell debris than in crude extracts indicating the presence of particle-bound enzymes which sedimented at low centrifugation speed. The highest activities found are marked by grey shadow in table 1. From the results obtained it is obvious that high differences of degree of deacetylations correspond to high enzymatic activity releasing acetate from colloidal chitin, with the only exception of culture 3.3. Therefore we conclude that some microorganisms growing in the

enrichment cultures partially deacetylate chitin to chitosan. In so far the results of Gooday and co-workers, who observed similar microbial activities in nature [3], were confirmed by our lab experiments under controlled conditions.

One important intention of the research project was the isolation of microorganisms responsible for the observed changes in degree of deacetylation of chitin and producing the enzymes of the "chitosan pathway". The preliminary results obtained are shown in table 2.

From the enrichment cultures 25 strains were isolated in pure cultures on agar with acetate as the sole source of carbon and energy (see table 2). It was expected that deacetylating bacteria will most probably grow on acetate as sole carbon and energy source.

After isolation in pure culture the growth of the bacteria was tested on chitin agar. About half of the strains were able to grow on chitin agar, the others didn't. Most of the "chitin positive" strains were grown in enrichment cultures with squid pen as substrate. The large number of positive strains may be explained by the fact that among all chitin sources applied squid pen had the lowest degree of deacetylation.

Table 2. Isolation of bacteria in pure cultures on acetate medium and growth test on chitin agar.

number of the isolates	isolates derived from enrichment	Source of chitin / chitosan	degree of deacetylation/ degree of fragmentation	growth on chitin agar	cleared zones on chitin agar
1a, 1b, 2	3.11	shrimp	73,0% / finely ground	--	--
3	3.3	squid	12,4% / coarse ground	++	--
4	3.3	squid	12,4% / coarse ground	++	--
5	3.3	squid	12,4% / coarse ground	++	--
6	3.3	squid	12,4% / coarse ground	++	--
7	3.3	squid	12,4% / coarse ground	--	--
8	3.4	shrimp	31,5% / finely ground	--	--
9 -15	3.5	shrimp	31,5% / finely ground	--	--
16	3.11	shrimp	73,0% / finely ground	--	--
17	3.11	shrimp	73,0% / finely ground	+	--
18	3.11	shrimp	73,0% / finely ground	++	++
19	3.11	shrimp	73,0% / finely ground	++	++
20	3.3	squid	12,4% / coarse ground	++	++
21	3.3	squid	12,4% / coarse ground	++	++
22	3.3	squid	12,4% / coarse ground	++	++
23	3.3	squid	12,4% / coarse ground	++	++
24	3.3	squid	12,4% / coarse ground	++	++
25	3.3	squid	12,4% / coarse ground	++	++

In addition to growth, the chitinolytic activity of the strains was identified by cleared zones of the colloidal chitin around the colonies. Most of the strains growing on chitin agar were able to hydrolyse chitin. Interesting enough 5 strains which grew quite well on chitin agar did not dissolve the chitin substrate. Therefore it seemed reasonable to assume that these strains can use only the acetate group of chitin as a carbon source and produce chitosan which is not further metabolised. However with the test for chitin deacetylase applied [6] no enzyme activity could be detected so far.

The enzyme test may not be sufficient to meet the requirements, currently we concentrate on optimizing the enzyme test to screen more strains with more efficiency.

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Kinetic behaviour of chitinase isozymes

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Summary
We analyzed the enzymatic reactions of yam (plant) chitinases and the silkworm (insect) chitinase and β-*N*-acetylglucosaminidase by a HPLC method to investigate the anomer formation and splitting pattern. As the results, the silkworm chitinase, which belongs to family 18 chitinases and is inhibited by allosamidin, hydrolyzed the substrates in the retaining mechanism and in an endo manner. The silkworm β-*N*-acetylglucosaminidase, which is not inhibited by allosamidin, also hydrolyzed the substrate in the retaining mechanism, but in an exo manner from the non-reducing end side. On the other hand, there are two endo/random types of chitinases in yam. The family 18 chitinase, which is inhibited by allosamidin, hydrolyzed the substrates in the retaining mechanism, whereas the family 19 chitinases, which are not inhibited by allosamidin, hydrolyzed in the inverting mechanism.

Introduction
Chitinases are widely distributed in living organisms, and play an individual and specific role in each organism. In insect and crustaceans, chitinases act for insect ecdysis by degrading the cuticle chitin [1]. In higher plants, chitinases play a role in self-defense against plant pathogens and pests [2]. On the basis of amino acid sequence, chitinases are classified into two families such as family 18 and 19 of glycosyl hydrolases [3]. Plant chitinases are further classified into about 6 classes [4,5]. On the other hand, some chitinases were successfully crystallized and their crystallographic structures have been analyzed. Referring to these three-dimensional structure, the hydrolytic mechanism was elucidated by computer simulation [6,7,8].

We have already purified 5 chitinase isozymes from yam [9,10] and 3 isozymes from *Bombyx mori* [11,12]. *B. mori* chitinases were all identified to be the family 18 chitinases on the basis of amino acid sequence and antigenicity, and inhibited by allosamidin [12,13]. There are, however, two families of yam chitinases. Chitinases E, F and G were identified to be the family 19 chitinases on the basis of amino acid sequence and/or antigenicity, and not inhibited by allosamidin. Chitinase A and H were identified to be the family 18 chitinases on the basis of antigenicity, and inhibited by allosamidin. By summarizing these data, we could easily find a relationship between the chitinase family and the inhibition by allosamidin. This inhibitor allosamidin is very similar to the reaction intermediate of the substrate in having the structure of oxazoline ring. If the enzymatic reaction would occur via the intermediate of the oxazoline ring, the product at the non-reducing end side may be β-anomer. Therefore, we tried to analyze the anomeric form of the product.

To analyze the anomeric form, ^{1}H-NMR analysis has been used [14,15] However, for ^{1}H-NMR analysis, it is required to exchange water protons in the enzyme solution with deuterium by lyophilization which sometimes inactivates the enzyme. Therefore, we tried to

find a simple HPLC method [16]. Furthermore, ^{1}H-NMR analysis gave us the information only on the anomeric form of the mixture of *N*-acetylchitooligosaccharides produced in the reaction, but not any information on the cleavage pattern. The splitting patterns are also important information for characterization and classification of chitinolytic enzymes. Previously we used artificially synthesized *p*-nitrophenyl *N*-acetylchitooligosaccharides to identify the splitting patterns [17]. However, it was demonstrated by our HPLC method the splitting patterns were not completely the same between the natural substrates and such artificial substrates.

In this study, therefore, we analyzed the enzymatic reactions of yam chitinases and the silkworm chitinases and β-*N*-acetylglucosaminidase by using our HPLC method to investigate the anomer formation and splitting pattern.

Materials and Methods

Enzymes and substrates: Yam chitinases E, F, H and G were purified by the methods of Tsukamoto *et al.* [18] with a little modification and Arakane *et al.* [10]. *Bombyx mori* 54-kDa chitinase and β-*N*-acetylglucosaminidase were purified by the methods of Abdel/Banat *et al.* [12] and Koga *et al* [19], respectively. *N*-Acetylchitooligosaccharides were generous gifts from Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan. All of the reagents were of analytical grade.

Enzymatic reaction and HPLC analysis: The enzymatic reactions were performed by the method of Koga et al. [16]. One hundred μL of 0.11 mM *N*-acetylchitooligosaccharide dissolved in 4.0 mM sodium acetate buffer, pH 4.0 - 6.0, was reacted with 10 μl of enzyme solution at 25°C. After the appropriate time less than 30 min, which is required to prevent the equilibration of the produced anomeric forms, the reaction mixture was immediately cooled in an ice bath, and a 10-μl portion was analyzed by HPLC (Shimadzu LC-10) on a Tosoh TSK-Gel amide-80 column (0.46 ID x 25 cm) at 28°C. The elution of *N*-acetylchitooligosaccharides was done with 70% acetonitrile at a flow rate of 0.7 ml/min, and monitored at 210 nm. The molar concentration of the oligosaccharides eluted was calculated using the relationship between the concentration of each oligosaccharide and its total peak-area of both anomers. Furthermore, the calculation of the anomer ratio was performed based on the method of Koga *et al.* [16] by comparing the peak-areas of both anomers in each *N*-acetylchitooligosaccharide.

Results and Discussion

Identification of α and β anomeric forms: There are two anomeric forms of *N*-acetylglucosamine and its oligosaccharides. These anomeric forms were separated into two peaks by the HPLC method, and could be identified by comparing with the peaks produced by authentic enzymes such as hen-egg lysozyme, which produces β anomer [20], and yam Chitinase E, which produces α anomer [15]. The results are shown in Fig. 1. By comparing the peaks, we could conclude that the early peaks are α anomers and the later peaks are β anomers. The ratios of α and β anomeric forms in naturally occurring *N*-acetylglucosamine and its oligosaccharides were similarly about 1 : 0.6.

Estimation of hydrolysis mechanism and cleavage site: To estimate the hydrolysis mechanism and cleavage site, a typical example is shown in Fig. 2. When the pentasaccharide was reacted with yam chitinase E, the disaccharide and trisaccharide were produced. Therefore, it is easily estimated that yam chitinase E hydrolyzes the pentasaccharide to disaccharide and trisaccharide. Furthermore to estimate the cleavage site, the anomer ratios of the produced oligosaccharides were compared. However, the high ratio of α anomer were

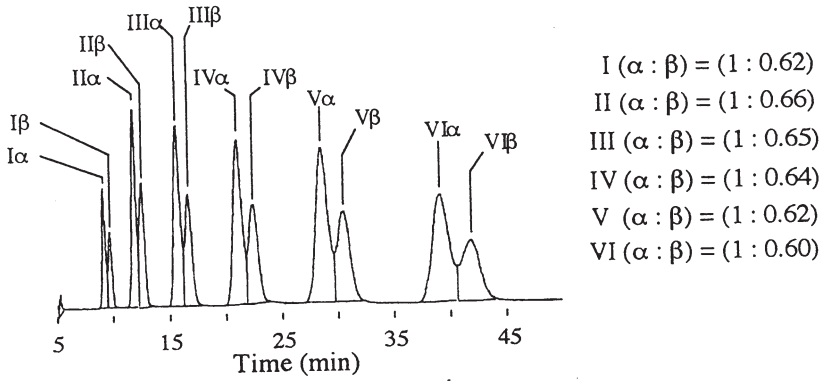

Figure 1. HPLC Analysis of anomeric forms of *N*-acetylchitooligosaccharides. *N*-Acetylchitooligosaccharides (0.1 mM each) were analyzed by HPLC as described in Materials and Methods. Their peaks were identified as represented near the elution peaks, and the ratios of the anomers are calculated and represented at the right side.

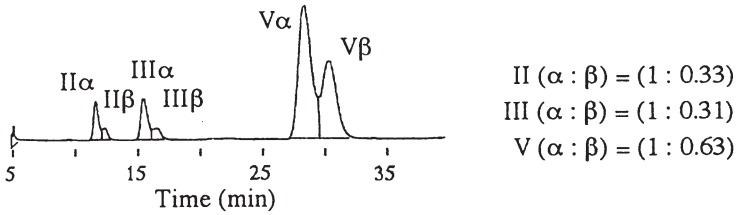

Figure 2. HPLC Analysis of the reaction of *N*-acetylchitopentasaccharide by yam chitinase E. *N*-acetylchitopentasaccharide (0.1 mM) was reacted with 40 nM yam chitinase E for 30 min in 4 mM sodium acetate buffer, pH 4.0, at 25°C. The reaction mixture was analyzed by HPLC as described in Materials and Methods. The ratios of α to β anomers of *N*-acetylchitooligosaccharides after the 30 min reaction are represented at the right side.

observed in both products, suggesting yam chitinase E hydrolyze the substrate in the inverting mechanism to produce the α amomeric forms. Therefore, we supposed that the pentasaccharide would be cleaved in two patterns such as OOIOOO (case 1, A%) and OOOIOO (case 2, B%), and tried to calculate the concentrations of both anomers of the products such as the disaccharide and trisaccharides under two assumptions. First, we assumed that the anomer ratios of the reducing end-side oligosaccharides produced from the substrate are the same as those of naturally occurring *N*-acetylchitooligosaccharides such as 1 : 0.66 for the disaccharide and 1 : 0.65 for the trisaccharide as shown in Fig. 1. Second, we assumed that the anomer ratios of the newly produced oligosaccharides with α anomer would change to 1 : 0.1 by mutarotation during the reaction, that is the ratio value of the disaccharide produced from the substrate trisaccharide in a 30-min reaction. Using these two assumptions, we made two equations on the ratio of α to β with respect to the disaccharide and trisaccharide. For the disaccharide that comes A% from case 1 with newly formed anomer

and B% from case 2 with equilibrated anomer, as its ratio of a to b was 1 : 0.33, the following equation comes.

$$[A/(1+0.1)+B/(1+0.66)] : [0.1A/(1+0.1)+0.66B/(1+0.66)] = 1 : 0.33 \quad (1)$$

For the trisaccharide that comes A% from case 1 with equilibrated anomer and B% from case 2 with newly formed anomer, as its ratio of α to β was 1 : 0.31, the following equation comes.

$$[A/(1+0.65)+B/(1+0.1)] : [0.65A/(1+0.65)+0.1B/(1+0.1)] = 1 : 0.31 \quad (2)$$

From equations 1 and 2, B/A was calculated to be 1.052 and 1.079, respectively. These values are very similar, indicating that these assumptions are reasonable. As A + B = 100%, we could calculate A and B to be 48.4% and 51.6%, respectively, using the average B/A value of 1.065. As the result, we could estimate that *N*-acetylchitopentasaccharide is cleaved to the disaccharide and trisaccharide at the second linkage (48.4%) and third linkage (51.6%) from the non-reducing end side of the substrate. Other reactions were similarly analyzed by this method.

Table 1. Splitting pattern and anomer formation.

		Trimer	Tetramer	Pentamer	Hexamer	Anomeric form	Inhibition by allosamidin
Yam							
Family 19 chitinase							
Chitinase E (Class IV)	(pH 4.0)	100	100	48.4 51.6	14.5 13.7 12.2 59.6	α	Not inhibited
	(pH 8.0)				7.5 9.1 10.4 73.0	α	Not inhibited
Chitinase F	(pH 4.0)	100	90.83 9.17	35.17 45.52 19.31	8.34 9.40 7.54 74.72	α	Not inhibited
	(pH 8.0)				5.3 5.3 6.7 82.7	α	Not inhibited
Chitinase G	(pH 4.0)		100	24.1 67.2 8.7	19.0 74.9 6.1	α	Not inhibited
Family 18 chitinase							
Chitinase H (Class III)	(pH 4.0)		100	or 5.6 94.4	3.0 61.3 35.7	β	ID_{50} = 1.26 mM
	(pH 8.0)				1.7 85.7 12.6	β	ID_{50} = 44.7 μM
Silkworm							
Family 18 chitinase							
54-kDa chitinase	(pH 6.0)	36.5 64.5	2.0 64.5 4.6	100	48.2 51.8	β	ID_{50} =0.552 μM
β-*N*-Acetyl-glucosaminidase	(pH 6.0)	100	100	100	100	β	Not inhibited

The percentages of cleaving rates are indicated below the arrows.

○ : *N*-Acetylglucosamine

Analysis of yam chitinases: For yam chitinases such as Chitinases E, F, H and G, the enzymatic reactions were analyzed by this HPLC method. The results are shown in Table I.

Chitinases E, F and G, which all belong to the family 19 chitinase, hydrolyzed the *N*-acetylchitooligosaccharides longer than disaccharide in the inverting mechanism to produce the α anomeric form. On the other hand, Chitinase H, which belongs to the family 18 chitinase, did in the retaining mechanism to produce the β anomeric form.

These all yam chitinases showed only one optimum pH around 4 toward *N*-acetylchitoligosaccharides. However, Chitinases E, F and H have two optimum pH toward a long substrate glycolchitin, whereas Chitinase G has only one. Therefore, we examined the splitting patterns at another optimum pH such as pH 8. The results show that the hydrolytic mechanism such as anomer formation and cleavage site are almost same between both pHs (Table I).

Analysis of the silkworm chitinases and N-*acetylglucosaminidase*: We has already purified and characterized another family 18 chitinase from the silkworm, *B. mori*, such as 54-kDa chitinase. Therefore, it is interesting to examine whether these insect family 18 chitinase hydrolyzes the substrate in the retaining mechanism as well as plant family 18 chitinase such as yam Chitinase H. The results are shown Table I. Although the cleavage sites are a little different, the anomer formations are the same between them.

On the other hand, the silkworm β-*N*-acetylglucosaminidase hydrolyzed *N*-acetylchitooligosaccharides in the retaining mechanism to produce β anomeric form as well as the family 18 chitinases, but in the exo manner. This cleaving direction was already proved by using *p*Np-conjugated *N*-acetylchitooligosaccharides [21]. The monomeric *N*-acetylglucosamine is successively released from the non-reducing end side.

Thus the family 18 chitinases such as yam Chitinase H and the silkworm 54-kDa chitinase hydrolyze the substrate in the retaining mechanism. However, there is a large difference between the family 18 chitinases and the exo-type chitinolytic enzyme N-acetylglucosaminidase. That is the inhibition by allosamidin. The family 18 chitinases were inhibited by allosamidin, whereas the silkworm β-*N*-acetylglucosaminidase was not. This would suggest that the family 18 chitinases may hydrolyze the substrate via the intermediate of the oxazoline ring, but the β-*N*-acetylglucosaminidase may hydrolyze like hen-egg lysozyme, which is not inhibited by allosamidin either.

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An acidic chitinase from gizzards of broilers (*Gallus gallus* L.)

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Summary
An acidic chitinase from the gizzards of broilers was purified to homogeneity using precipitation with $(NH_4)_2SO_4$, ion exchange chromatography, gel filtration, chromatofocusing and hydrophobic interaction chromatography. The enzyme was purified 180 fold with a yield of 5% enzyme activity. The molecular weight of the purified enzyme was 48.2 kDa as assessed by SDS-PAGE and 48.7 kDa by gel filtration chromatography. Chromatofocusing resulted in a pI of 3.1 for the 48.2 kDa chitinase. The purified enzyme was an endochitinase devoid of β -*N*-acetylglucosaminidase and lysozyme activity. Kinetic studies using [^{3}H]-chitin indicate that the enzyme has a K_m for chitin of 1.97 mg/ml and a V_{max} of 185 mg/mg protein/h at optimal pH and temperature (pH 4.0-5.0 and 60°C). The first 25 NH_2-terminal residues of the chitinase shared 55-64% homology with animal chitinases and some other animal proteins, but little homology was found with either microbial and plant chitinases or egg white lysozyme.

Introduction

The enzymes responsible for the hydrolysis of chitin consist of two hydrolases, endochitinase (EC 3.2.1.14) and β -*N*-acetylglucosaminidase (EC 3.2.1.30). The chitinases from bacteria, molds, insects and plants have been purified and are relatively well characterized [1]. This is mainly due to their vital roles in energy extraction from the environment, modification of the chitin components in fungi and arthropods [2], and the stress response system in plants [3].

In contrast, chitinases from the gastrointestinal tracts of vertebrates have been poorly studied, excepting those from fish [4]. However, studies of the digestive tracts of birds have shown that the sparrow (*Passer domesticus*), the Japanese nightingale (*Liothrix lutea*), adult chicken (*Gallus gallus*), and the barn owl (*Tyto alba*), all have high chitinolytic activity. Conversely, the wood pigeon (*Columba palumbus*) and the African grey parrot (*Psittacus erithacus*) do not exhibit any chitinolytic activity [5]. Furthermore, these studies demonstrated that 20-50% of the ingested chitin was digested by *Gallus gallus* and *Liothrix lutea*. Despite their important role in poultry nutrition, bird chitinases have not been extensively investigated.

Following the report of a crude enzyme prepared from chicken [6], we have recently investigated the chitinolytic activity of the gizzard and the chyme from *Gallus gallus* for use in industrial applications [7]. The crude preparations from gizzards of *Gallus gallus* showed

high endochitinolytic activity with good thermostability. In this paper, we describe the purification, enzymatic properties, and NH_2-terminal amino acid sequence of an acidic endochitinase from the gizzards of broilers.

Materials and Methods

Materials and extraction of the crude enzymes: Fresh broiler gizzards were obtained from a local poultry-processing factory. They were stored at -70°C until use. Crude extracts of gizzard were prepared as previously described [7]. The protein content was determined by the method of Lowry, et al. [8]

Purification procedures: The crude preparation was adjusted to pH 4.5 and loaded onto a Source S (Pharmacia) column. The column was extensively washed prior to elution using a linear gradient of NaCl. The unbound fraction containing the enzyme activity was concentrated by ultrafiltration. The sample was applied onto a Source Q (Pharmacia) column. The column was washed with 0.02M Tris/HCl and eluted with a step-wise gradient of NaCl (0.18M and 0.38M). After freeze-drying, protein-containing fractions were applied to a Superdex G200 (Pharmacia) gel filtration column. The fractions showing enzyme activity were pooled and freeze-dried. The active fractions were loaded onto a Mono-P HR (Pharmacia) chromatofocusing column. The column was successively eluted with diluted Polybuffer®74 (Pharmacia) at pH 3.2 and 2.0, respectively. Solid $(NH_4)_2SO_4$ to a concentration of 1 M was added to the active fractions. The resulting solution was applied to a Phenyl-Sepharose HP (Pharmacia) hydrophobic interaction column and eluted with a linear gradient of $(NH_4)_2SO_4$ (1.0 M to 0.0 M). The eluted chitinases were pooled and kept at 4°C.

Electrophoresis and detection of chitinases and proteins on gels: 12.5% SDS-PAGE was performed in the presence of 7.5% glycol chitin according to Laemmli [9] and the enzyme activity was detected as described by Trudel [10]. Proteins were visualized according to the method of Wray et al. [11].

Enzyme assays: Chitinase activity was measured using [^{3}H]-chitin according to the method reported previously [7]. 588 dpm were equivalent to 1 μg of dry chitin. One unit of enzyme activity is defined as the amounts of enzyme required to produce 1 μmol of monomers per min at 50°C. β-*N*-acetylglucosaminidase assays were carried out by adding 100 μl of the purified enzyme solution to 0.2 ml of 4 mM *p*-nitrophenyl-β-*N*-acetylglucosamine in McIlvane buffer (0.05 M, pH 5.0). After 1h incubation at 37°C with shaking, 2 ml of 0.2M sodium carbonate were added to the reaction mixture and the liberated *p*-nitrophenol was measured at 420 nm.

Lysozyme activity was measured according to the method described by Martin [12].

Determination of NH_2-terminal amino acid sequence: The purified enzyme was blotted onto a PVDF membrane. Automated Edman degradation was performed using a protein/peptide sequencer 471A (Applied Biosystems, USA) according to the method described by the manufacturer.

Results and Discussion

Purification of an acidic chitinase: As seen in Fig. 1A, more than 95% of the total chitinase activity was found in the unbound fractions. When the unbound chitinases (SS1) were chromatographed on the Source Q column, they were separated into two major peaks (QF1 and QF2) containing chitinase activity (Figure 1B). Although QF1 contained 43% of the total activity and had higher specific activity and yield (0.63mg/mg protein/h, 43%) than QF2 (0.35mg/ mg protein/h, 16%), QF2 was further purified based on its protein composition as assessed by SDS-PAGE (data not shown). Gel filtration of the QF2 fraction from the Source

Q column resulted in two peaks. GF2 contained most of the chitinase activity (Fig.1C). No protein was eluted at pH 6.3 after chromatofocusing of GF2 on a Mono-P column. The first fraction with enzyme activity (CF1) was eluted at pH 4.1, but showed reduced enzyme activity. After stabilizing the column at pH 3.8, a second linear pH gradient (3.8 to 2.6) was carried out. Immediately after gradient formation, CF2 was eluted, and contained 80% of the total chitinase activity (Fig.1D). CF2 from chromatofocusing was subjected to HIC. HF1 and HF2 were obtained (Fig.1E), HF1 showed a single band upon SDS-PAGE (Fig.2A). HF1 had a molecular weight of 48.2kDa (Fig.2B). This purified enzyme showed a molecular weight of 48.7kDa by gel filtration (data not shown). These results indicate that the acidic chitinase functions as a monomeric enzyme similar to other chitinases [1-3]. Animal chitinase molecular weights range from 35kDa to 88kDa depending upon their origin. A chitinase of 50kDa was isolated from the stomach of Japanese eel (*Anguilla japonica*) by Kono, et al. [13]. Koga, et al. [14] purified two chitinases (65 kDa and 88 kDa) from larvae of *Bombyx mori.* Lundblad, et al. [15] have purified a 47kDa chitinase from bovine serum.

The results of the purification are summarized in Table 1. After the final purification step, the chitinase was purified 180 fold with a recovery of 4.9%.

Table 1. Purification of the acidic chitinase

Treatment	Protein (mg)	Activity (U)	Specific activity (mU/mg)	Recovery (%)	Purification (fold)
Crude extract	3,006	11.52	3.8	100	1
$(NH_4)_2SO_4$	1,058	9.3	8.8	81.1	2.3
Source-S	352	8.4	24.0	73.3	6.7
Source-Q	63.3	1.84	29.0	15.9	7.6
Superdex G200	22.1	1.46	66.0	12.7	17.2
Mono-P	6.2	1.16	187.0	10.0	48.7
Phenyl-Sepharose	0.82	0.57	688.0	4.9	179.6

Enzymatic properties of the purified chitinase: The optimal pH of the chitinase (HF1) ranged from 4.0 to 5.0. The purified chitinase has maximal activity at 60°C and loses most of its activity at 80°C (data not shown). As the crude enzyme preparation from gizzards maintained 40% of its activity at 80°C [7], some thermostabilizing factors must be lost during purification. β-*N*-acetylglucosaminidase or lysozyme activity was not observed, even after prolonged incubations. The lack of β-*N*-acetylglucosaminidase and lysozyme activities is in good agreement with properties reported for other animal chitinases [1]. The K_m and V_{max} of the purified chitinase (HF1) using [^{3}H]-chitin was 1.97mg/ml and 185mg/mg protein/h, respectively.

NH_2-terminal amino acid sequence: HF1 chitinase shared little homology with the known NH_2–terminal sequences of chitinases from bacteria and plants [1]. As shown in Fig.3, however, HF1 shares 64, 63, 59 and 54% homology with animal chitinases from Brugia malayi (SwissProt P29030), silk worm (Bombyx mori) [14], and chitotriosidases from humans [16] and Caenorhabditis elegans (SwissProt Q11174), respectively. Furthermore, the

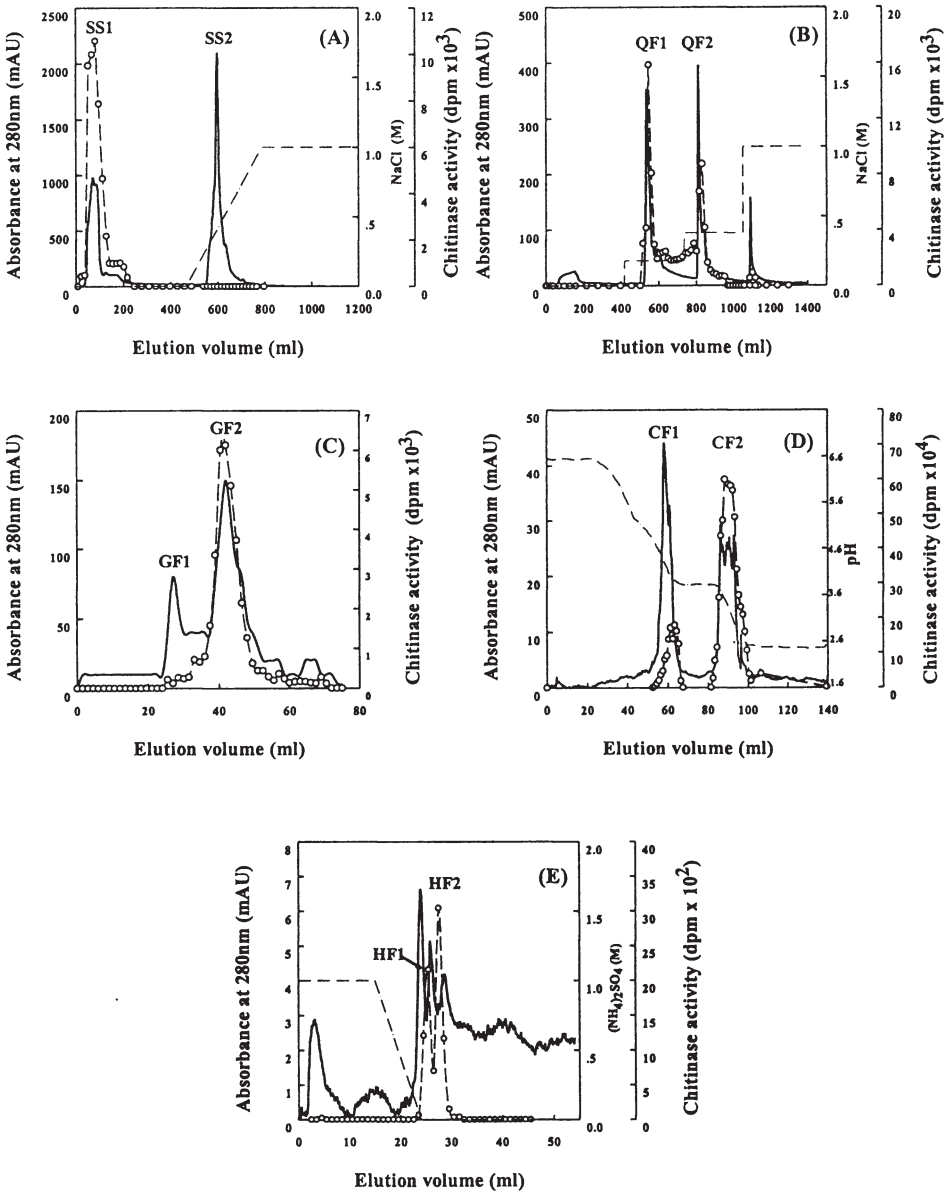

Figure 1. Purification of an acidic chitinase from gizzard of broilers by different chromatographic methods. (A) Source S chromatogram of 20,000g supernatant, (B) Source-Q chromatogram of fraction SS1, (C) Superdex G-200 gel filteration of fraction QF2, (D) Mono-P chromatofocusing of GF2 with Polybuffer 74, (E) HIC of CF2 with Phenyl-Sepharose

——— : Absorbance at 280nm, —□—□— : Chitinase activity,

— - — : Salt/pH gradient.

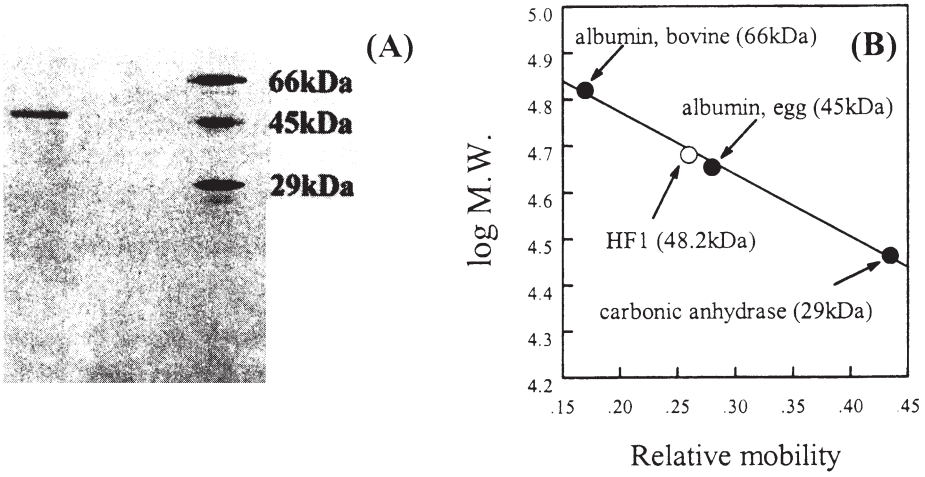

Figure 2. SDS-PAGE of HF1 and estimation of its molecular weight. (A) Electrophoretogram of HF1, (B) Estimation of the molecular weight

purified chitinase also shares 60 and 55-56% homology with bovine cartilage glycoprotein-39 (SwissProt P30922) and oviduct-specific glycoproteins of several species (SwissProt Q62010, P36718, Q28042, Q12889). The fact that there is little homology with egg white lysozyme agrees with the finding that the 48.2kDa chitinase did not have lysozyme activity. This may suggest that in animals the roles of chitinases and lysozymes are highly specific.

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Protein		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	%
Broiler HF1		Y	V	L	S	X	Y	F	T	N	W	A	Q	Y	R	P	G	V	G	S	F	M	P	D	N	I	100
B. malayi hitinase		Y	V	R	G	C	Y	Y	T	N	W	A	Q	Y	R	D	G	E	G	K	F	L	P				64
B. mori chitinase		A	R	I	V	X	Y	F	S	N	W	A	V	Y	R	P	G										63
Chitotriosidase		A	K	L	V	C	Y	F	T	N	W	A	Q	Y	R	Q	G	E	A	R	F	L	P				59
Cartilage gp-39		Y	K	L	V	C	Y	Y	T	S	W	S	Q	Y	R	E	G	D	G	S	C	F	P				50
wiss Prot	Q62010	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	
		Y	K	L	V	C	Y	F	T	N	W	A	H	S	R	P	G	P	A	S	I	M	P	H	D	L	56
	P36718						27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	
							Y	F	T	N	W	A	H	S	R	P	G	P	A	S	I	L	P	H	D	L	55
	Q28042						24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	
							Y	F	T	N	W	A	F	S	R	P	G	P	A	S	I	L	P	R	D	L	55
	Q12889						27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	
							Y	F	T	N	W	A	H	S	R	P	G	P	A	S	I	L	P	H	D	L	55
Hen lysozyme		K	V	F	G	R	C	E	L	A	A	A	M	K	R	H	G	L	D	N	Y	R	G	Y	S	L	18

Figure 3. NH_2-terminal amino acid sequence and alignment with the chitinase protein family

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On the contribution of conserved acidic residues to catalytic activity of chitinase B from *Serratia marcescens*

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Summary

Acidic residues in the active site of chitinase B from *Serratia marcescens* were replaced by their corresponding amides and/or alanine. Mutation of the catalytic acid (Glu144) gave the largest reduction in activity (k_{cat}). Mutation of Asp140, Asp142 and Asp215 also reduced activity considerably. Mutation of the relatively buried Asp140 was highly deleterious for activity, presumably as a result of the loss of specific interactions with Asp142 and the loss of the negative charge. The data support the notion that the importance of Asp142 for activity is due to its interactions with Glu144 and with the C2 N-acetyl group of the –1 sugar. Asp215, which has received little attention in previous studies, also seems to have important interactions with the substrate during catalysis.

Introduction

Serratia marcescens is one of the most efficient bacteria for degradation of chitin and it has been shown that this organism produces up to five different chitinases [1]. The crystal structures of two of these chitinases are known: chitinase A (ChiA), a family 18 [2] endochitinase [3], and chitobiase, a family 20 chitinase [4]. Another family 18 chitinase produced by *Serratia marcescens* is chitinase B (ChiB), which contains 499 amino acid residues and presumably acts as an exochitinase (chitobiosidase) [5-7]. A three-dimensional model of the catalytic domain of ChiB has been built using the ChiA structure as a template (35 % sequence identity [8]).

The catalytic domains of family 18 glycosyl hydrolases have a typical $(\beta/\alpha)_8$ barrel-fold (TIM-barrel), which, in the case of ChiA and ChiB, is supplemented with a so-called $\alpha+\beta$ domain inserted between strand 7 and helix 7. Catalytically active members of family 18, including ChiA and ChiB, contain a conserved DXXDXDXE motif that spans strand 4 ([9] Fig. 1). Site-directed mutagenesis as well as crystallographic studies have indicated that the glutamate residue located at the C-terminus of strand 4 is the catalytic acid that protonates the glycosidic bond during catalysis [3,9,10,11]. Since ChiB and other chitinases are active in the pH 4 – 8 range, the pK_a of the catalytic glutamate must be considerably higher than usual during parts of the catalytic cycle. Disturbance of this pK_a as well as its fluctuation during catalysis [12] are presumably at least in part achieved by a complex interplay with several (conserved) acidic residues that are close to the catalytic center [9,12], including (some of) those in the DXXDXDXE motif. These acidic residues also play a role in stabilizing the postively charged transition states and/or intermediates that emerge during catalysis [11-16].

Hydrolysis of glycosidic bonds by family 18 chitinases results in retention of the anomeric conformation [3, 17]. According to the classical view on glycosidase mechanisms, retention of the configuration would be achieved by a double displacement mechanism. This

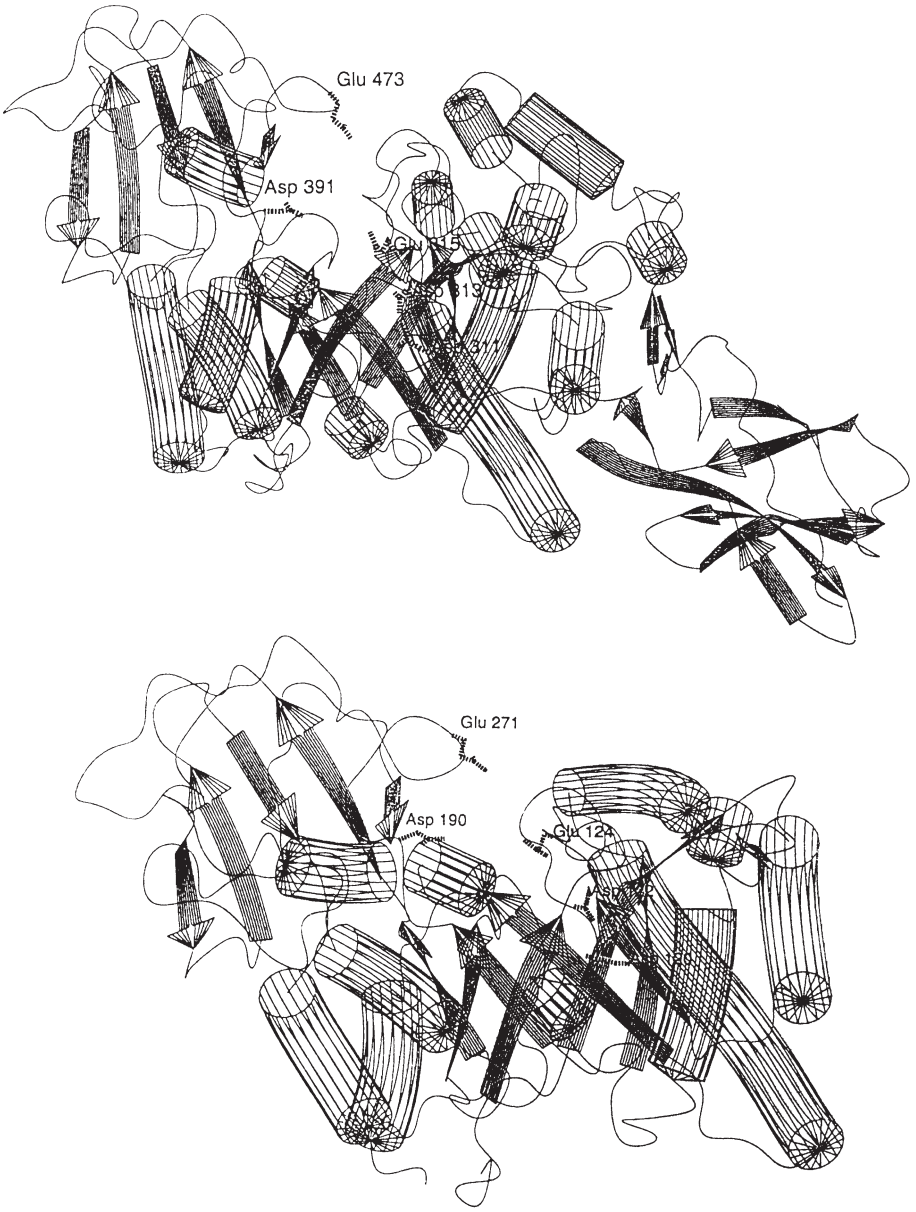

Figure 1. Ribbon drawing of the structure of ChiA (upper panel) and the model of ChiB (lower panel) in which side chains of the residues corresponding to Asp140, Asp142, Glu144, Asp215 and Glu330 in ChiB are indicated. Note that the model of ChiB lacks 78 residues that presumably constitute a linker followed by a chitin-binding domain [7] as well as 68 residues divided over nine insertions spread throughout the displayed model. Because of the nine missing insertions, residue numbers in the lower panel differ from those used in the text (120, 122, 124, 190 and 271 correspond to 140, 142, 144, 215 and 330, respectively).

mechanism requires the presence of a second acidic residue that acts as a nucleophile in the first part of the catalytic cycle (formation of a covalent enzyme-substrate intermediate via a oxycarbenium-like transition state [12-14,18]). Such a residue needs to be located in close proximity of the catalytic acid (approximately 5.0 Å between the carboxylic oxygens [14]). The situation in family 18 chitinases is somewhat different because they lack an acidic residue that is sufficiently close to the catalytic glutamate and because the substrate contains an N-acetyl group at C2 which may affect the mode of substrate-binding. It is now generally accepted that catalysis occurs in a substrate-assisted way, with the carbonyl oxygen atom of the substrate's N-acetyl group acting as the nucleophile [11,15,16]. This mechanism proceeds via formation of an oxazoline ion and does not involve covalent intermediates.

To gain further insight in the mechanism of catalysis in ChiB, we have studied the effects of mutating acidic residues near the active site on catalytic activity. In addition to residues in the DXXDXDXE motif, we mutated Asp215 which is fully conserved in family 18 chitinases and whose carboxyl oxygens are located at only 7 Å from the carboxyl group of the catalytic glutamate. Glu330, in close proximity to Asp 215, was also mutated.

Materials and methods

Genetic techniques

Plasmid pMAY2-10 carrying the *chiB* gene from *Serratia marcescens* JBL200 has been described previously [6]. For site-directed mutagenesis, fragments of the *chiB* gene were subcloned in plasmids pGEM5Z(+) or pGEM3Z(+) (Promega, Madison, WI). Site-directed mutagenesis was performed using the QuikChange™ Site-directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA), essentially as decribed by the manufacturer. Sequences of mutated *chiB* fragments were verified using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 377 DNA Sequencer (PE Applied Biosystem, Foster City, CA). Fragments having the correct sequence were used to construct pMAY2-10 variants containing intact *chiB* with the desired mutation. pMAY2-10 variants were transformed to competent *E. coli* DH5α™ (Life Technologies, Rockville, MD). All recombinant strains were grown in Luria Broth (LB) medium supplemented with 50 μg ampicillin ml^{-1}. For plates, LB was solified with 1.5 % (w/v) agar.

Production and purification of wt and mutated ChiB:

The wild type and mutants of ChiB were produced in *E. coli* DH5α and purified from a periplasmatic extract by hydrophobic interaction chromatography, as described previously [7]. Columns were washed extensively between purifications, to prevent crosscontamination of low activity mutants. The purity of the enzyme preparations was verified using SDS-PAGE. protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA).

Enzyme assays

The activity of ChiB variants was determined using the $(GlcNAc)_3$ analogue 4-methylumbelliferyl-β-D-N.N´-diacetylchitobioside [4-MU-$(GlcNAc)_2$] as substrate. The enzyme concentrations used varied, since they had to be adapted to the varying activities of the ChiB variants. In a standard assay [7], 100 μl of a mixture containing enzyme, 20 μM substrate, 50 mM citrate-phosphate buffer, pH 6.3, and 0.1 mg/ml BSA was incubated at 37 °C for 10 minutes, after which the reaction was stopped by adding 1.9 ml 0.2 M $NaCO_3$. The amount of 4-MU released was determined using a DyNA 200 Fluorimeter (Hoefer Pharmacia Biotech, San Francisco,CA). Kinetic properties were determined by initial rate measurements, using various substrate concentrations. Product formation was monitored during time and found to be linear

with time for at least 10 minutes, at all substrate concentrations and for all mutants. K_m and k_{cat} were determined using a S/V against S plot.

Results and discussion

In ChiB the DXXDXDXE motif contains Asp137, Asp140, Asp142 and Glu144 (Fig. 1). Across the active site cleft, opposite of Glu144, ChiB contains an aspartate (Asp215; Fig. 1). Another conspicuous acidic residue near the active site in ChiB is Glu330, which is close to Asp215 (Fig. 1). These acidic residues were mutated to their corresponding amides and/or Ala, as indicated in Table I. All ChiB variants were expressed in normal amounts, with the exception of D137N, for which no protein could be detected. All mutants behaved as the wild-type during the purification process.

Mutation	Specific activity		K_m	k_{cat}	
	(nmol s^{-1} mg^{-1})	(%)	(μM)	(s^{-1})	(%)
D137N*					
D140N	0.21	0.18	49.4	0.025	0.13
D140A	0.18	0.15			
D142N	4.4	3.7	3.8	0.32	1.6
D142A	0.14	0.12			
E144Q	0.035	0.029	19	0.0037	0.019
E144A	0.0044	0.0037			
D215N	12.0	10	26.7	1.47	7.6
D215A	0.043	0.036			
E330A	53	44	20	6.5	34
wild type	120	100	31.5	19.4	100

Table I. Activity of ChiB variants. *For the D137N mutant no protein could be detected.

The results (Table I) show that, with the exception of E330A, all mutations severely affected activity. Glu330 is apparently located too far away from the catalytic center to affect hydrolysis of the short substrate used in this study.

A major effect of both types of mutations (acid-to-Ala or acid-to-amide) is the loss of a negative charge close to the catalytic glutamate (positions 140, 142 and 215) or the ability to act as a proton-donor during catalysis (position 144). Although this major effect would be more or less similar for the two types of mutations, introduction of an alanine was considerably more deleterious for activity at positions 142, 144 and 215. One explanation for this difference could be that the activity observed for the amide mutants is at least in part resulting from some degree of deamidation in the enzyme preparations, thus restoring the wild-type acidic residue. This explanation is unlikely, however, since Ala mutants and their corresponding amide mutants displayed similar pH-activity profiles (S. Gåseidnes, B. Synstad and V.G.H. Eijsink, unpublished observations). Thus, it would seem that the extra loss in activity observed upon replacing the amides at positions 142, 144, 215 by alanine is due to the loss of specific interactions with the substrate and/or local disturbances of the structure.

From previous studies on chitinases [3,9,10,11,19] it is clear that Glu144 plays an essential role in catalysis by donating the proton necessary for initial protonation of the scissile

glycosidic bond. Thus, it is not surprising that E144Q was the most deleterious acid -> amide mutation in this study.

The side chain of Asp142 is at hydrogen-bonding distance of the side chain of Glu 144 and can clearly modulate the pK_a of Glu144. Another important property of Asp142 may be its ability to interact with the N-acetylgroup in the –1 sugar and to stabilize positive charge that emerges during catalysis [11,15,16]. Both these roles can in part be fullfilled by an asparagine, which may explain why D142N, in contrast to D142A, left a considerable amount of activity (1 – 4 %). It is interesting to note that the D142N mutation resulted in an 8-fold reduction of the K_m. A similar effect on K_m was observed after mutation of the corresponding residue in chitinase A1 from *B. circulans* [20]. Thus, it would seem that an uncharged asparagine is optimal for binding of the (uncharged) substrate, whereas a charged aspartate is optimal for stabilizing the positively charges emerging during catalysis.

Despite its location relatively deep in the TIM-barrel core, mutation of Asp140 was almost as deleterious for enzyme activity as mutation of the catalytic Glu144. Asp140 may affect catalysis through direct interactions, since it makes hydrogen bonds to Asp142. Residue 140 is likely to also contribute to catalysis through electrostatic interactions that do not involve hydrogen bonds, since we did not observe a significant difference in activity between D140N and D140A. In accordance with this notion, replacement of the corresponding Asp in chitinase A1 from *B. circulans* by Glu had no effect on k_{cat} [10].

Replacing Asp215 by asparagine reduced activity approximately 10-fold, whereas replacing this residue by alanine was highly deleterious. Thus, as previously suggested for ChiA [3], this residue opposite of the catalytic glutamate contributes significantly to catalysis. The moderate effect of D215N indicates that residue 215 is hydrogen bonding with the substrate along the reaction pathway (such hydrogen bonds could be conserved upon the D215N mutation). Residue 215 could also make a contribution to modulation of the pK_a of the catalytic glutamate and/or to stabilizing partial positive charges emerging during catalysis. Such contributions are probably of limited importance because of the rather large distance to Glu144 and the N-acetyl group in the -1 sugar.

Combining the present results with the results of the various structural studies cited above, it becomes clear that catalysis in ChiB and other family 18 chitinases depends on a rather extended network of interactions, involving residues from inside the TIM-barrel (e.g. Asp140) as well as residues in more exposed loops on the catalytic surface of the barrel (e.g. Asp215). More detailed conclusions, and, eventually, acquisition of the ability to rationally manipulate the catalytic properties of chitinases await further studies of the present as well as new mutants (measuring pH profiles, calculating electrostatics, X-ray crystallography). Such studies are currently in progress in our laboratories.

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Detection, isolation and preliminary characterisation of a new hyperthermophilic chitinase from the anaerobic archaebacterium *Thermococcus chitonophagus*

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Summary
Thermococcus chitonophagus is a novel isolate from deep sea vents and the first Achaeon to be studied for chitin degradation mechanisms. We have grown it under strictly anoxic and high temperature conditions. The presence of a cell-associated, chitin- inducible chitinoclastic enzyme system has been verified by replacing the organic source of carbon with chitin and the consequent strong expression of the genes encoding for chitinolytic enzymes, as it has been demonstrated with several kinetics experiments. The highest levels of expression were obtained after 50-60 h of incubation at 85°C in the presence of chitin. The activities of chitinase and chitobiase have been measured using chromogenic and fluorogenic substrates on whole, untreated cells and on periplasmic extracts. The enzymes have been found to exhibit the optimum activity at temperatures higher than 80°-85°C and at pH 7.0. Chitinase has been identified as a clear band of a single polypeptide of approximate size of 70 kDa on SDS-PAGE gels and activity gels using as substrates CM-chitin-RBV or fluorogenic mono/oligosaccharides.

Introduction

Chitin, the second most abundant polysaccharide, a cellulose-like biopolymer is widely distributed in marine and terrestrial biotopes. This homopolymer of N-acetyl-D-glucosamine is not only a major constituent of the fungal cell wall and the arthropod exoskeleton, but also consists an important nutrient source of carbon and nitrogen in the marine environment. Both chitin and chitin- related enzymes are widespread in nature, occuring in bacteria, plants, fungi, vertebrates and invertebrates and are of major biotechnological interest. Because of the potential application of this natural polymer for various purposes in industry, several chitin-related enzymes are the subject of intensive research [1].

Chitinases are a class of enzymes that hydrolyse chitin into smaller oligosaccharides, which are further degraded by *chitobiase* to monosaccharides. *Chitin deacetylase* partially removes acetyl groups from chitin , converting it to chitosan. Chitinases (E.C. 3.2.1.14.) have been classified into families 18 and 19 of glucosyl hydrolases. The predominant product of their enzymatic activity is N,N'-diacetylglucosamine – chitobiose - which is the substrate for chitobiase (E.C. 3.2.1.30), the enzyme that has been classified into family 20 of glucosyl hydrolases [2,3] .

Chitin degrading/modifying enzymes are important as potent inhibitors of fungal growth in plants and could especially protect commercially important crops from insect host

and pathogenic fungi, providing in this way an environment friendly alternative of the chemical pesticides. For this reason, but also thanks to the other well promising industrial applications, chitinases have acquired, in the recent years, a major biotechnological and commercial interest [4,5,6]. During the last five years the number of studied chitin hydrolysing / modifying proteins – genes has been dramatically increased. However, the number of published chitin-hydrolysing enzymes from bacteria is by far smaller compared to plant and fungal chitinolytic enzymes.

Most chitin degraders are aerobes and very little is known about the anaerobic degradation of chitin. Untill recenlty, Archaea with chitinolytic activity were unknown. Within the Archaea domain the ability to use chitin as a growth substrate is a peculiar property of *Thermococcus chitonophagus*, a novel, anaerobic, hyperthermophilic archaebacterium isolated from a hydrothermal vent site off the west Mexican coast at the depth of 2,600 m [7]. At deep-sea hydrothermal sites, the dominant megafauna contains chitinous structures, such as clam shells, crab carapaces and pogonophoran tubes. These types of invertebrates can be grown at unusually high rates providing substantial amounts of substrates for organisms with special substrate specificities, such as the new chitin-degrading isolate [8]. Members of the Thermococcales represent a unique group of hyperthermophilic microorganisms belonging to the Euryarchaeota branch within the Archaea domain.. During the last years a variety of extracellular proteolytic and saccharolytic enzymes from the Thermococcales have been purified and characterised. In general, these enzymes are oxygen-stable and exhibit high thermostabilities, being active at temperatures of up to about 140°C [9,10].

Chitinolytic enzymes produced by hyperthermophilic organisms are expected to present increased thermostability that could also be accompanied by chemical stability. Since many industrial enzymes are used at temperatures above 50^0C, there is a considerable commercial demand to develop thermostable forms as biocatalysts in modern biotechnology [11,12].

The new isolate grows between 60° and 90°C (optimum 85°C), from pH 3.5 to 9 (optimum: pH 6.7) and from 0.8 to 8% NaCl (optimum: 2%). The isolate is an obligate organotroph that can use chitin, yeast extract, meat extract and peptone for growth. Chitin is fermented to H_2, CO_2, NH_3, acetate and formate, while H_2S is formed in the presence of sulfur. The chitinoclastic system is oxygen stable, cell- associated and inducible by chitin. Cellulose and chitosan are not degraded, indicating that the chitinolytic system of *Thermococcus chitonophagus* exhibits high substrate specificity [7].

Materials and Methods

Thermococcus chitonophagus was grown under strictly anoxic and high temperature conditions in 120- ml serum bottles containing 60 ml of medium, in the presence of sulfur and 2% NaCl and at pH 7.0. The medium for non-induced cultures was supplemented with 0.1% yeast extract and 0.5% peptone. For the chitin-inducible medium peptone was replaced with approximately 0.5% chitin.

Cultures were incubated at 85° C in the presence of 300kPa N_2 for several days. Cells were harvested at 10, 20, 30, 40, 50 and 60 h intervals, pelleted and washed with PBS. Cell density was determined spectrophotometrically at 600 nm and chitobiase and chitinase activities were monitored on intact cells, free of culture medium, using the chromogenic substrates p -nitrophenyl-n-acetyl-a-D-glucosaminide [pNP-GlcNAc], for chitobiase, and p-nitrophenyl-a -D-N,N'-diacetyl-chitobiose [pNP-$(GlcNAc)_2$] for chitinase, and measuring the increase of absorbance (release of the p-nitrophenyl group) spectrophotometrically at 405 nm [13,14]. A substrate concentration of 80 μM was used to determine reaction rates of both chitinase and chitobiase in the presence of a variety of different cell densities (ranging from

0.01 to 0.1 OD_{600nm}/ml culture), pH (ranging from 4.0 to 12), temperatures (ranging from 40° to 95°C) and denaturing reagents (like, urea and guanidine-HCl).

Cells were also harvested after incubation at 85°C for 10, 20, 30, 40, 50 and 60 h, pelleted and mixed vigorously, boiled for 10 min. and sonicated for 30 min. in a hot ultrasonic bath in a biffer containing 20 mM Tris, pH 7.5, 1% CHAPS, 1% Triton X-100, 1 mM EDTA and 0.1 mM PMSF. The cell extracts were centrifuged at 16,000xg, for 30 min, at 4°C and samples from the supernatants and pellets were electrophoresed on 12% SDS-PAGE, coomassie- stained, as well as on 12% SDS-PAGE, either containing 0.7 mg/ml of the blue substrate CM-chitin-RBV, or overlayed with 0.4 mM of the fluorogenic subtrates 4MU-$(GlcNAc)_2$ for chitinase and 4MU-GlcNAc, for chitobiase [15,16,17]. The analysed supernatants and pellets were also transferred on nitrocellulose membranes and incubated with antibodies raised against purified chitinase A and chitobiase from *Serratia marcescens*.

Supernatants and pellets of treated cell extracts from non-induced cultures were used in every experiment as negative controls, whereas purified chitinase A and chitobiase from Serratia marcescens were added as positive controls.

Results and Discussion

Thermococcus chitonophagus was grown at 85°C, in a nitrogen atmosphere and in the presence of sulfur and chitin or peptone as a carbon and energy source. The two constituents of the chitinoclastic system of *Thermococcus chitonophagus* -chitinase and chitobiase- were detected using the chromogenic (pNP-$(GlcNAc)_2$ and pNP-GlcNAc) and fluorogenic (4MU-$(GlcNAc)_2$ and 4MU-GlcNAc) substrates. The enzymes were biochemically characterised and cell density-, pH- and temperature- dependence was determined. Optimal activity of both enzymes was observed at pH 7.0 and at temperatures ranging between 70° and 80°C. Pre-incubation of the cells at 70°, 80° and 85°C, for a time period ranging between 5 min. and 12 h preserved or even increased activities. In the presence of denaturing reagents, such as 0.5-4 M urea, chitobiase activity was highly preserved, while chitinase activity was reduced to half of the initial levels. The last finding suggests that chitinase is located on the outer surface of the cell, being more exposed to external reagents, whereas chitobiase is presumably located in the periplasmic space.

The presence of a chitin – inducible, cell - associated chitinoclastic enzyme system [5] has been verified by the addition of chitin in the culture medium, in absence of any otrher organic source of carbon and energy, and the consequent strong induction of the expression of the genes coding for chitinolytic enzymes. Chitin-degradating enzymes of mesophilic bacteria are induced by chitin oligomers and N-acetylglucosamine and the expression of the chitinolytic enzymes of *Thermococcus chitonophagus* seems also to be genetically regulated. This is in contrast to the constitutive formation of proteolytic and amylolytic enzymes of various hyperthermophiles and makes *Thermococcus chitonophagus* an interesting tool for further genetic studies [10,18].

A clear band of approximate molecular size of 70 kDa, corresponding to the novel chitinase was identified on SDS-PAGE and activity gels and was observed only in the clarified and 60%-enriched supernatant of the treated cell extract, thus indicating the release of the chitinase protein from the outer cell surface following treatment with detergents, such as Triton X-100 and CHAPS. This confirms the hypothesis that both enzymes are cell surface-associated, with chitinase being most likely anchored on the outer surface of the cell, whereas chitobiase, which, so far, has not been visualised on SDS-polyacrylamide or activity gels, is assumed to be in the periplasmic space.

The highest amounts of chitinase were produced after 50-60 hrs of incubation at 85°C, in the presence of 0.5% chitin, exhibiting strong expression of the chitinase gene when cells enter the stationary phase.

Preliminary immunoreaction experiments of analysed soluble supernatants and insoluble pellets from all cultures produced faint signals, suggesting low affinity of the enzyme(s) with antibodies raised against the purified chitinase A and chitobiase from *Serratia marcescens*. Large scale cultures have been prepared to produce several 100s of purified protein. Preliminary peptide mapping, and internal peptide sequencing results has shown no identity to other known proteins showing that we have isolated a novel hyperthermophilic chitinase.

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Biochemical and genetic engineering studies on chitinase A from *Serratia marcescens*

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Summary
Chitinase A (ChiA) is an endochitinase from the chitinolytic soil bacterium *Serratia marcescens*, which cleaves chitin into oligomeric derivatives of N-acetyl-D-glucosamine (NAG). The ChiA gene has been isolated, cloned and sequenced, recloned into expression vectors, introduced into suitable *E. coli* expression hosts, overexpressed and the protein has been purified. The ChiA structure has been solved at 2.3 Å, previously refined to 1.9 Å, and its biochemical and biophysical properties have been thoroughly studied. We are currently investigating ways to modify this enzyme towards a smaller, more stable and presumably a more active form. As a first step towards this direction, we have performed selectively several deletions of structurally distinct domains of the protein. These new forms have been recloned into expression vectors, introduced into *E. coli* expression hosts, overexpressed and purified for further characterisation.

Introduction
Chitin is not only the major constituent of the fungal cell wall and the arthropod exoskeleton, but also an important nutrient source for bacteria. Chitin is a homopolymer of N-acetyl-D-glucosamine (NAG) in β(1,4) linkage and its enzymatic hydrolysis to N-acetyl-D-glucosamine is performed by the chitinolytic system that involves several hydrolases. The first chitin hydrolase is a chitinase (endohydrolase), which splits the chitin polymer randomly into small oligomers of N-acetyl-D-glucosamine. The second hydrolase is a β-N-acetyl-D-glucosaminidase or chitobiase (exohydrolase) which further degrades these oligomers to N-acetyl-D-glucosamine. Hydrolysis of chitin to monosaccharides and larger oligomeric saccharides usually takes place extracellularly by the action of this specific enzymatic system.

Chitinases are widespread in nature and have been found in bacteria, fungi, plants, invertebrates (mainly nematodes, insects and crustaceans) and all classes of vertebrates. The role of chitinases in these organisms is diverse and subject to intensive investigation. In vertebrates, chitinases are usually part of the digestive tract. In insects and crustaceans, chitinases are associated with the need of partial degradation of old cuticle and their secretion is controlled by a complex hormonal mechanism. In higher plants, chitinases serve as a defense mechanism against fungal pathogens. The way in which chitinases, as well as other pathogen–related proteins contribute to pathogen resistance is of great biotechnological interest. Chitinases in fungi are thought to have autolytic, nutritional and morphogenetic roles. Finally, in bacteria, the degradation products of chitin are used solely as a nitrogen, carbon and energy

source. The number of chitinases detected from a variety of organisms is rapidly growing and in most cases the corresponding gene has been isolated, cloned and primary structure of the enzyme has been determined.

Chitinases are classified into two different families. These correspond to family 18 and 19 of glucosyl hydrolases. The members of family 19 are generally highly conserved in primary structure and consist only of plant chitinases. Family 18 is diverse in evolutionary terms and contains enzymes from plants, bacteria and fungi. There are representative structure analysis for both these families: for family 19, chitinase from the plant *Hordeum vulgare* has been solved at 2.8 Å resolution and for family 18, Chitinase A and hevamine.

In our laboratory we are trying to modify ChiA, a family 18 enzyme, from the Gram–negative soil bacterium *Serratia marcescens*. The biochemical properties of ChiA are well known: the enzyme has optimum activity at pH 6.0–6.5 and at temperature 40–45°C. The enzyme acts as an endochitinase, producing NAG oligomers consisted of 2 up to 6 NAG monomers. The main product of chitin cleavage is diacetylchitobiose and, for higher enzyme/substrate ratios, free NAG.

The gene encoding Chitinase A was isolated, cloned and sequenced. The X-ray structure of ChiA is currenntly available to 1.9 Å resolution. The enzyme is transferred extracellularly, facilitated by leader peptide, which is 23 amino acid residues long and it is cleaved upon secretion. The matured enzyme comprises 540 amino acids and has a calculated molecular weight of 58.7 kDa.

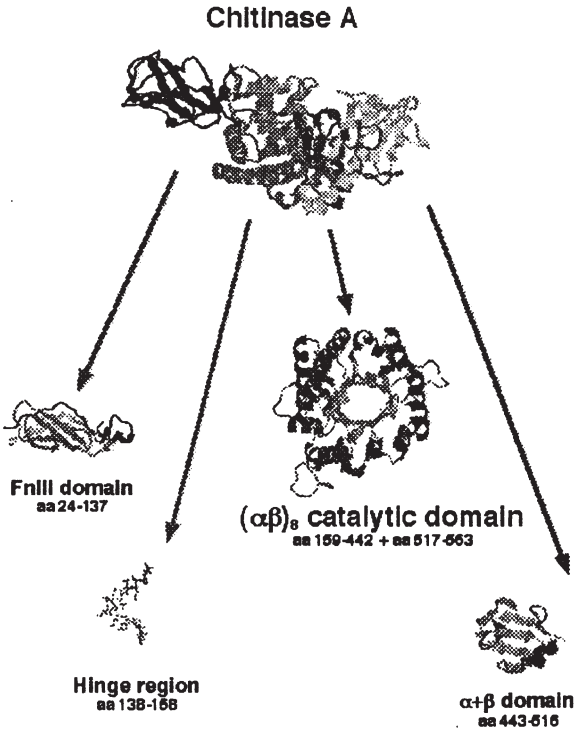

Figure 1. Protein domains of chitinase A from *Serratia marcescens*.

ChiA 3D-structure consists of three domains (Fig. 1). The amino-terminal domain (residues 24-137), which consists only of β-strands and has a fold that resembles the fibronectin III module (FnIII domain), connects through a hinge region (residues 138-158) to the main $(\alpha\beta)_8$-barrel domain (residues 159-442 and 517-563). This eight-stranded $(\alpha\beta)_8$-barrel is the catalytic domain of the enzyme. It is well known that Glu315 is the amino acid residue responsible for the catalytic activity of the enzyme. The third domain, which has an $(\alpha+\beta)$ fold, is formed by an insertion in the $(\alpha\beta)_8$-barrel motif (residues 443 – 516).

Materials and methods

The initial step was to construct deletions of ChiA structurally distinct domains. By applying the PCR technology we have constructed various forms of ChiA. We have obtained deletions of major parts of ChiA, including those of the FnIII domain, the $(\alpha+\beta)$ in various combinations. All these forms have been cloned using TA Cloning System (InVitrogen) into the pCR 2.1 vector, recloned into expression vectors, introduced into several *E. coli* host strains and overexpressed. The most adequate expression system, in terms of obtaining sufficient quantities of soluble, active protein, was found to be the pET-15b vector which contains a Tag of 6xHistidines introduced into the AD494 (DE3) host cells.

The various constructs required particular incubation and induction conditions in order to produce soluble protein in large scale cell cultures (in Luria-Bertani Broth Miller medium). The construct pET-15b-ChiADel$(\alpha+\beta)$ produced abundant soluble protein from a 2 L cell culture, when induced with 1mM IPTG at 37°C for 3 hours. Under the same conditions the two other constructs, pET-15b-ChiADelFnIII and pET-15b-ChiADel$(\alpha+\beta)\Delta$FnIII produced large amounts of protein which formed inclusion bodies. However, smaller amounts of soluble protein from these constructs was obtained by lowering the growth and induction temperature of the cell culture to 18°C and the final concentration of IPTG to 0.25mM. In all cases the induction triggered at a cell density of 0.6-0.8 OD_{600nm}.

After cell harvesting, sonication and centrifugation, the supernatant was applied onto a High Trap Ni^{2+}-Chelating column (Pharmacia) (Buffer: 20mM PO_4^{3-}, 100mM NaCl, 10mM Imidazole, pH 8.0). The elution of bound proteins was performed by applying a linear gradient of Imidazole between 10mM and 300mM. Further purification was obtained via an FPLC chromatography system, using the Resource Q column (Pharmacia) (Buffer: 20mM Tris-Cl, pH 8.0). The bound protein was eluted within a gradient of NaCl from 0M to 1M. In addition, in selected samples, the 6xHis-Tag was cleaved using thrombin and the protein was re-applied on High Trap Ni^{2+}-Chelating column under the same conditions. The purification steps were followed by 12% SDS-PAGE, immunobloting using a polyclonal antibody raised against the ChiA wt as well as enzymatic activity assays.

Results and Discussion

It is clear that the three different forms of ChiA show differential solubility in the *E. coli* cytoplasm which can be correlated to their pI values which were theoretically calculated. The wild type ChiA has a pI value of 6.7 and shows rather high solubility. In addition, ChiADel$(\alpha+\beta)$ has a pI value of 7.2, while ChiADelFnIII has a pI of 5.8 and finally ChiADel$(\alpha+\beta)\Delta$FnIII has a pI of 5.9. The FnIII domain has considerably high pI value of 9.3 and it is very soluble, fact which is also supported by its low molecular weight. Moreover, the $(\alpha+\beta)$ domain has a pI value close to 7.0. These observations point to the suggestion that in the reduced *E. coli* cytoplasmic environment the low pI overexpressed proteins are regarded

by the cells as toxic, presumably by lowering the interior pH value. *E. coli* cells response to this undesirable change by trapping these proteins into inclusion bodies.

The deletion of FnIII and (α+β) domains, both individually and simultaneously, have severe effect on the enzyme catalytic activity. Preliminary studies give evidence that the (α+β) domain affects the size of the substrate molecule being catalyzed and that the FnIII domain is possibly involved in the substrate recognition by the enzyme. ChiADelFnIII shows much lower activity with chromogenic pNP-$(NAG)_2$ as substrate than the wild type ChiA. ChiADel(α+β) shows no detectable activity with chromogenic pNP-$(NAG)_2$ as substrate but seems to have some activity with fluorogenic 4MU-$(NAG)_3$ and 4MU-$(NAG)_4$ as substrates. ChiAΔ(α+β)DelFnIII shows no detectable activity.

The objectives of our laboratory are to modify ChiA towards smaller, more stable and perhaps more active forms and even to alter its substrate specificity. Our experiments are not only of biotechnological interest, since chitinases are contributing in plant pathogen control, but also have a basic research interest. TIM-barrel enzymes, such as chitinases, cover a wide range of catalytic activities. Apparently, this topology is a good framework for active sites catalyzing very different reactions. Generating new protein forms and examining their altered biochemical and biophysical characteristics can lead us to gain knowledge that could be of importance for the redesign of protein function.

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Induction of chitinase production by *Serratia marcescens*, using a synthetic N-acetylglucosamine derivative

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Summary
N-acetylglucosamine derivatives (2-acetamido-2-deoxy-6-*O*-acyl-D-glucopyranose) were chemically synthesized in a 60% yield one-step reaction, and assayed on *S. marcescens* cultures. Both 6-*O*-octanoyl- and 6-*O*-stearoyl- derivatives behaved as carbon substrates for the bacteria while only the former caused chitinase induction. The chitinolytic activity produced by this way, was 8 ± 3 U/ml, which accounts for about 20% of the activity obtained with the chitin-based medium reference. Surface tension measurements confirmed the surfactant properties of this compound.

Introduction
The production of chitinases by microorganisms or plants need to be induced, generally by chitin, or more exactly by soluble oligomers deriving from chitin degradation [1-3]. The monomer, N-acetylglucosamine (NAG) plays a particular role since it causes induction at low concentration, with an optimum value which was estimated about 6.10^{-3} g/l [2], and since it causes repression at high concentration. When NAG is used in place of chitin as carbon substrate, the chitinase activity produced remains low, about 1.8 unit / ml [1] after conversion into our unit system.

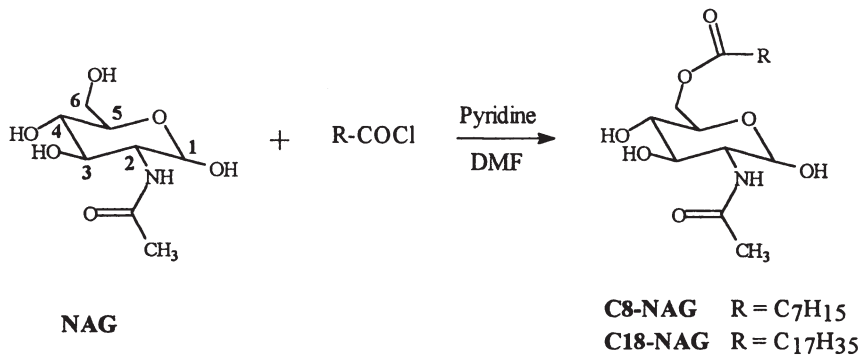
The understanding of the induction/repression mechanism is quite important for optimizing fed-batch or continuous chitinase production processes [4] or for utilizing various carbon substrate sources. In that context, the behaviour of NAG derivatives towards chitinase producing microorganisms could help to explain biological phenomena. By performing regioselective esterification of the C6 alcohol function it is possible to prepare carbohydrate amphiphiles retaining the functional environment of C1 and C2, characteristic of NAG. Such a synthesis have been described yet [5,6], but properties of the products obtained then, were not mentioned. In the present work, an improved version of this last synthesis is proposed for preparing 2-acetamido-2-deoxy-6-*O*-octanoyl-D-glucopyranose (noted C8-NAG) and 2-acetamido-2-deoxy-6-*O*-stearoyl-D-glucopyranose (C18-NAG). Some biological properties of these compounds towards *Serratia marcescens* cultures are presented. Surface activity of C8-NAG is also reported.

Materials and Methods
Analytical methods. Melting points were determined with a Kofler-block apparatus and were uncorrected. ^{1}H and ^{13}C spectra were measured on a Brucker 200 MHz spectrometer in DMSO-d_6. Chemical shifts were expressed in parts per million (ppm) downfield from TMS.

Mass spectra were recorded on a quadrupole Ribermag R 1010 spectrometer by DCI/NH_3. Thin layer chromatography were carried out using aluminium precoated plates of silica gel 60 F_{254} (Merck). Microanalyses were performed by the laboratory of Microanalysis CNRS (Gif-sur-Yvette, France).

Synthesis of 2-acetamido-2-deoxy-6-*O*-octanoyl-D-glucopyranose (noted C8-NAG). This compound has been synthesized as shown in Scheme 1. This is a one-step reaction synthesis modified from a previously reported method [5]. A mixture of NAG (2.5 g) with one equivalent of anhydrous pyridine (0.88 g) in dimethylformamide (DMF) (30 ml) was stirred and cooled. Octanoyl chloride (2 ml = 1 equivalent) was then slowly added. After one night at room temperature, 5 ml water were added for completing the hydrolysis of octanoyl chloride. Evaporation under reduced pressure afforded a gum which was solubilized in ethyl acetate and washed with Na_2CO_3 saturated water. Evaporation of ethyl acetate provided a slightly coloured powder which was further purified by recrystallization from dichloromethane / pentane mixture (white powder, 2.4 g, 60% yield). ^1H NMR δ 0.86 (t, 3H, CH_3), 1.25 (m, 8H, CH_2), 1.52 (m, 2H, CH_2), 1.83 (s, 3H, CH_3), 2.29 (t, 2H, CH_2), 3.07-4.32 (m, 8H, CH, CH_2, OH), 4.9 (m, 1H, OH), 6.55 (d, 1H, OH), 7.66 (d, 1H, NH). ^{13}C NMR δ 14.2, 22.3, 22.9, 24.7, 28.7, 31.4, 33.7, 38.0, 54.4, 64.0, 69.5, 70.6, 71.4, 91.0, 169.6, 173.2. MS m/z 348 $[M+H]^+$ and 330 $[M+H-H_2O]^+$. The consideration of the elementary analysis results suggests the presence of water : Calcd for $C_{16}H_{29}NO_7$, H_2O : C, 52.6; H, 8.49; N, 3.84. Found: C, 51.8; H, 7.66; N, 3.77.

Synthesis of 2-acetamido-2-deoxy-6-*O*-stearoyl-D-glucopyranose (noted C18-NAG). The same method as for C8-NAG synthesis was followed, replacing octanoyl chloride by stearoyl chloride (3.8 ml). 3g of a fine white powder was recovered (54% yield).



NAG

C8-NAG R = C_7H_{15}
C18-NAG R = $C_{17}H_{35}$

Scheme 1.

Synthesis of Allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (noted Allyl NAG). The method followed is a Fischer glycosidation described previously [7]. A mixture of 2-acetamido-2-deoxy-D-glucose (35 g), distilled allyl alcohol (350 ml) and boron tri-fluoride etherate (3.5 ml) was boiled for 3 h under reflux and under argon atmosphere. Evaporation (40°C, reduced pressure) led to a solid which was washed overnight in diethyl ether and filtered off. After drying, 40 g of a white powder was recovered (yield 97%). Single major spot appeared in thin layer chromatography in (9:1) dichloromethane-methanol (R_F 0.3). Melting point 144-147°C. ^1H NMR δ 1.83 (s, 3H, CH_3), 3.12-4.0 (m, 8H, CH, CH_2), 4.67 (s,

4H, 3OH, CH), 5.13 (dd, 1H, CH), 5.30 (dd, 1H, CH), 5.8 (m, 1H, CH allyl), 7.78 (d, 1H, NH). ^{13}C NMR δ 22.8, 54.0, 61.0, 67.1, 70.8, 71.1, 73.1, 96.2, 116.7, 134.8, 169.8.

Determination of static surface tension. The surface tension of C8-NAG solutions were measured at 25°C with a Wilhelmy tensiometer (Tensimat n°3 - Prolabo). The critical micellar concentration (CMC) was determined from the break point of surface tension versus concentration (on log-scale) curve.

Chitin preparations. Two kinds of chitin were prepared, according to their use in fermentation or for enzyme assays.

Purified chitin : 100 g of crude chitin obtained from Sigma was mixed with 600 ml of 2M HCl for 1 h at 4°C. After filtering and washing, the chitin was treated with a solution of 1M NaOH for 1 h at 100°C. After filtering and washing, this purified chitin was dried and used as substrate in culture medium.

Colloidal chitin : It was prepared by adding 5 g of purified chitin to 50 ml 14M H_3PO_4 at 4°C for 24 h. The product was suspended in 650 ml of deionized water and stirred vigorously. After centrifugation (20 min, 11000 g), the pelleted chitin was resuspended in 8L of deionized water and filtered through a paper filter. The chitin was then freeze dried and sieved through a standard sieve between 150 and 90 μm (100-170 mesh) to give an average particule diameter of 120 μm.

Enzyme activity assay. The activity of chitinases was determined by following the amount of reducing ends released by the cleavage of glycosidic bonds. The reaction mixture, consisting of enzyme solution (0.5 ml) and colloidal chitin (20 mg) in 1.5 ml of 100 mM phosphate buffer (pH 6.6), was shaken for 1 h at 50°C. The reaction was stopped by adding 0.1 ml 10% (m/v) trichloroacetic acid to 0.6 ml of the mixture. After centrifugation, 0.5 ml supernatant was heated for 3 min at 100°C with 1.5 ml dinitrosalicylic (DNS) reagent (0.69 g DNS, 1.18 g NaOH, 20 g sodium potassium tartrate per 100 ml water). After dilution, the absorbance was measured at 530 nm. The amount of reducing moieties released, expressed as NAG equivalents, was determined from a calibration curve. One unit (U) of chitinase activity was defined as the amount of enzyme required to produce 1 μmol NAG equivalent in 1 h at 50°C and pH 6.6.

Microorganism, media and culture conditions. Experiments were performed using the bacterial strain *Serratia marcescens* BCCM 18541. The microorganism was pregrown in LB medium (g/l : tryptone 10, yeast extract 5, NaCl 10), at 32°C for 24 h. Growth and chitinase induction assays were carried out at 32 °C, in baffled Erlenmeyer flasks containing Reese medium base (g/l : yeast extract 0.5, $(NH_4)_2SO_4$ 1, $MgSO_4,7H_2O$ 0.3, KH_2PO_4 1.36). The pH was adjusted to 8.0 with 2M NaOH before sterilization (121°C-30 min). Each compound to be tested was added to this medium before inoculation. Cell concentration was determined by live cell counts on LB/agar plates using the spread plate technique.

Results and Discussion

Synthesis of C8-NAG and C18-NAG. The utilization of DMF, which solubilizes NAG better than pyridine, led to an increased reaction yield which reached about 60% instead of the 26-29% previously reported [5]. The compounds obtained were pure (95%), according to analysis data. However, some polyesters, as the *O*-1,6-di-acyl-NAG [6], were most probably formed. Though it was not evidenced by NMR or mass spectra, this hypothesis will be strengthened by the study of interfacial properties.

Surface active properties of C8-NAG. Surface tension vs. concentration plots for C8-NAG are shown in Figure 1. C8-NAG exhibits surfactant properties, since the surface tension of water is reduced by 14 mN/m. However, it has to be noted that the critical micellar concentration which can be deduced from the present curve (CMC ~ 10^{-6} mol./l) remains far from the value (6.10^{-3} mol./l) measured with a batch of C8-NAG prepared by a route in which successive protection and deprotection steps prevented from di-ester formation (results to be published elsewhere). The presence of even traces of di-ester, greatly more hydrophobic than the monoester, could explain the decrease of the CMC of the solutions assayed. At this occasion, this underlines the potency of surface tension measurements as additional means for purity control of amphiphiles.

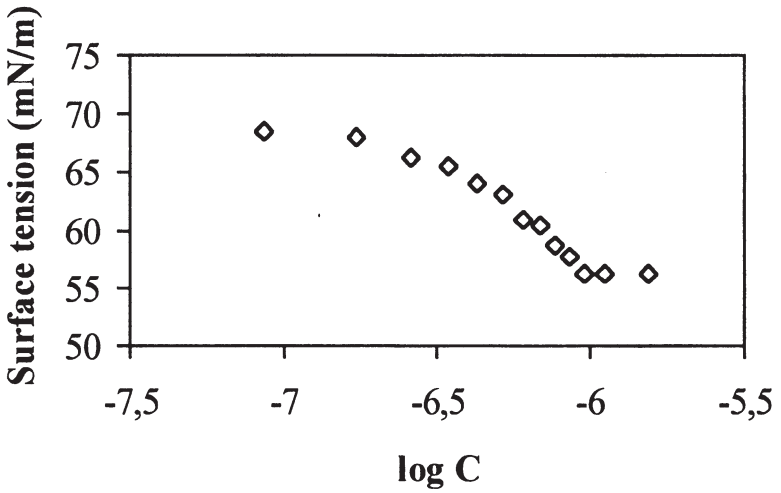


Figure 1. Surface tension of aqueous solutions of C8-NAG vs. log C (C mol./l) at 25°C.

***Serratia marcescens* cultures.** Series of *S. marcescens* cultures have been performed in presence of different carbohydrate compounds which were added to sterile medium just before inoculation (inoculum size 2% v/v). In each series, experiments have been duplicated. Table 1 shows a comparison of the effect of different compounds on growth (live cell concentration) and chitinase production after 5 days of culture. A first result is the consumption of C8-NAG by the bacteria leading to a significant increase of cell concentration, in a similar way as purified chitin : multiplication of the cell population by 50 from initial time, to be compared with the little growth allowed by the Reese medium base in which yeast extract is the only carbon source (multiplication by 4). It can be noted that C18-NAG behaves also as a substrate, though less efficient than C8-NAG and that Allyl NAG does not.

Concerning the induction of chitinases, only C8-NAG caused a significant production of chitinases, though lower than that obtained with chitin. The 8 U/ml obtained with C8-NAG after 5 days represented about 24% of the production of reference (chitin 10 g/l). Concerning NAG, the high concentration (10g/l) present in the broth exerted a strong repression on chitinase production, as expected [1,2].

Table 1. Live cell concentration and chitinolytic activity of *S. marcescens* cultures at t = 5 days ; cell concentration at t = 0 : 0.25 10^9 /ml.

	Additive Conc. (g/l)	**Additive Conc. (M)**	**Cell Conc. (10^9 /ml)**	**Chitinases (U / ml)**
No additive	0	0	1	0
Chitin	10	0.045 NAG eq.	10 - 15	33 ± 2
C8-NAG	4	0.011	10 – 15	8 ± 2
C18-NAG	4	0.008	2 - 5	0
Allyl NAG	4	0.015	1	0
NAG	10	0.045	6	0

In order to confirm the previous results and to study the influence of the amount of additive in the broth, other series of cultures containing the same molar concentration of additive (0.045 mol./l corresponding to 10g/l NAG equivalent) were performed. The ratios : (value at the end of culture) / (value at the end of chitin culture reference), are reported in Table 2, so as to compare different series. Relatively large intervals of uncertainty are noted, due to variation of results between series. However, clear trends show that an increase of available compound causes an increase of cell growth, confirming that C8-NAG and C18-NAG really behave as substrates. It could even be underlined that C8-NAG seems more efficient than chitin when considering the cell production per gram of compound initially added. The biodegradability of these surfactants could also be an advantage in various uses.

Table 2. Ratios of final cell concentration and ratios of final chitinolytic activity reported to reference (chitin), for different series of cultures.

	Additive Conc. (g/l)	Additive Conc. (M)	Cell Conc. (ratio to Ref.)	Chitinases (ratio to Ref.)
Chitin	10	0.045 NAG eq.	1	1
C8-NAG	1	0.003	0.25	0.17 ± 0.02
C8-NAG	4	0.011	0.7 – 1.5	0.22 ± 0.04
C8-NAG	16	0.046	2 - 3	0.15
C18-NAG	4	0.008	0.15 – 0.5	0
C18-NAG	22	0.045	1	0

On the other hand, the results concerning chitinase induction are quite different. C18-NAG does not induce measurable chitinase activity, even at high concentration, while C8-NAG seems to produce approximatively the same final activity : 8 ± 3 U/ml in all the tests, which accounts for about 20% ± 6% of that obtained with 10 g/l chitin reference. These phenomenon should be related to the actual concentration level of each compound near the site of induction which depends on its intrinsic solubility in aqueous media and depends also on transmembrane transports. It could be supposed that C18-NAG is too scarcely present inside

the bacteria – in comparison with C8-NAG - or that the hindrance due to its hydrophobic tail prevents it from interacting with the induction site. In the hypothesis that C8-NAG would not be the actual inducer but had to be modified by the bacterial metabolism, it could be supposed, for example, that it slow degradation could release sufficiently low NAG concentration, suitable for chitinase induction [2].

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Libraries of chito-oligosaccharides of mixed acetylation patterns and their interactions with chitinases

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Summary
Chito-oligosaccharide libraries of mixed acetylation patterns were prepared by *N*-acetylation of pure GlcN oligosaccharides with substoichiometric amounts of acetic anhydride. Libraries of the 50% *N*-acetylated DP5 and DP6 show strong inhibition of *Serratia marcescens* chitinase B. A non-covalent complex of the enzyme with a $GlcN_1$-$GlcNAc_5$ component of the library was detected by nano-ESI mass spectrometry.

Introduction

Chito-oligosaccharides are β-1,4 linked homo or hetero oligomers of 2-acetamido-2-deoxyglucose (GlcNAc) and 2-amino-2-deoxyglucose (GlcN). Numerous reports describe remarkable biological activities of fully or partially *N*-acetylated chitosans and chito-oligosaccharides [1], e.g. immune stimulation through activation of macrophages [2], chemotaxis for polymorphonuclear cells [3], and signaling [4] or elicitor functions in plant cells [5]. Evidence is accumulating that chito-oligosaccharides are possibly involved in hyaluronan biosynthesis and possess morphogenetic activity in vertebrates [6]. In several cases, chito-oligosaccharides of mixed acetylation pattern show higher biological activities than homo oligomers of either GlcN or GlcNAc [7,8]. Chito-oligosaccharides containing a GlcN residue at the non-reducing end are strong inhibitors of chitobiase (EC 3.2.1.52) from *Serratia marcescens* [9]. Furthermore, chito-oligosaccharides of mixed acetylation patterns are of interest for the determination of the substrate specificities and mechanisms of chitinases (EC 3.2.1.14) [10] and lysozymes (EC 3.2.1.17) [11].

Chito-oligosaccharides have been studied by NMR spectroscopy [12] or by enzymatic methods [13], yielding statistical information on the frequency of diads and triads in monosaccharide sequences. Mass spectrometry which offers high sensitivity and speed of analysis, was applied for the investigation of GlcNAc oligomers, using FAB [14] or MALDI TOF MS [15] techniques, and for GlcN oligomers by means of FAB^+ and FAB^- techniques [16]. Mixtures of partially acetylated GlcN oligomers were mapped by MALDI TOF MS [7,17]. Here, we report on the preparation of chito-oligosaccharide libraries of defined DP with mixed acetylation patterns and their interaction with chitinases from *Serratia marcescens*.

Materials and Methods

Chitosan (DD 50) was obtained from fishcontract bremerhaven GmbH, Germany. THAP (HPLC-grade for mass spectrometry) was from Fluka. Biogel P-2 and P-4 were from Bio-Rad.

Chromatography. – GPC: Biogel P-4, fine grade (45-90 µm), two columns in series: 850 x 25 mm and 600 x 15 mm; elution with a 0.05 M NH_4OAc solution in 0.23 M acetic acid; flow: 24 mL/h (cf. ref [18]); detection: Waters Differential Refractive Index Detector R401; fraction size: 4 mL. - HPLC: Amide-80 column from TOSO-HAAS (4.6 mm i.d. x 25 cm, particle size 5 µm). Mobile phase: acetonitrile/0.01% Et_3N in H_2O 1:1; flow: 0.7 mL/min for 5 min, then a linear gradient to give finally acetonitrile/0.01% Et_3N in H_2O 2 : 3 within 25 min.

Preparation of GlcN oligomers: Fully deacetylated chitosan was prepared according to the procedure given in ref. [18]. The DD was determined by potentiometric titration [19]. Conc. HCl (25 mL) was added to 0.50 g chitosan (DD >98). The reaction vessel was sealed and the mixture was stirred at 72°C (thermostat) for 2 h. The reaction vessel was immersed for a few min into liquid nitrogen and the brown solution was evaporated to dryness *in vacuo*, redissolved in 25 mL of water and evaporated again. This step was repeated twice. The residue was then dissolved in H_2O and the solution was adjusted to pH 4 by means of aq. NaOH. The solution was introduced into a 200 mL Amicon® ultrafiltration cell 8200 equipped with a YC 05 membrane. The cell was washed with 1 l of H_2O. The volume remaining in the cell was removed, concentrated to 15 mL, and freeze-dried. GPC of 30.1 mg of the residue afforded 1.52 mg (4.5 µmol) GlcN oligomer of DP 2, 2.45 mg (4.9 µmol) of DP 3, 2.19 mg (3.3 µmol) of DP 4, 2.39 mg (2.9 µmol) of DP 5, 1.44 mg (1.5 µmol) of DP 6, 1.46 mg (1.3 µmol) of DP 7, 1.03 mg (0.8 µmol) of DP 8, 0.99 mg (0.7 µmol) of DP 9, 0.72 mg (0.4 µmol) of DP 10, and 15.68 mg mixture of higher oligomers (total recovery: 29.87 mg).

Partial N-*acetylation of GlcN oligomers*: A solution of 0.5 µmol $GlcN_4$ in 10 µL of H_2O/MeOH 1:1 was agitated for 6 h at room temp. with 5, 10 or 15 µL of a 0.1 M solution of acetic anhydride in MeOH. Addition of 5, 10 or 15 µL, respectively, of a 0.1 M aq. NH_3 solution was followed by freeze drying. Higher oligomers were *N*-acetylated in H_2O as the solvent. The *N*-acetylated homologues were separated by HPLC on an Amide-80 column.

Mass spectrometry. MALDI-TOF MS: Sample Preparation: Solutions of samples in H_2O (1 µL) were placed onto the target and mixed with 1 µL of a 5 % solution of THAP in MeOH. After drying at room temp., the sample was redissolved in 1 µL of MeOH to yield a thin layer of very fine crystals when dried at room temp.. Mass spectra were recorded with a Bruker Reflex II Instrument (Bruker Instruments, Bremen, Germany). Ionization was achieved using a conventional nitrogen laser (337 nm, 3 ns pulse width, 3 Hz), attenuation 35 – 45 (THAP) or 45 – 55 (DHB). All spectra were measured in the reflector mode, ions being accelerated at 20 kV and reflected with 21.5 kV. In this mode the instrument was externally calibrated on the same target using a ladder of chito-oligosaccharides, obtaining an error in mass of less than 0.1 mass units. If not noted otherwise, monoisotopic peaks are labeled in all mass spectra.

Nano ESI MS: The nano electrospray Q-TOF instrument fitted with a Z-spray source (Micromass, Manchester, U.K.) was used in MS mode. The ions were detected after passing through the quadrupole as a broad band filter and orthogonal extraction into the TOF analyzer in a microchannel plate detector and converted in a time-to-digital converter (TDC). The nano spray capillaries were produced in-house as described previously [20]. The protein sample was dissolved in 5 mM NH_4OAc to a concentration of 1 pmol • μL^{-1} and the oligosaccharide library was dissolved in the same buffer to a concentration of 50 pmol • μL^{-1}.

Results and Discussion

MALDI TOF MS Analysis of oligosaccharides prepared from deacetylated chitin (50 % DD): In order to optimize the analytical conditions, various matrices, such as DHB, ATT, and THAP were tested. Formation of matrix clusters in the low mass range could be kept at a minimum using THAP.

Analysis of oligosaccharides prepared from fully deacetylated chitin: Chitosan (DD >98) was hydrolyzed by means of 12 N HCl, followed by ultrafiltration over a microporous membrane (exclusion limit 500 Da). In this step, most of the sodium chloride, as well as GlcNAc and $GlcNAc_2$ are removed from the mixture. A MALDI TOF mass spectrum of the filtration residue revealed that this was composed mainly of oligomers of DP 3 - 12. However, chromatography on a Biogel P-2 column and MALDI-TOF MS analysis showed that the crude product mixture contains besides low molecular weight oligomers of DP 3-15 (Fig. 2) also components of DP 10-50 (Fig. 3). This is the first example for the analysis of oligomers of GlcN of DP > 17 by MALDI TOF MS. These data are of general importance, as they demonstrate the feasibility of an absolute method for the determination of the molecular masses of higher chito-oligosaccharides.

Preparation and analysis of chito-oligosaccharide libraries of mixed acetylation patterns: Isolation of pure GlcN oligomers (DP 2 – 10) was achieved by GPC on a Biogel P-4 column [18]. *N*-acetylation of GlcN oligomers of defined DP, using *sub-stoichiometric* amounts of acetic anhydride (cf. [7]), affords mixtures of partially acetylated chito-oligosaccharides of different DD (i.e. homologs) and isobars (i.e. sequence isomers). The number of compounds resulting from partial acetylation of $GlcN_n$ is theoretically 2^n. For illustration, the mass spectrum of the reaction mixture obtained from $GlcN_6$ is depicted in Fig. 3.

DP-defined oligosaccharide mixtures obtained by partial acetylation of $GlcN_5$ and $GlcN_6$ show strong inhibition of the hydrolysis of 4-methylumbelliferyl *N,N′,N′′*-triacetylchitotrioside [21], specific to chitinase B, not to chitinase A, from *Serratia marcescens* (Table 1).

Table 1. Inhibition of chitinases from *Serratia marcescens* with partially *N*-acetylated chitosan oligomers. Substrate: 12 µM 4-methylumbelliferyl-*N,N′*-diacetylchito-bioside; enzyme concentration in assay: Chitinase A: 0.5 nM; Chitinase B: 1.0 nM .

	Chitinase A [IC_{50} (µM)]	Chitinase B [IC_{50} (µM)]
DP4; 25% acetylation	2000	500
DP4; 50% acetylation	3000	500
DP5; 50% acetylation	500	15
DP6; 50% acetylation	1000	15
DP7; 50% acetylation	800	40

The enzyme binds preferentially a penta-*N*-acetylated hexasaccharide, as revealed by nano-ESI MS. The calculated molecular mass $[M+H]^+$ of chitinase B is 55,333 Da (Fig. 4a) which was confirmed by mass spectrometry (Fig. 4b). Nano-ESI MS of an incubation mixture of chitinase B with the 50% *N*-acetylated DP6 library (Fig. 5) shows a major peak at 56,526 ± 2 which corresponds within the limits of error to the sum of the mass of the native enzyme plus that of $GlcN_1$-$GlcNAc_5$ (calcd. 1194.5). The low molecular weight range of the spectrum reveals a decrease of the relative intensity of the peak coresponding to $GlcN_1$-$GlcNAc_5$ as compared to the peak intensities of the homologs (Fig. 6).

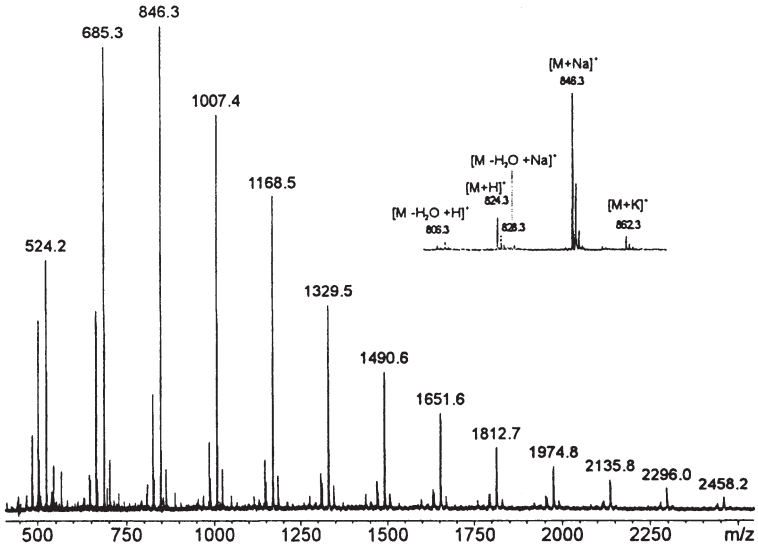

Figure 1. MALDI TOF MS of the low molecular weight fraction of GlcN oligomers obtained after separation of the ultrafiltration residue on Biogel P-2 (matrix: DHB). The labeled peaks refer to the $[M+Na]^+$ ions of $GlcN_n$ (n = 3 – 15). The smaller peaks indicate the presence of protonated and potassiated species as shown in the insert. The peaks at *m/z* = 806 and 828 in the insert indicate partial loss of water.

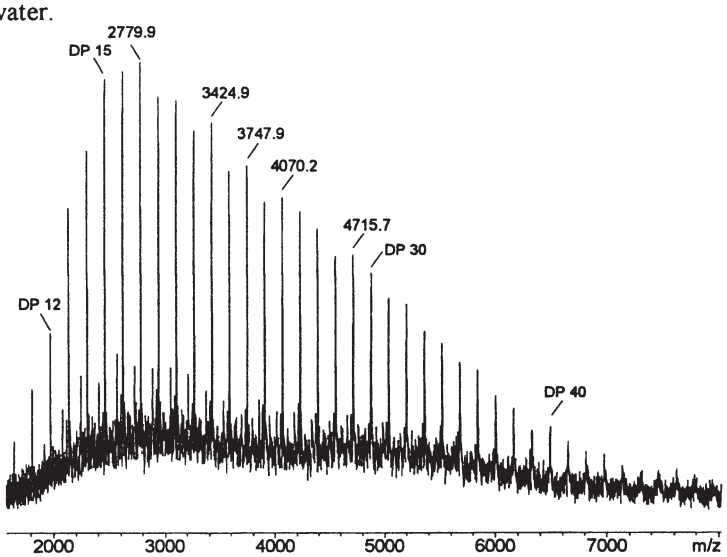

Figure 2. MALDI-TOF MS of the high molecular weight fraction of GlcN oligomers obtained after separation of the ultrafiltration residue on Biogel P-2 (matrix: THAP). The labeled peaks refer to $[M+Na]^+$ ions of the $GlcN_n$ series.

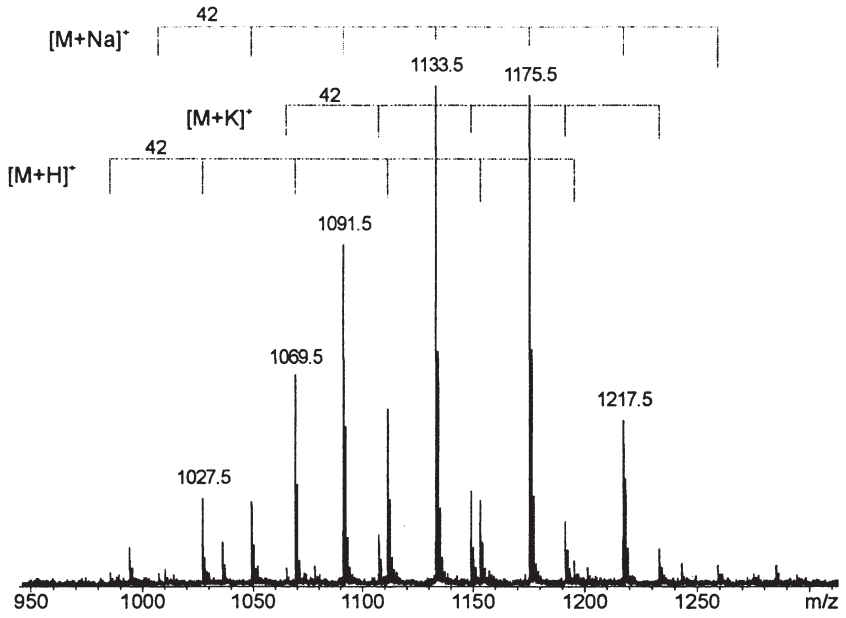

Figure 3. MALDI-TOF MS of the product mixture obtained after *N*-acetylation of $GlcN_6$ with 3 equiv. Ac_2O (i.e. 50% of the number of amino groups). Peak assignment: *m/z* [M+Na]: D_6: 1007.4; D_5A: 1049.5; D_4A_2: 1091.5; D_3A_3: 1133.5; D_2A_4: 1175.5; DA_5: 1217.5; A_6: 1259.5

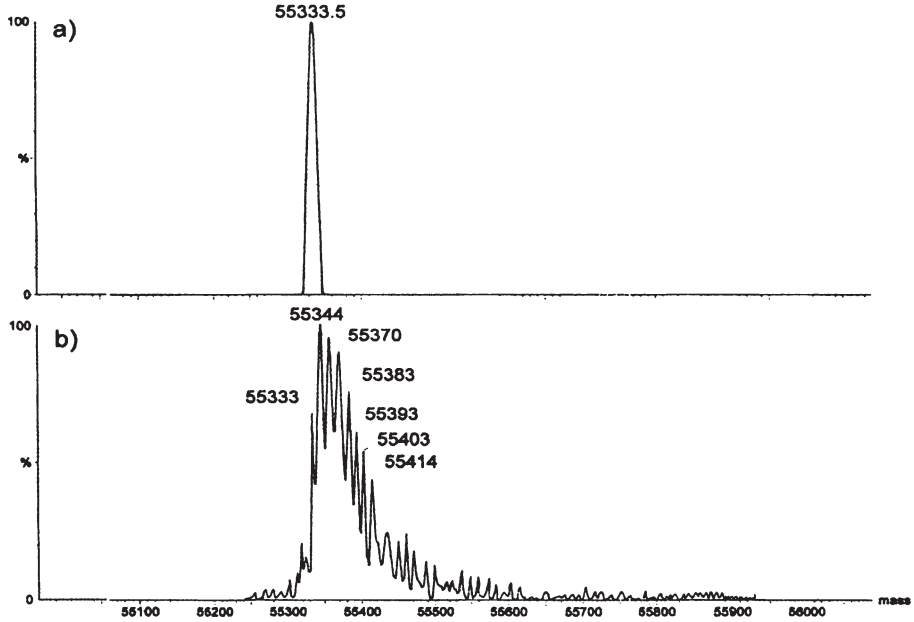

Figure 4. a) Calculated monoisotopic molecular mass of chitinase B $[M + H]^+$; b) deconvolution of the nano-ESI MS of chitinase B.

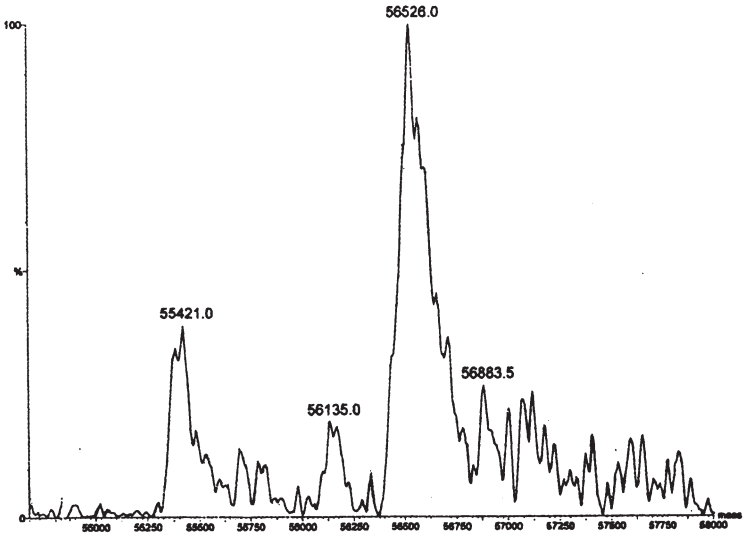

Figure 5. Deconvolution of the nano-ESI MS of a mixture of chitinase B and a DP 6 GlcN library (50% acetylated). The noncovalent complex of $GlcN_1$-$GlcNAc_5$ with chitinase B appears at 56,526 $[M + H]^+$.

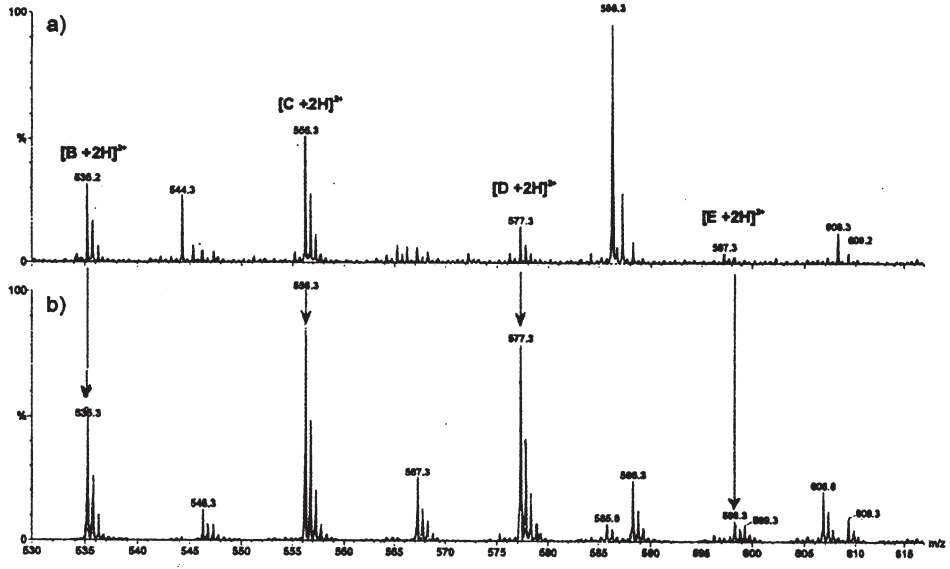

Figure 6. a) Low molecular weight region of the nano-ESI MS of a mixture of chitinase B (1 pmol • μL^{-1} in 5 mM NH_4Ac) and a DP 6 GlcN library (50% acetylated) (50 pmol • μL^{-1} in 5 mM NH_4Ac); b) mass spectrum of the original DP6 library. Peak assignments *m/z* $[M + 2H]^{2+}$: B ≙ $GlcN_4$-$GlcNAc_2$, C ≙ $GlcN_3$-$GlcNAc_3$, D ≙ $GlcN_2$-$GlcNAc_4$, E ≙ $GlcN_1$-$GlcNAc_5$. The relative intensities of D and E are lower in spectrum a) than in spectrum b), indicating preferential binding of $GlcN_2$-$GlcNAc_4$ and $GlcN_1$-$GlcNAc_5$.

Conclusions

Partially acetylated chitosan of DP5 and DP6 are strong inhibitors of chitinase B of *Serratia marcescens*. Nano-ESI MS is a powerful tool to detect non-covalent complexes of potent enzyme inhibitors in chemical libraries, as is shown in this work by analysis of chitinase B in the presence of a DP6 library of partially *N*-acetylated chitosan oligosaccharides. Work on the sequence analysis of the binding component is in progress.

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Approaches towards the design of new chitinase inhibitors

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Summary
New heterocyclic chitooligosylamides **3** were obtained via coupling of chitooligosylamines **1** with activated heterocyclic and amino carboxylic acids and subsequent deprotection. Pseudotrisaccharides **4** were prepared from protected GlcNAc-NH_2 and 4-*O*-succinoyl-GlcNAc. Based on their substitution patterns, compounds **3** and **4** were expected to show inhibiton of chitinases. Assays with *Serratia marcescens* chitinases A and B revealed, that some glycosyl amides of hetereocyclic amino acids **3** indeed were moderate inhibitors of chitinases. Chitotriosyl amides are, in general, stronger inhibitors than *N*-acetylglucosaminyl-, chitobiosyl- and spacer coupled derivatives.

Introduction

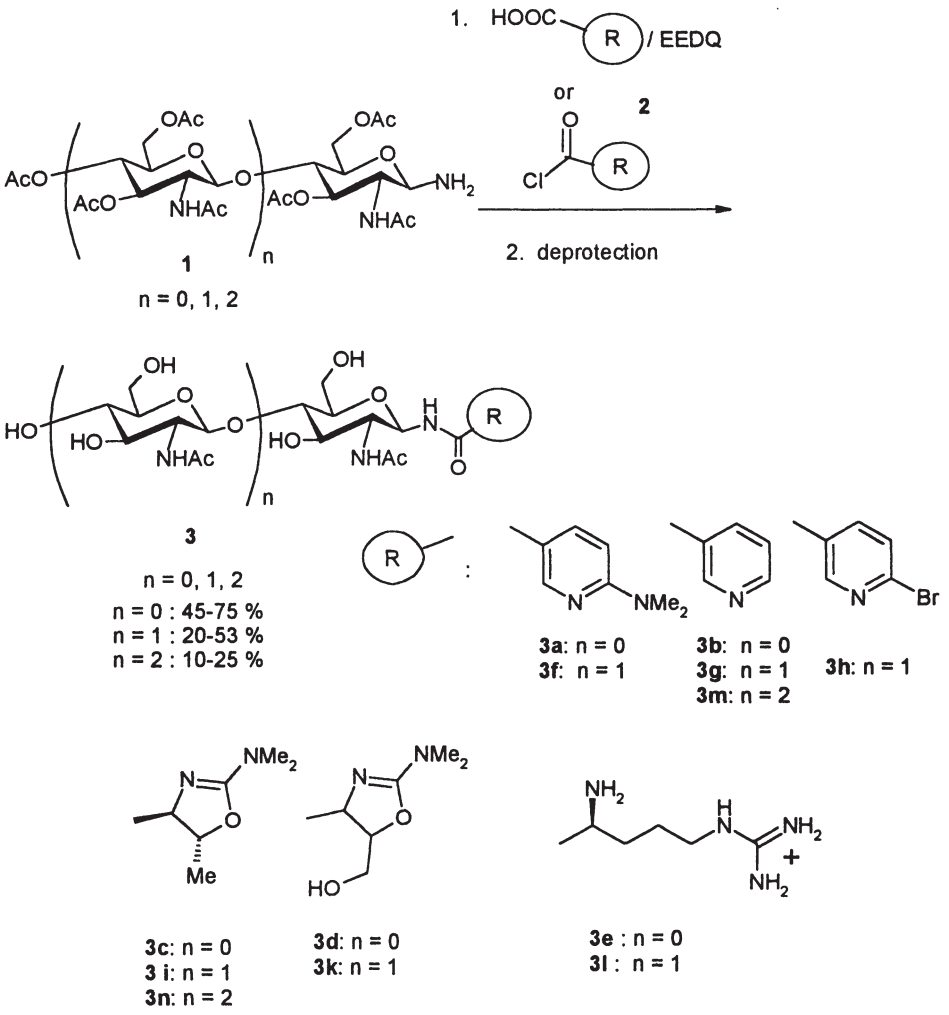
The enzymatic degradation of chitin is effected by endo- and exochitinases. The design of chitinase inhibitors is of interest for approaches towards an understanding of the structures and mechanisms of chitinolytic enzymes as well as for potential applications in medicine and agriculture [1,2].

Allosamidin, consisting of *N,N'*-diacetyl-allosaminbiose and allosamizoline, inhibits family 18 chitinases [3]. Obviously, the allosamizoline unit of allosamidin resembles the transition state towards formation of the oxazoline intermediate which appears in the course of cleavage of the glycosidic bond between the +1 and the -1 GlcNAc residues [4,5,6]. The crystal structure of a complex of allosamidin and the family 18 plant chitinase hevamine shows that binding occurs with hydrophobic interactions and hydrogen bonds between Tyr183, Trp255, Asp125 and the dimethylamino-oxazoline group of allosamizoline, between Ala224, Gly81 and the allosamizoline hydroxy groups located at the -1 binding site, between Ile82 and the AllNAc residue occupying the -2 binding site, and between Asn45, Gln9, Asn34 and the AllNAc residue occupying the -3 binding site [4]. Except the contacts with Gly81 and Asn45, analogous interactions have also been observed in the crystal structure of hevamine complexed with *N,N',N''*-triacetyl-chitotriosyl-L-histinine amide [7] which is a moderate inhibitor of an insect chitinase [2, 8].

The following items are considered to be important for the desig of new substrate-analouge chitinase 18 - inhibitors.

- The glycosidic bond at the reducing end (C-1) should be resistant to enzyme catalyzed hydrolysis.

- The presence of a strong proton acceptor, such as methylamino or guanidino groups, should interfere with proton transfer in catalysis.
- Aromatic or heteroaromatic residues should enhance binding due to stacking with aromatic amino acid residues which are conserved in the active site of all family 18 chitinases on which structure information is available so far.
- A hydroxymethyl group should be present as a hydrogen bond donor at the -1 binding site.

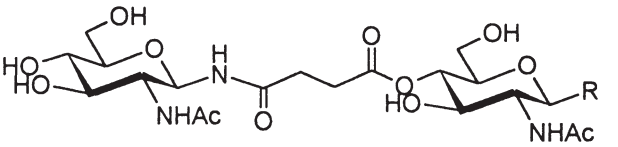


Scheme 1. Synthesis of chitooligosylamides of carboxylic acids **3**.

Results and Discussion

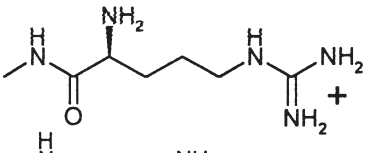
We have synthesized a number of new GlcNAc mono- and oligosaccharide derivatives **3** containing a heterocyclic or basic unit, respectively (Scheme 1). Chitooligosaccharides were linked *N*-glycosidically to various heterocyclic carboxylic acids or L-arginine. The carboxylic acids were converted into the acid chlorides or activated with EEDQ and coupled with acetyl protected chitooligosylamines. Deprotection gave the desired chitooligosylamides **3** [9,10].

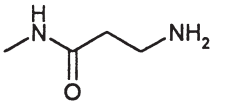
The crystal structure data of hevamine suggest that the binding interactions at the -2 site probably do not contribute much to binding affinity. Therefore, it was of interest to investigate pseudotrisaccharides, such as succinic acid containing sugar hybrids of type **4** [10] (Scheme 2). These compounds should not be cleavable by endo- or exo-chitinases.



4

a: R = ~OH

b: R = 

c: R = 

Scheme 2. Structures of pseudetrisaccharides **4** [11].

Molecular modelling studies of chitobiosyl derivatives show, that the sugar units of several of the compounds could bind to hevamine in a conformation similar to allosamidin while the heterocyclic or the L-arginine units would fit into the active site in nearly the same position as the oxazoline group of allosamidin [12].

Compounds **3** and **4** were tested for inhibition of bacterial chitinases A and B from *Serratia marcescens,* using 12 μM 4-methylumbelliferyl *N,N'*-diacetylchitobioside as the substrate (Table 1) [13]. Moderate or no inhibition was found for most of the GlcNAc mono- and disaccharide derivatives. The chitotriosylamide **3m** is remarkably effective with chitinase A and B (IC_{50} 200 μM), while **3n** inhibits chitinase B (IC_{50} 100 μM) much stronger than chitinase A (IC_{50} 500 μM). In all other cases, the IC_{50} values are higher with chitinase B than with chitinase A. Unexpectedly, the L-arginine derivatives show no or only very poor inhibition.

The spacer-coupled 1-hydroxy-trisaccharide **4a** as well as the *N*-glycosidically coupled amino acid amides of L-arginine **4b** and β-alanine **4c** show practically no inhibition of chitinases A and B. Heterocyclic derivatives of **4** could not be investigated because of difficulies encountered during protecting group cleavage.

Table 1. Inhibition of chitinases A and B from *Serratia marcescences* by compounds **3** and **4**; substrate concenration: 12 μM 4-methylumbelliferyl-*N,N'*-diacetyl-chitobioside, ChiA: 0.5 nM, ChiB: 1.0 nM. (no I. means no inhibition at c ≥ 4 mM).

Compound	Chitinase A (IC_{50}/mM)	Chitinase B (IC_{50}/mM)
3a	1.0	2.0
3b	3.0	3.5
3c	3.0	4.5
3d	no I.	no I.
3e	no I.	no I.
3f	1.0	1.5
3g	0.5	2.5
3h	0.5	> 1
3i	1.0	2.5
3k	no I.	no I.
3l	≈ 1.0	no I.
3m	0.2	0.2
3n	0.5	0.1
4a	no I.	no I.
4b	14% at 1.0 mM	no I.
4c	no I.	15% at 1.0 mM

Investigations on the inhibition of several chitinases (*inter alia* hevamine) by selected compounds are in progress. Prelimiary data indicate species differences in the susceptibility of family 18 chitinase to some inhibitors of type **3** [10].

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Allosamidin inhibits the fragmentation and autolysis of *Penicillium chrysogenum*

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Summary
The pseudotrisaccharide allosamidin, which is a specific and potent inhibitor of chitinases, retarded significantly the fragmentation and autolysis of hyphae in ageing carbon-depleted cultures of an industrial β-lactam-producing strain of *Penicillium chrysogenum*. Moreover, allosamidin also hindered, even after the addition of an extra dose of glucose, the outgrowth of new tips from the surviving hyphal fragments. In spite of this, no difference between the glucose utilisation rates of allosamidin-treated and control cultures was found. Under antibiotic producing conditions, allosamidin did not have any effect on either the growth or the penicillin yields.

Introduction
All chitin-containing fungi produce chitinases which are thought to have important nutritional and morphogenetic functions [1,2]. As far as the possible morphogenetic roles are concerned, fungal chitinases may be involved in swelling and germination of spores, branching and apical extension of growing hyphae, tailoring chitin microfibrils in rigidifying wall, vegetative anastomoses, sexual fusion between gametic cells, dissolution of septa during dikaryon formation in Basidiomycetes, fusion of hyphae during clamp connections, cell separation of yeasts, release of spores including the gross autolysis of ink-cup mushrooms as well as in age-related fragmentation and autolysis of hyphae [1-3]. Although the involvement of chitinases in fungal morphogenesis seems to be self-evident the experimental data supporting these presumptions are often scarce or controversial [2]. Nevertheless, *in vivo* enzyme inhibition experiments [1,4,5] and the disruption of genes coding for fungal chitinases [2,6,7] have given us some valuable pieces of information about the morphogenetic functions of these hydrolases. For example, the participation of chitinolytic enzymes in the idiophasic breakage of *Acremonium chrysogenum* hyphae has been demonstrated more recently by Sándor *et al.* [5]. In their experiments, neither the growth nor the cephalosporin-C production of the fungus was affected but the fragmentation of hyphae was significantly hindered by allosamidin, one of the most effective chitinase inhibitors [5]. In present paper we report on how the fragmentation and autolysis of the other main β-lactam producer fungus, *Penicillium chrysogenum*, was influenced by allosamidin.

Materials and Methods
The effect of allosamidin on the growth, morphology and penicillin V production of *P. chrysogenum* was tested under penicillin producing conditions using standard cultivation methods [8]. *P. chrysogenum* NCAIM 00237 was grown in shake flasks (500 ml) containing

100 ml of a standard culture medium. The mycelia were separated by filtration on sintered glass, were washed and transferred immediately into a medium (100 ml) containing 101 mM glucose, 1% sodium glutamate, 0.4% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4.6H_2O$, 0.5% (32.9 mM) phenoxyacetic acid and also supplemented with 9.6 μM allosamidin as required. The starting mycelial dry weight was 5.0 mg/ml in each experiment, and all the cultures were incubated with shaking at 25 °C and at 250 rpm.

The age-related fragmentation and autolysis of *P. chrysogenum* hyphae was studied as before [9-11]. In this case the microorganism was grown in a complex medium consisting of 51 mM glucose, 0.4% casein peptone, 0.4% yeast extract, 0.24% corn steep liquor, 0.2% KH_2PO_4, 0.8% $Na_2HPO_4.12H_2O$, 0.05% $MgSO_4.6H_2O$. The culture medium was not supplied with any penicillin side-chain precursor. Culture flasks (500 ml) containing 100 ml medium were inoculated with 10^8 spores and were incubated at 25 °C at 250 rpm. To investigate the effect of allosamidin and glucose on carbon-depleted cultures, 9.6 μM allosamidin and 70 mM glucose were added to selected flasks at 35 and 115 h fermentation times, respectively.

The cell morphology was examined under an OLYMPUS BH-2 microscope equipped with a SPlan 20NH phase contrast objective and a PM-CBAD photomicrography apparatus. The penicillin V [12] and glucose [13] concentrations as well as the mycelial dry weights [9] were determined by the methods indicated in parentheses.

Results and Discussion

Similar to *A. chrysogenum* [5], allosamidin did not have any effect on the growth and antibiotic production of *P. chrysogenum* either (Fig. 1). In general, this chitinase inhibitor does not seem to influence the metabolic activity of growing fungi, independently of the actual growth forms [4,5,7,14,15]. In addition, the allosamidin-treated *P. chrysogenum* cultures showed typical pelleted morphology as compared to the controls (data not shown).

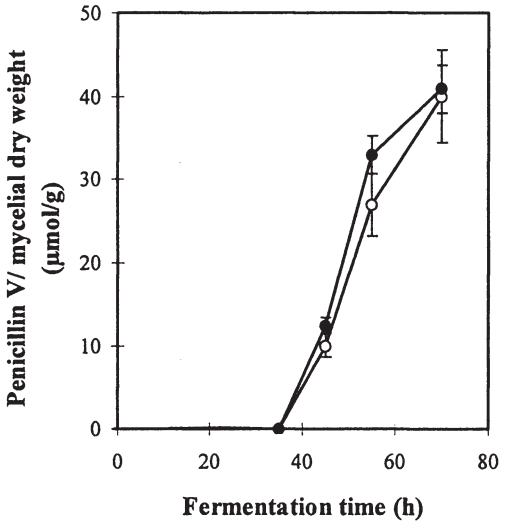

Figure 1. Specific penicillin V production of *P. chrysogenum* cultures in the presence (•) and in the absence (○) of 9.6 μM allosamidin. Results are expressed as mean±S.D., which were calculated from two independent experiments.

On the other hand, allosamidin added to *P. chrysogenum* cultures at the deceleration phase of growth affected significantly both the fragmentation and autolysis of ageing mycelia (Figs. 2-4). The morphological differences were most spectacular between 66 and 82 h fermentation times. In this period some structural elements of the pellets were still observable in allosamidin-treated cultures (Fig. 3) when control cultures had gone through an extensive fragmentation (Fig. 4). Under carbon depletion, round-ended hyphal fragments consisting of two cells were found to be the dominant surviving forms of *P. chrysogenum* (Fig. 5) [10]. Later, the breakage of allosamidin-treated mycelia also resulted in very similar round-ended fragments (Fig. 6) but the autolysis in the presence of chitinase inhibitor always lagged behind that observed in control cultures (Fig. 2).

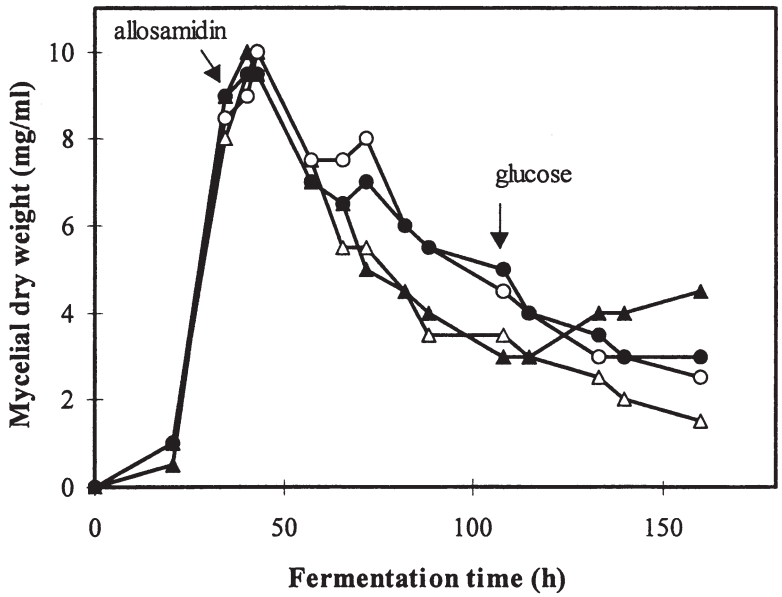

Figure 2. Changes in the mycelial dry weights in control cultures (Δ) and after addition of 9.6 μM allosamidin (○; 35 h fermentation time), 70 mM glucose (▲; 115 h fermentation time) or 9.6 μM allosamidin (35 h fermentation time) + 70 mM glucose (115 h fermentation time) (●) into carbon-depleted *P. chrysogenum* cultures. A typical set of curves is presented here.

After addition of an extra dose of glucose to the carbon-depleted cultures, the dormant hyphal fragments germinated mostly at both ends and reverted to vegetative growth (Fig. 5). As a consequence, the autolytic loss of biomass was reversed (Fig. 2). Surprisingly, the addition of the same quantity of glucose did not reinitiate any increase in the mycelial dry weight in the presence of allosamidin (Fig. 2). As shown in Fig. 6., new narrow tips emerged only very rarely in this case, and usually only at one end of the surviving fragments. Moreover, the apical extension of young hyphae was negligible even after a prolonged incubation period (Fig 6). The outgrowth of hyphae from the dormant fragments presumes the local softening of the wall by chitinases. Most likely, the inhibition of this process by

allosamidin resulted in a considerably reduced outgrowth frequency and tip extension rate although the glucose utilisation rates of the allosamidin-treated and control cultures were very similar (Fig. 7). It is well-known that the germination of some fungal spores is also allosamidin-sensitive [1,2,7].

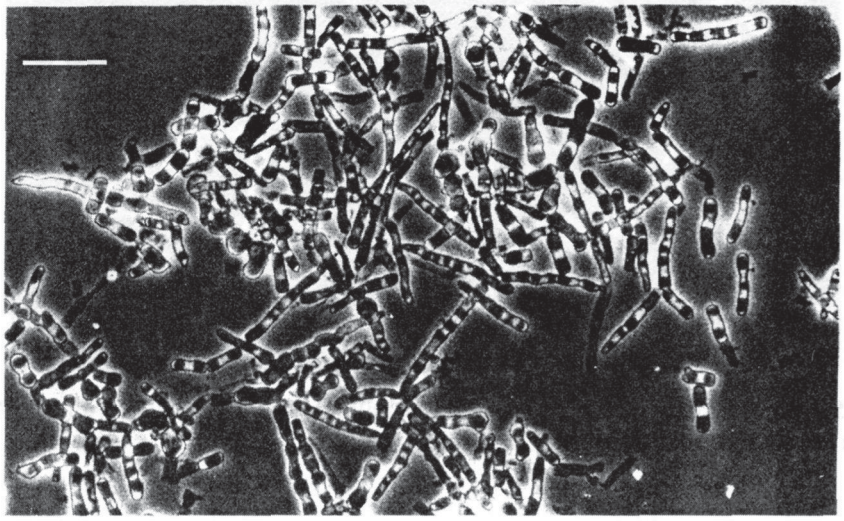

Figure 3. Ageing (72 h) *P. chrysogenum* culture supplied with 9.6 μM allosamidin in the deceleration phase of growth (35 h). Bar = 40 μm.

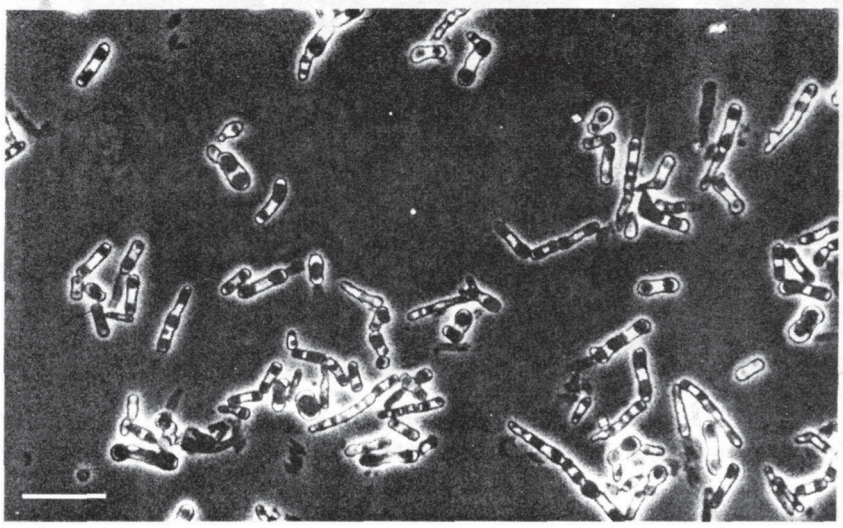

Figure 4. Ageing (72 h) *P. chrysogenum* culture – control. Bar = 40 μm.

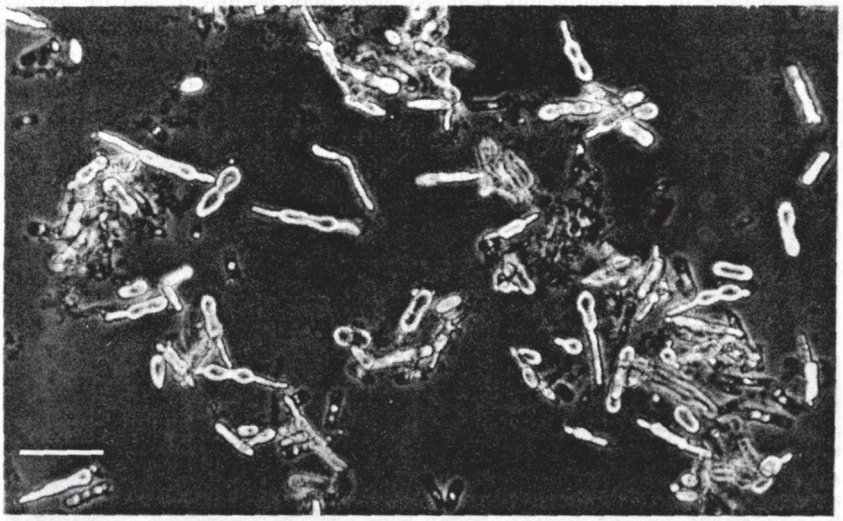

Figure 5. Outgrowing hyphae in *P. chrysogenum* cultures (160 h) supplied with 70 mM extra glucose at 115 h fermentation time. Bar = 40 μm.

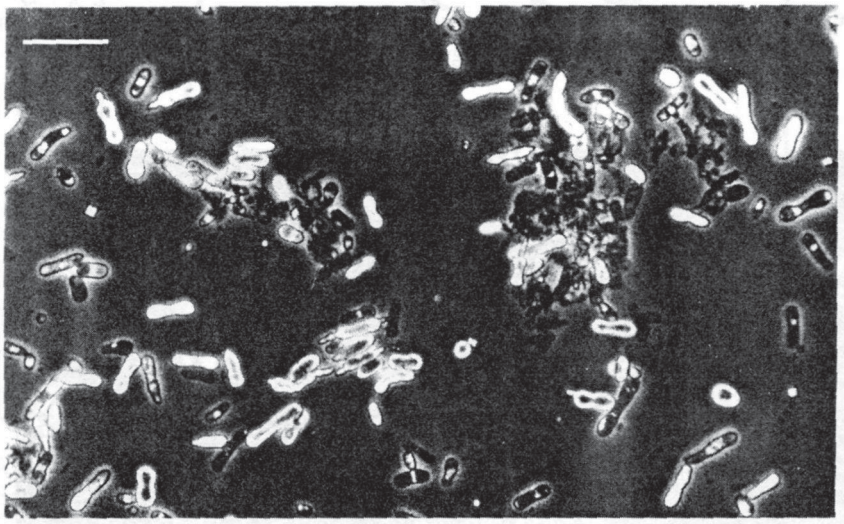

Figure 6. A *P. chrysogenum* culture (160 h) supplemented with 9.6 μM allosamidin (35 h fermentation time) and 70 mM glucose (115 h fermentation time). Bar = 40 μm.

To sum it up, the morphological effects of allosamidin on ageing *P. chrysogenum* gave a clear-cut evidence of the involvement of age-related chitinases in the fragmentation and autolysis of the fungus. A similar causal connection between chitinase production and

autolysis has been considered for other fungi as well but the evidence has remained mainly circumstantial thus far [16-21]. Moreover, allosamidin-sensitive chitinase(s) have also been shown to play a crucial role in the outgrowth of new hyphal tips from dormant hyphal fragments, which may therefore contribute to the maintenance of the cryptic growth observed in *P. chrysogenum* cultures under carbon starvation [10].

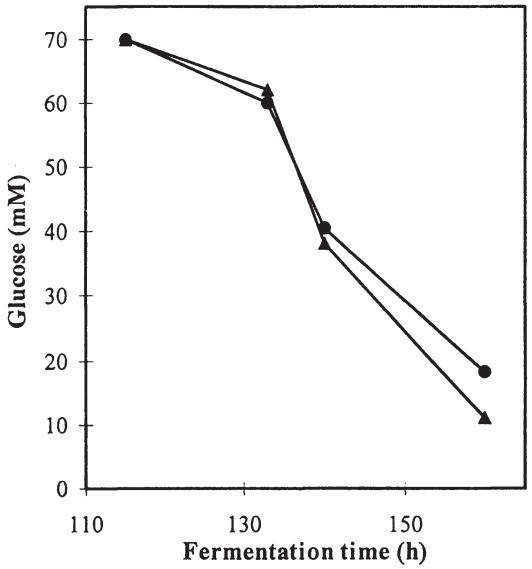

Figure 7. Glucose utilisation rates of allosamidin-treated (●) and control (▲) *P. chrysogenum* cultures supplemented with 70 mM glucose at 115 h fermentation time. A typical set of curves is presented here.

Acknowledgements

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cDNA encoding chitinase in the midge, *Chironomus tentans*

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Summary
From a cDNA library of *Chironomus tentans* prepupae a clone was isolated with identical nucleotide sequence to the cDNA already described from the cell line of this species, except 10 amino acids within the putative signal peptide and a prolonged 3'-nontranslated region. This domain contains thymidine rich sequences which are known to be important for RNA stability and adenylation. The cDNA encodes for a protein with the typical characteristics of a family 18 chitinase. According to Southern blots and *in situ* hybridisation on giant chromosomes only one gene seems to be present in *Chironomus tentans*.

Introduction
Insect chitinases are not only mandatory for the moulting process, but they are also considered as suitable target for the development of pesticidal strategies (1-4). Nevertheless, only a few insect chitinase specific cDNAs have been sequenced (5-10) and the structure of chitinase genes is only known from two insect species (9, 11). Recently, we described the sequence of a chitinase specific cDNA from the epithelial cell line of *Chironomus tentans* which was expressed in a functional form in the baculovirus/*Spodoptera* system (12). Here, we describe a second cDNA derived from prepupae of the same species and the localisation of the chitinase gene on giant chromosomes.

Materials and Methods
PCR based library screening: Two oligonucleotides which are specific for the chitinase of the *Chironomus tentans* cell line, Chir 1: 5'- GGGTGGAATGAGGGCTCA-3'(sense) and Chir 2: 5'- CCCATCGAAATTGTGCTGCTTAAT-3'(antisense) were designed to amplify a Digoxigenin-labelled probe (111bp) by PCR using cDNA of the cell line as template and dig-dUTP (Boehringer Mannheim, Germany) for labelling. This probe was used for screening of a λ-Zap-cDNA-library from *Chironomus tentans* prepupae (kind gift of Prof. M. Lezzi, ETH Zürich, Switzerland) according to the instructions of the manufacturer.

Sequencing: Plasmids or PCR products were sequenced in both directions using the didesoxy-method according (13). Cycle sequencing with fluorescence labelled oligonucleotides (MWG Biotech, Ebersberg, Germany) and the Thermo Sequenase fluorescent-labelled primer sequencing kit with 7-deaza-dGTP (Amersham-Buchler;

Braunschweig, Germany) was performed according to the manufacturer. Samples for sequencing were separated on acrylamide gels (Long Ranger, FMC Bioproducts; Rockland, USA) containing 6 M urea (Licor 4000 DNA sequencer, MWG Biotech, Ebersberg, Germany).

In situ hybridisation: Chitinase encoding cDNA from *Chironomus tentans* prepupae was used as probe for *in situ* hybridisation experiments on giant chromosomes from salivary glands of *Chironomus tentans*. Labelling of the probe with digoxigenin and hybridisations were performed as described (14). Identification of the position of the signal was kindfully done by Prof. Dr. Keyl (Institut für Genetik, Ruhr-Universität Bochum).

Results and Discussion

Screening of a cDNA library obtained from prepupae of *Chironomus tentans* revealed a clone (clone 2), whose coding region is identical to the cDNA isolated from the cell line (clone 1) with the exception of 10 amino acid residues at the 5′-end within a putative signal peptide. The leader sequence of clone 2 seems to be incomplete; the first methionin is not preceded by a stop codon and the triplet is not surrounded by a favourable environment (15). Attempts to complete this region by 5′-RACE failed. The 3′ untranslated region of clone 2 is extremely rich in thymidine. More than 50 % of all nucleotides are T. However, no poly-A tail and no polyadenylation signal is present. In the remaining overlapping regions of both cDNAs only two nucleotides are exchanged, but without consequences for the deduced amino acid sequence (Fig. 1).

```
                    5′ AAT TCC GTG CTT TTG ATA TAC ATT TTT AAT TAT   33
                          V   L   L   I   Y   I   F   N   Y

ATG ACT ATT TTT TAC CAA GCA CAG GCA CAA ACA GGA CCG CAA CAT AAT   81
M   T   I   F   Y   Q   A   Q   A   Q   T   G   P   Q   H   N    16

.....................//.....................TTA CTG TAA AAA AAA 1425
.....................//.....................L   L   *           461

AGA TTG AGA ACA CTT CGA GGT AGC ACT TCC TTT TTA AAT ATA ATT TTT 1473
CAT TTA TTT CTC TTT TTT TCT TAC AAT TTT TTT TTT TTA TTT TTT CCA 1521
CTT TTT TTC TTA TAA TTT TAT TTT TTT GTT TCC TCT TTT TTA TAA TAA 1569
TAA TTT TCT TTA TGC TTT TTT CAT TCT CTA AAA AGA AGA GAA ATA GAA 1617
ACT TTT GTG TAT ACA CCT AAA GTT GAT TAG CAA CTC TTC ACG GAA CAC 1665
AAA TTA ATT ATT CCC CAT CTA TTT TAT GAG CCC CGG 3′               1701
```

Figure 1. Nucleotide and deduced amino acid sequence of the 5'- and 3'-ends of cDNA encoding chitinase from *Chironomus tentans* prepupae. Nucleotides or amino acid residues, identical to chitinase from the cell line, are marked in bold. The middle region of both clones is identical, except positions 126 (A-G) and 558 (T-A), corresponding to positions 262 and 694 in the clone from the cell line. These exchanges are without effect on the amino acid sequence. The first ATG (Met) present in clone 2 is probably not used for inition of translation (see text); the preceeding nucleotides (the corresponding amino acid residues are in parentheses) are typical for a leader sequence, although the sequence is still incomplete.

An interesting feature of chitinase clone 2 is the predominance of thymidine in the 3'-untranslated region. The same phenomenon has also been found in the chitinase cDNAs from *Manduca sexta* (5), *Bombyx mori* and *Hyphantria cunea* (8), *Anopheles gambiae* (7) and the cuticular chitinase from *Penaeus japonicus* (16), but not in that from hepatopancreas of the same species (17). The functional significance of the corresponding U-rich area of chitinase mRNA is unknown, but from *Drosophila* and vertebrate mRNAs it is known that comparable motifs are specific binding sites for RNA-binding proteins. For example, one class of RNA-binding proteins, the Elav (embryonic lethal abnormal vison) family, binds to U-rich parts of the 3′ untranslated region of various mRNAs and was first described in *Drosophila* (18) and later in humans (19, 20). These proteins are present not only in neuronal tissue and they are involved in regulation of growth and differentiation. A second U-rich motif is important for mRNA localisation (21) and a third one – $(U)_5AU$ – is a cytoplasmic adenylation control element (22, 23). This motif is present twice in the 3′ untranslated region of the *Chironomus tentans* chitinase clone 2. Chitinase activity is regulated by ecdysteroids during the moulting cycle. Another ecdysteroid-responsive gene, 3-dehydroecdysone - 3ß - reductase from *Spodoptera littoralis* (24) also contains AU-rich elements typical for many unstable mRNAs, which may indicate regulation of these hormone dependent genes on the posttranscriptional level.

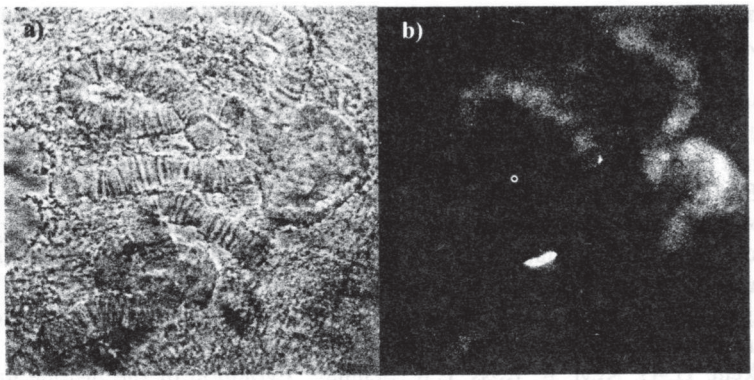

Figure 2. *In situ* hybridisation of the *Chironomus tentans* chitinase gene. The probe hybridizes on chromosome III, 7C7 (b). a) Phase contrast picture.

Since the major part of the coding region (except part of the leader sequence) of clone 2 encodes for the same amino acid sequence as the chitinase clone from the cell line, only one chitinase gene may be present in *Chironomus* and the differences may be of allelic origin. This is partly supported by *in situ* hybridisation, where only one band at chromosome III, position 7C7 is visible (Fig. 2). Of course, we can not exclude that a gene duplication event has generated closely neighboured gene copies, since the resolution of the *in situ* hybridisation is only about 100 kb. Southern blots (data not shown) also confirm these results and are in agreement with data from *Manduca sexta* which also point to a single gene (11).

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Extraction and purification of chitosanase from *Bacillus cereus*

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Summary
Chitosanase from a wild strain of *Bacillus cereus* was produced and optimal conditions for chitosan hydrolysis were observed to be at pH 5.8 and 54 °C; however, hydrolysis activity drastically decreased at pH 7.0. The enzyme was purified (single-electrophoretic band) by partitioning in an aqueous two-phase system (ATPS), followed by cation-exchange chromatography with a 66% yield. Chitosanase was mainly collected in the top phase (K = 129) of a 22% PEG 1,500, 13% phosphate (pH = 5.8) and 12% NaCl (w/w) solution, and the main protein contaminants were evenly distributed between the phases (K = 1.07). The apparent molecular weight and the isoelectric point of the chitosanase, determined by SDS-PAGE electrophoresis and by isoelectric focalization, were 47 kDa and 8.8, respectively.

Introduction
Chitosan is a linear polysaccharide composed of β-1,4 linked D-glucosamine residues, which can be obtained by deacetylation of chitin. This transformation can be catalyzed by different enzymes, including proteases, some lipases and chitosanases. Chitosanase represents a class of hydrolytic enzymes found in bacteria, fungi and plants [1]. Oligosaccharides from chitosanase action have industrial, medical and agricultural applications and transformation of chitosan into chitooligosaccharides could be potentially commercialized, as long as the hydrolysis processes were well controlled and reproducible [2]. Chitosanases able to produce oligosaccharides have been purified by conventional operations in several steps such as ammonium sulfate fractionation, gel filtration and ion exchange chromatography and by preparative isoelectric focusing, but most of the reported recovery processes include several chromatographic steps [1, 3, 4]. Economic analysis shows that protein separation and purification procedures are commonly responsible for up to 40% of the total cost of production. This is particularly true for protein processing which, because of the complexity of the starting material, often requires many steps to reach the levels of purity required for medical and food applications. The separation specialists' task is to develop safe and simple processes to achieve products with a high level of purity. A substantial research effort has been directed toward the use of aqueous two-phase systems (ATPSs) to replace the initial steps in protein purification.

Protein extraction in an aqueous two-phase system (ATPS) is a rapid procedure which avoids most of the problems of denaturing fragile molecules in conventional downstream processing. ATPS provides a gentle environment for biologically active proteins and may be employed on a large scale. In order to have a high yield, recovery and also a good purification factor for a target protein, a composition has to be selected for the ATPS to quantitatively extract the desirable protein from one of the phases with minimal concentration of

contaminant molecules. It has been found that the debris are usually removed in one of the phases and that whole cells and large particles such as chromosomes partition at the interface. A single extraction step is usually sufficient for removal of some contaminants, but others can be eliminated by countercurrent extraction using fresh phase from identical systems without the sample or by a chromatographic step. It is desirable to find the highest difference values between their partition coefficients, and the volume ratio of the two phases has to be considered as it also affects the yield of enzyme purification [5,6].

This article reports on the extraction and purification of chitosanase produced by *Bacillus cereus* from the main protein contaminants by the non-conventional process of partitioning in PEG-phosphate systems.

Material and Methods

Chitosanase production and activity: An isolated wild strain identified as *Bacillus cereus* was maintained on Chitosan Detector Agar. *B. cereus* was first grown on Tripticase Soytone (TSB) media for 14 hours at 30^0C and 200 rpm. Erlenmeyer flasks containing medium with fixed amounts of KH_2PO_4 (0.31g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}) and $Na_2HPO_4.12H_2O$ (1.7 g l^{-1}); trace element solutions of $FeSO_4.7H_2O$ (0.001 g l^{-1}), $ZnSO_4.7H_2O$ (0.0005 g l^{-1}), $MnSO_4.H_2O$ (0.001 g l^{-1}) and $CuSO_4.5H_2O$ (0.0001 g l^{-1}); and variable concentrations of $(NH_4)_2SO_4$ and chitosan (Sigma, St. Louis, MO, USA). The culture media composition was optimized after a fractional experimental. The inoculated flasks were shaken continuously at 200 rpm and 30 °C for 16 and 32 hours. The fermented broth was then centrifuged and a clear supernatant was obtained. Hydrolysis studies were proceeded in a 25.0 mL stirred, jacketed reactor and terminated by the removal of aliquots, followed by the addition of 1 mL of Somogyi reagent and subsequent heating to 100 °C for 6 min. The samples were left in an ice bath and the soluble-reducing sugars were measured. Chitosanase activity was assayed by using a 0.2% chitosan solution (w/w) in 0.05 M of acetate buffer at pH = 5.6 as the substrate. The release of reducing sugars in 40 min. at 30 °C was measured [7]. One unit of chitosanase activity (U) is defined as the amount of enzyme that produces 1μmol of reducing sugars measured as glucosamine equivalent per min. at given assay conditions.

Purification of chitosanase by partitioning in aqueous two-phase systems (ATPS) was studied in five systems, according to [5,6], as described in Table 1. Ten grams of ATPS were prepared in beakers by weighting solid PEG 1500, 40% phosphate stock solution at pH = 5.8 (w/w) and solid sodium chloride, and the remainder was the clear fermented broth. The systems were mixed and centrifuged for two minutes at 1,000 g to achieve phase separation. The partition coefficient (K) of the chitosanase was calculated as the ratio of the enzyme concentration in the top phase to that in the bottom phase at room temperature. In order to improve the separation of chitosanase from the protein contaminants, 16 mL of the diluted isolated top phase of the 22% PEG 1,500 + 13% phosphate + 12% NaCl system was applied to a HR 5/5 column packed with S-Sepharose (Amershan-Pharmacia), equilibrated with 50 mM sodium acetate buffer, pH 5.8. Elution was then achieved with a gradient of NaCl (0 to 1.5 M), and the fractions collected were analyzed for chitosanase activity and total proteins [8].

Results and Discussion

The best composition of the culture medium for *Bacillus cereus* was found after a fractional factorial design where the five two-level factors studied were ammonium sulfate concentration, chitosan concentration, aeration, pH and fermentation time. It was observed that chitosanase production is greatly affected by the concentration of ammonium sulfate,

aeration, pH and the interaction between the first two factors. The best culture medium composition for the highest level of chitosanase was obtained with 2% chitosan, 4% ammonium sulfate, an aeration of 10 (Erlenmeyer volume/culture media volume) at pH = 5.0 and 16 hours of fermentation. It was observed that the enzyme level decreased after 32 hours of fermentation indicating that denaturation or proteolysis may have occurred during the last hours of fermentation, since the pH remained approximately constant. An important decrease in the production of chitosanase was obtained in the medium with the lowest aeration, despite the fact that it had the highest ammonium concentration (4%) added to the culture media.

The conditions for chitosanase activity were studied for pH, temperature and hydrolysis time. The results showed that a maximum of chitosanase activity was reached at pH = 5.8 for temperatures above 50 °C. It was found that the highest chitosanase activity was obtained at 54 °C and pH = 5.8 after 40 min. of hydrolysis and that it decreased drastically above 54 °C and its stability was also lowered. A possible denaturation of the enzyme may have occurred at pH values above 7.0, as its activity was almost lost after a ten-minute incubation at this pH.

In order to separate the chitosanase from the main contaminant proteins, five different compositions of ATPS, which are usually employed [5] for the purification of biomaterial, were investigated. Two of the aqueous systems tested were unable to increase the concentration of enzyme in one of the phases (systems 2 and 4). However, systems 1, 3 and 5 separated chitosanase from the contaminant proteins. A 1,800-fold increase in the partition coefficient of chitosanase was observed (from 0.04 to 72) with the addition of 12% NaCl (systems 2 and 3) but the K of the main protein contaminants was not strongly affected (from 0.92 to 1.72). The addition of 12% NaCl to a 22% PEG 1,500 + 13% phosphate increased the $K_{chit.}$ almost 645-fold (from 0.2 to 129), with the main protein contaminants distributed between the two phases ($K_{prot.}$ =1.07), but the purification factor remained the same (Table 1). Sixteen mililiters of isolated material from the top phase of system 5 were diluted 200-fold and applied to the ion exchanger chromatographic media S-Sepharose. Chitosanase was strongly adsorbed by the cationic chromatographic media at pH 4.0, 5.0 and 5.8 and was eluted with 1.2 M, 0.7 M and 0.5 M of NaCl, respectively, suggesting that the isoelectric point (pI) of the chitosanase is located above 5.8, which was confirmed by isoelectric focalization. The pI and molecular weight were observed to be 8.8 and 47 kDa, respectively. A photograph of a SDS-PAGE gel (Figure 1) shows the protein profile of the crude fermentation broth, the isolated material from the top and bottom phases of an ATPS and the two step purified chitosanase (partitioning + chromatography). Chitosanase yield and the purification factor were 66% and 20 after just two simple purification procedures (partitioning + ion exchange adsorption).

Table 1: Purification of Chitosanase by Partitioning in ATPS

System	PEG (%)	Phosphate (%)	NaCl (%)	$K_{chit.}$	$K_{prot.}$	Purification Factor	Chitosanase recovery* (%)
1	16	13	12	90.7	0.95	2.8	105
2	22	10	0	0.04	0.92	5.2	91
3	22	10	12	72	1.72	2.2	101
4	22	13	0	0.20	0.64	1.9	81
5	22	13	12	129	1.07	1.9	102

* in the predominant phase

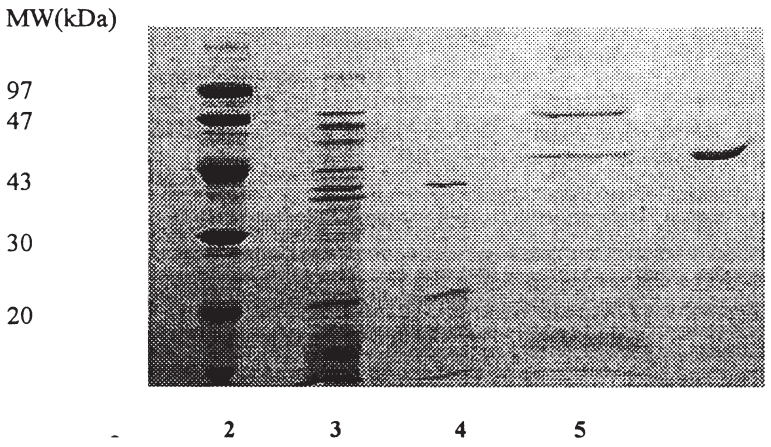

Figure 1: Coomassie-stained SDS-PAGE of standard markers: phosphorilase B, bovine serum albumin, ovalbumin, carbonic anhydrase and trypsin inhibitor (lane 1); crude fermented broth (lane 2); top phase of system 4 (lane 3), bottom phase of system 4 (lane 4) and purified material collected after partitioning followed by IEC (lane 5)

Conclusions

The best composition for chitosanase production was a culture medium containing 2% chitosan and 4% ammonium sulfate, with an aeration of 10 (Erlenmeyer volume/culture medium volume) at pH = 5.0 for 16 hours of fermentation. An important decrease in the production of chitosanase was obtained in the medium with the lowest aeration, despite its having the highest ammonium concentration (4%) added to the culture media. The best conditions for chitosan hydrolysis were pH = 5.8 and 54 °C, and enzymatic activity drastically decreased at pH = 7.0. The enzyme was partially purified by partitioning in 22% PEG 1,500 + 13% phosphate + 12% NaCl at pH 5.8. A high yield of recovery of chitosanase was obtained in the top phase (102%), and the main protein contaminants were evenly distributed between the phases. Cation exchange chromatography was employed in a second purification step to produce a pure enzyme, confirmed by a single electrophoretic band in SDS-PAGE, achieving a 66% yield of pure chitosanase.

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Substrate binding mechanism of chitosanase from *Streptomyces* sp. N174

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Summary
Substrate binding mechanism of chitosanase from *Streptomyces* sp. N174 was investigated by oligosaccharide digestion experiments, theoretical calculation, site-directed mutagenesis, and biomolecular interaction analysis. From the substrate $(GlcN)_6$, $(GlcN)_3$ was predominantly produced by the chitosanase. Numerical calculation based on the reaction model for the oligosaccharide degradation by the (-3)(-2)(-1)(+1)(+2)(+3)-type enzyme successfully gave a theoretical time-course well fitted to the experimental one. Mutation of Asp57 of the chitosanase to alanine reduced the activity toward $(GlcN)_6$ to 0.48% of that of the wild type. By monitoring surface plasmon resonance, the interaction ability with chitosan was found to be significantly affected by the mutation. Theoretical analysis of the time-course of $(GlcN)_6$ degradation by the mutant enzyme suggested that Asp57 is responsible for the glucosamine residue binding at subsite (-2).

Introduction

Substrate binding cleft of most glycosyl hydrolases is composed of several subsites, each of which binds a monosaccharide unit of corresponding substrates. Splitting specificity of the enzymes is mostly dominated by the subsite arrangement, hence elucidation of the subsites is essential for understanding the reaction mechanism. Hen egg white lysozyme has a well known subsite arrangement, so called subsites A, B, C, D, E, and F, which is now represented by (-4)(-3)(-2)(-1)(+1)(+2) [1]. Trp62 plays an important role of sugar residue binding at subsite (-2) in the lysozyme [2]. However, such a substrate binding mechanism of other chitinolytic enzymes has not been understood yet. Recently, Honda and Fukamizo reported that barley chitinase has binding subsites, (-3)(-2)(-1)(+1)(+2)(+3), which is very similar to those of goose egg white lysozyme [3]. This is consistent with the structural similarity found in between these enzymes [4]. *Streptomyces* sp. N174 chitosanase was found to have similar folds to those of these enzymes, suggesting that the chitosanase would have a similar substrate binding mechanism. In this study, we investigated the substrate binding mechanism of chitosanase from *Streptomyces* sp. N174 chitosanase, and found that the binding subsites are represented by (-3)(-2)(-1)(+1)(+2)(+3) and that Asp57 is important for sugar residue binding at subsite (-2).

Materials and Methods

Materials: Chitosanase from *Streptomyces* sp. N174 and its mutants (D57A and D57N) were obtained by the method previously described [5]. The global conformations of the chitosanase mutants did not differ from that of the wild type enzyme as determined by circular dichroism spectroscopy. Other reagents used in this study were of analytical grade commercially available.

Activity toward polymer substrate: Chitosan (d.a.= 0.21) was dissolved in 50 mM sodium acetate buffer, pH 5.0. Chitosanase activity was determined by measuring the reducing sugar concentration produced by the enzymatic hydrolysis of the chitosan.

Chitosan binding ability to chitosanases: The binding ability was determined using a biomolecular interaction analyzer, BIAcore 1000 (Biosensor). Each chitosanase was immobilized on the sensor chip, and the chitosan (d.a.<0.01) solution dissolved in 50 mM sodium acetate buffer pH 5.5 was flushed onto the chitosanase molecule on the sensor chip. A profile exhibiting the chitosan association and dissociation processes was obtained by monitoring surface plasmon resonance.

HPLC determination of the reaction time-course: Each chitosanase solution was added to $(GlcN)_6$ solution, and the mixture was incubated in 50 mM sodium acetate buffer pH 5.0 and at 40 °C. The substrate and product concentrations were determined by HPLC using a gel-filtration column of TSK-GEL G2000PW (Tosoh). The elution was performed with 0.5 M NaCl at a flow rate of 0.3 ml/min and at room temperature.

Theoretical calculation of the reaction time-course: Theoretical calculation based on the reaction model shown in Fig. 1 was done by the method of Honda and Fukamizo [3]. Considering the structural similarity between barley chitinase and the chitosanase, we assumed that the chitosanase has a subsite arrangement similar to that of barley chitinase; (-3)(-2)(-1)(+1)(+2)(+3). The time-course calculation was repeated by modifying the value of the binding free energy change of each subsite to obtain the minimum of the cost function,

$$F = \sum_i \sum_n [\,(GlcN)^c_{n,i} - (GlcN)^e_{n,i}\,]^2 \qquad (1)$$

Here, e and c are the experimental and calculated values, n is the size of the chitooligosaccharides, and i the reaction time. In calculation of the time-course, rate constant value of k_1 (for cleavage of glycosidic linkage) was assumed to be dependent upon the substrate size, and fixed at the values estimated from steady state kinetics; $50 s^{-1}$ for $(GlcN)_4$, $50\ s^{-1}$ for $(GlcN)_5$, and $200\ s^{-1}$ for $(GlcN)_6$. The value, $100\ s^{-1}$, was tentatively allocated to the rate constant for hydration, k_2.

Results and Discussion

Time-course of $(GlcN)_6$ hydrolysis by the chitosanase: HPLC determination successfully afforded the reaction time-course of the $(GlcN)_6$ hydrolysis as shown in Fig. 3A. The major product was $(GlcN)_3$, and $(GlcN)_2$ and $(GlcN)_4$ were the minor ones. $(GlcN)_4$ produced was further decomposed to $(GlcN)_2$+$(GlcN)_2$. It is very likely that the chitosanase hydrolyzes $(GlcN)_6$ predominantly in the middle producing $(GlcN)_3$+$(GlcN)_3$. From the product composition, the subsite arrangement of the chitosanase was estimated to be (-3)(-2)(-1)(+1)(+2)(+3).

Theoretical calculation of the reaction time-course: Based on the reaction model shown in Fig. 1, theoretical calculation was repeated changing the values of the binding free energy changes of the individual subsites. Finally, the best fitted time-course was obtained

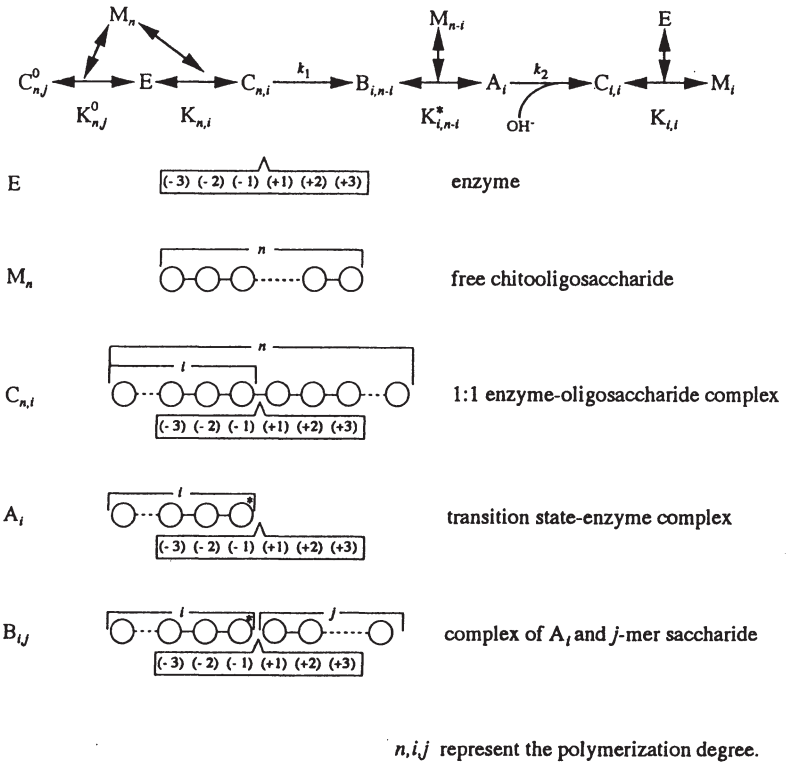

Figure 1. Reaction model for oligosaccharide hydrolysis catalyzed by the chitosanase.

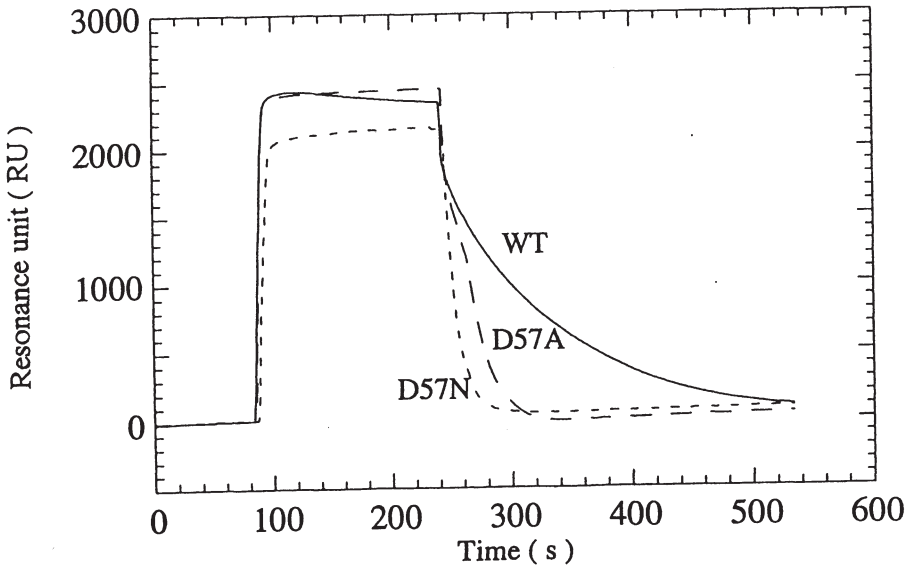

Figure 2. Sensorgram exhibiting chitosan association and dissociation processes to the chitosanases obtained by a BIAcore 1000.

with the free energy values listed in the first row of Table 2, and is shown in Fig. 3B. The chitosanase reaction toward $(GlcN)_6$ could be rationalized by the subsite arrangement, (-3)(-2)(-1)(+1)(+2)(+3).

Estimation of amino acid residue responsible for sugar residue binding: As reported in the previous paper [3], among the six subsites of barley chitinase, subsite (-2) has the highest affinity to the GlcNAc residue, -5.0 kcal/mol. From the crystal structure [6] and site-directed mutagenesis study of barley chitinase [7], Asn124 is considered to be one of the amino acid residues responsible for sugar residue binding at subsite (-2). Asn124 is located at the forth α-helical structure from the N-terminus, helix D. In the crystal structure of *Streptomyces* sp. N174 chitosanase [8], an α-helical structure is found at the position corresponding to helix D of barley chitinase. The helical structure in the chitosanase which consists of the amino acid residues from 56th to 69th residues might be important for sugar residue binding. Sequence comparison of the residues from 56th to 69th indicated that only Asp57 is conserved in all chitosanases sequenced thus far. Thus we paid attention to Asp57 as an amino acid residue responsible for sugar residue binding.

Relative activities of Asp57-mutated chitosanases: The relative activities of Asp57-mutated chitosanases were determined using the substrates, chitosan and $(GlcN)_6$, and are listed in Table 1. The activities of mutant chitosanases, in which the catalytic residues Glu22 and Asp40 are substituted, are also listed for comparison. The activities of the catalytic residue mutants were less than 1% of that of the wild type for both substrates. However, the activities of D57A and D57N were 5.7 and 85 % for the substrate chitosan, and 0.48 and 72 % for the oligosaccharide substrate, respectively. The substitution of $-CH_2-COO^-$ with $-CH_3$ strongly affected the enzymatic activity, but the substitution with $-CH_2-CO-NH_2$ did not so strongly affect the activity. Thus the -CO- moiety of Asp57 is very important for the chitosanase activity.

Table 1. Relative activities of the mutant chitosanases toward chitosan and (GlcN)6.

enzyme	Relative activities (%)	
	chitosan	$(GlcN)_6$
wild type	100	100
E22Q	0.04	not detected
E22D	0.23	not detected
E22A	0.03	not detected
D40N	0.21	0.23
D40E	0.78	0.47
D57A	5.7	0.48
D57N	85	72

Biomolecular interaction analysis using a BIAcore: As shown in Fig. 2, by flushing the chitosan solution onto the immobilized chitosanase (80–240 sec), resonance units instantaneously increased, due to a difference in the refractive index between the buffer solution and the chitosan solution. Then, the dissociation of the chitosan from immobilized chitosanase could be observed by flushing the buffer solution without chitosan (after 240 sec). The dissociation curve for the wild type enzyme was gradual, indicating a significant

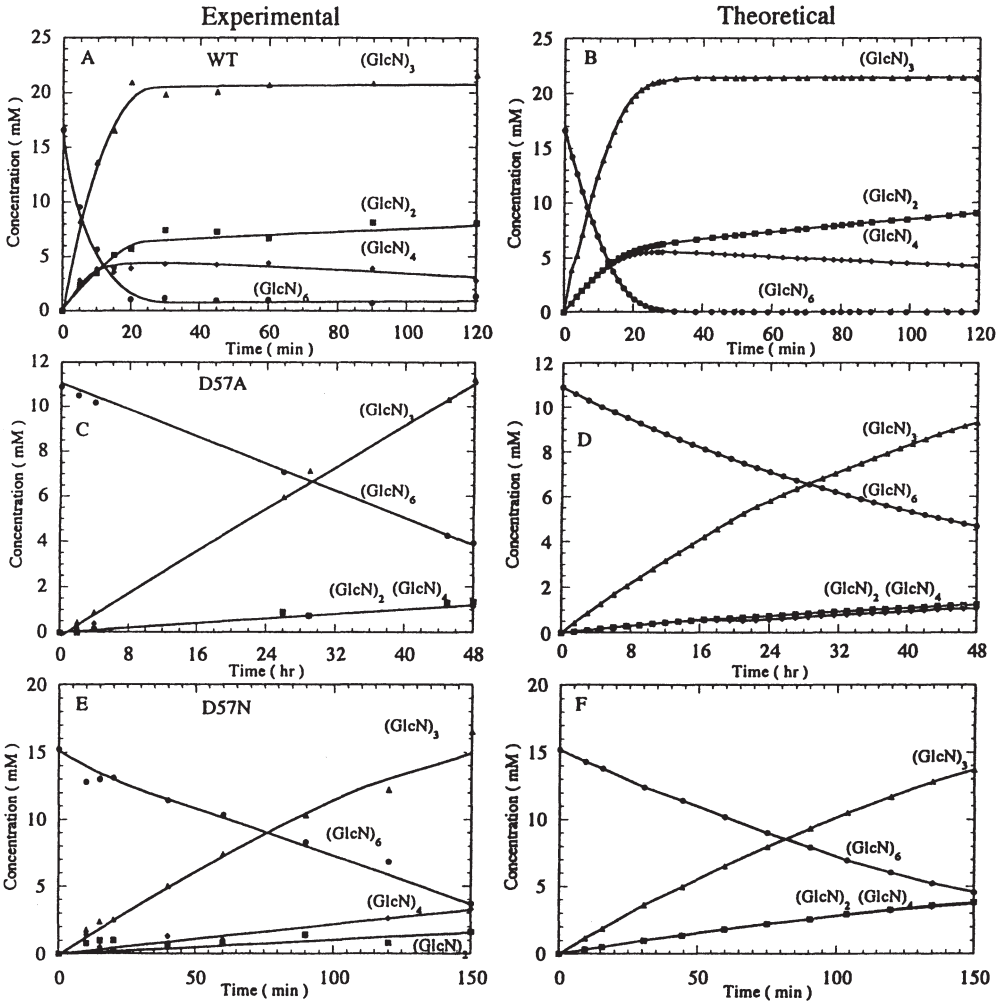

Figure 3. Experimental (A, C, and E) and theoretical time-courses (B, D, and F) of hexasaccharide hydrolysis catalyzed by the wild type chitosanase (A and B), D57A (C and D), and D57N (E and F). Enzyme concentrations were 0.38 μM for the wild type, 0.25 μM for D57A, and 0.04 μM for D57N.

interaction between chitosan and the chitosanase. On the other hand, when D57A or D57N was used instead of the wild type, the dissociation curves were steep. This clearly indicated that the mutation of Asp57 significantly affects the interaction ability. It is very likely that Asp57 is responsible for sugar residue binding.

Time-course experiments and theoretical calculations for Asp57-mutated chitosanases: HPLC determination of the time-course of $(GlcN)_6$ hydrolysis was done using Asp57-mutated chitosanases. The results are shown in Figs. 3C and 3E. In the case of D57A, a long incubation time is required for obtaining the profile of time-course, because of its extremely low activity. The product distribution in each experimental time-course was similar to that observed for the wild type enzyme. For estimation of the binding free energy change of each subsite, theoretical calculation was conducted changing the value of binding free energy changes of the subsites. The theoretical time-courses best fitted to the experimental ones were obtained with the free energy values listed in the second and third rows of Table 2, and are shown in Figs. 3D and 3F. The mutation of Asp57 was found to affect the sugar binding ability at subsite (-2), and more weakly at neighboring subsites. These results clearly indicate that Asp57 is responsible for sugar residue binding at subsite (-2). The interaction might be due to hydrogen bond, but not to electrostatic forces, because the binding free energy change was intensively affected in D57A, but not so affected in D57N.

Table 2. Binding free energy changes of individual subsites of *Streptomyces* sp. N174 chitosanase and its mutants.

chitosanase	binding free energy changes (kcal/mol)					
	(-3)	(-2)	(-1)	(+1)	(+2)	(+3)
wild type	-0.7	-4.7	+3.4	-0.5	-2.3	-1.0
D57A	-1.7	0.0	+4.5	-0.5	-2.3	-1.0
D57N	-0.7	-3.8	+3.4	-0.5	-2.3	-1.0

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Chitosanase-catalyzed hydrolysis of 4-methylumbelliferyl β-chitotrioside

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Summary

4-Methylumbelliferyl β-chitotrioside [$(GlcN)_3$-UMB], a versatile synthetic substrate for the assay of chitosanase, was prepared by enzymatic deacetylation of 4-methyl-umbelliferyl tri-*N*-acetyl-β-chitotrioside [$(GlcNAc)_3$-UMB] using chitin deacetylase from *Colletorichum lindemuthianum* . The chitosanase from *Sttreptomyces* sp. N174 hydrolyzed specifically the glycosidic linkage between GlcN residue and UMB. The k_{cat} and K_m values for the substrate $(GlcN)_3$-UMB were determined to be 8.1 x 10^{-5} s^{-1} and 201 μM, respectively. A high sensitivity of fluorescence detection of the released UMB molecule from $(GlcN)_3$-UMB would enable a more accurate determination of kinetic constants for chitosanases.

Introduction

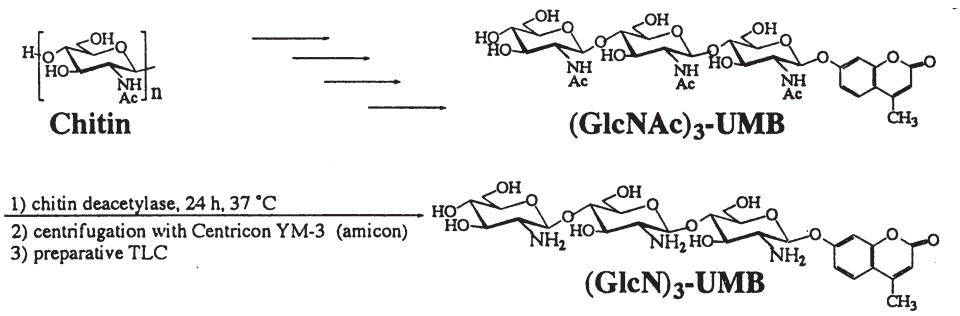
We have been investigated reaction mechanism of chitosanases from various enzyme sources [1]. Evaluation of chitosanase activity has been done in terms of the increase in reducing sugar concentration during the chitosan hydrolysis . Acetylation degrees of most chitosans, however, are in the intermediate range, and the *N*-acetylglucosamine residues (GlcNAc) are randomly localized in the chitosan chain, resulting in chemical diversity of the polysaccharides. Thus the kinetic constants obtained for the chitosan substrate contain some uncertainty, hence it is highly desirable to establish a strategy for obtaining definitive values of kinetic constants for chitosanases.

Recently, we tried to determine the kinetic constants of chitosanases using $(GlcN)_n$ substrate. The time-course for oligosaccharide degradation was successfully obtained by separating and quantifying $(GlcN)_n$ with a gel-filtration HPLC system [2]. Although

information concernig the mode of enzymatic hydrolysis could be obtained by the HPLC determination, the sensitivity of the oligosaccharide detection using the refractive index was insufficient to allow an accurate determination of kinetic constants. On the other hand, synthetic chromophoric substrates have been used for the kinetic analysis of various hydrolases. In particular, 4-methylumbelliferyl (UMB) glycoside substrate enables the most sensitive assay of glycosyl hydrolases (3-6), because the enzymes release a fluorescent UMB moiety from the corresponding synthetic substrate.

To release the fluorescent aglycon from the synthetic substrate by the action of chitosanase, the design of the substrate should be based on the minimum structural requirements of natural substrates. We previously reported that the enzyme mainly produces $(GlcN)_3$ or $(GlcN)_2$ from $(GlcN)_n$ (n=4, 5, and 6) (2). From the functional information, we synthesize $(GlcN)_3$-UMB because this should be the minimum structural requirement for the release of the UMB molecule by chitosanase action. We at first synthesized $(GlcNAc)_3$-UMB, and then tried to deacetylate it chemically. However, it was quite difficult to eliminate *N*-acetyl groups chemically without affecting the UMB glycosidic bond. Thus, we employed chitin deacetylase from *Colletorichum lindemuthianum* to prepare $(GlcN)_3$-UMB as shown Scheme 1.

In this report, we investigated kinetically the chitosanase-catalyzed hydrolysis of $(GlcN)_3$-UMB in comparison with the kinetic data for the substrate $(GlcN)_n$ (n=4, 5, and 6).



Scheme 1

Materials and Methods

Streptomyces sp. N174 chitosanase was produced by the expression system of *Streptomyces lividans* TK24 and purified according to the method of Boucher et al. [7]. Chitin deacetylase from *Colletotrichum lindemuthianum* was obtained by the method previously reported [8, 9].

Other reagents were commercially available and of analytical grade. $(GlcNAc)_3$-UMB was synthesized according to the method of Delmotte et al. [10]. Deacetylation of $(GlcNAc)_3$-UMB was carried out with chitin deacetylase according to the method of Tokuyasu et al (8, 9) with slight modification.

$(GlcN)_3$-UMB was dissolved in 0.6 ml of 50 mM sodium acetate buffer (pH 5.5) to obtain a 43-630 μM substrate solution. Enzyme solution was added to the substrate solution and the mixture was incubated at 40 °C. The final enzyme concentration of 0.353 μM. A portion (0.1 ml) of the reaction mixture was withdrawn at an appropriate reaction time, and mixed with 0.5 ml of 0.4 M Na_2HPO_4-NaOH buffer, pH 11.9, to terminate the enzymatic reaction. Fluorescence intensity of the resultant solution was measured at 450 nm with excitation at 360 nm using a Shimadzu-RF1500 spectrofluorometer. Concentration of UMB released from $(GlcN)_3$-UMB was calculated from the calibration curve obtained with an authentic solution of UMB. The values of kinetic constants were calculated from double reciprocal plots of initial velocity versus substrate concentration.

The substrate, $(GlcN)_n$ (n=4, 5, or 6), was dissolved in 50 mM sodium acetate buffer, pH 5.5, to obtain 1-16.6 mM substrate solution. The enzyme solution was added into the substrate solution, and the reaction mixture was incubated at 40 °C. The final enzyme concentrations were 0.37 μM for $(GlcN)_4$ substrate and 0.053 μM for $(GlcN)_5$ or $(GlcN)_6$. After an appropriate reaction time, a portion of the reaction mixture was withdrawn and mixed with an equal volume of 0.1 N NaOH to terminate the enzymatic reaction. The reaction products obtained were analyzed by HPLC on a gel-filtration column of TSK-GEL G2000PW (TOSOH, 0.5 × 600 mm). The elution was performed with 0.5 M NaCl at a flow rate of 0.3 ml/min and at room temperature using a Hitachi L-6200. The product oligosaccharides were detected with a Hitachi L3350 RI monitor and the oligosaccharide concentrations were calculated from peak area using standard curves obtained from pure oligosaccharide solution. Initial velocity was determined from decrease in the substrate concentration. The values of the kinetic constants were calculated from double reciprocal plots of initial velocities versus the substrate concentrations.

Results and Discussion

The deacetylation of $(GlcNAc)_3$-UMB catalyzed by chitin deacetylase was confirmed by measuring the ^1H-NMR spectrum. The mass spectorometry analysis indicated that the enzymatic product to have a molecular weight 659 (data not shown).

When the $(GlcN)_3$-UMB obtained was incubated with chitosanase, the fluorescence intensity at 450 nm obtained by 360 nm was found to increase with the reaction time in the presence of chitosanase, but not in its absence (data not shown). Thus, the increase in fluorescence intensity indicates that the chitosanase hydrolyzes the glycosidic linkage between the GlcN residue and UMB moiety of the substrate releasing the fluorescent UMB molecule.

Kinetic behaviour for the substrate $(GlcN)_3$-UMB was investigated in comparison

with the substrate $(GlcN)_n$ (n= 4, 5, and 6). As shown in Fig. 1, substrate inhibition was

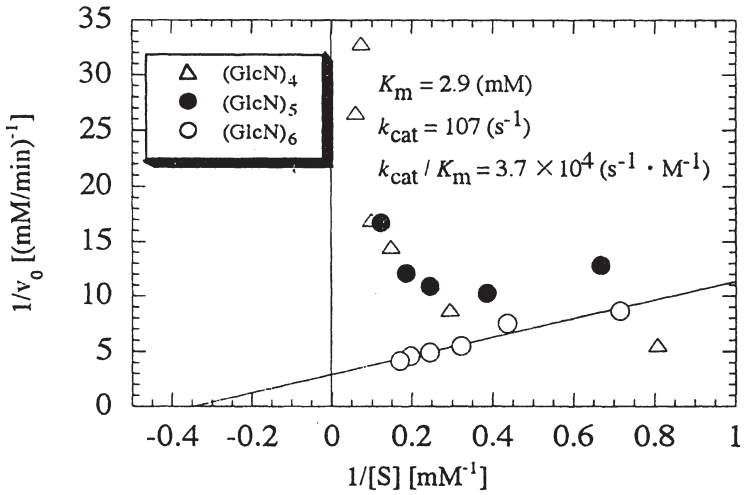


Fig. 1 **Double reciprocal plot for $(GlcN)_n$ (n=4,5, and 6) hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase**

Experimental conditions are described in the text.

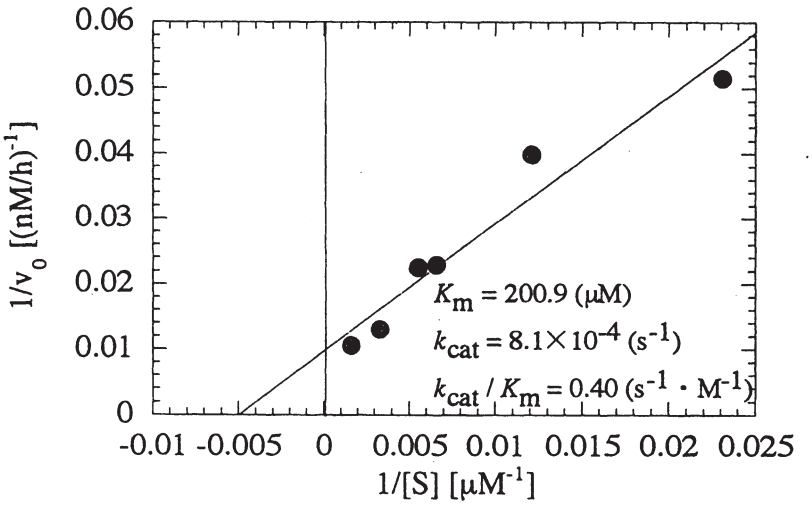


Fig. 2 **Double reciprocal plot for $(GlcN)_3$-UMB hydrolysis catalyzed by *Streptomyces* sp. N174 chitosanase**

Experimental conditions are described in the text.

observed with the substrates $(GlcN)_4$ and $(GlcN)_5$. The shorter the substrate chain length, the stronger the substrate inhibition. The shorter oligomeric substrate might be able to bind to vacant subsites [11], producing a non-productive enzyme-substrate complex. In the substrate concentration range below 1 mM, the hydrolytic rate of the chitosanase cannot be evaluated because of the low sensitivity of the refractive index detection used in HPLC analysis. Thus, kinetic constants could not be obtained with the shorter chain length substrates. On the other hand, the substrate inhibition was not observed when $(GlcN)_3$-UMB was used as the substrate, and the steady state kinetic constants were successfully obtained with the synthetic substrate (Fig. 2).

The k_{cat} value obtained for $(GlcN)_3$-UMB was much lower than that for $(GlcN)_6$. The difference in the k_{cat} values might be rationalized from substrate size dependence. The substrate size dependence of the rate constant has been reported for hen egg white lysozyme, goose egg white lysozyme, and barley chitinase [12, 13]. *Streptomyces* sp. N174 chitosanase, whose structure is similar to those of goose egg white lysozyme and barley chitinase, might exhibit a similar substrate size dependence as well resulting in the much lower k_{cat} value for $(GlcN)_3$-UMB. Another explanation of the difference in k_{cat} value can be made from the splitting specificity; that is, chitosanase can hardly attack the terminal glycosidic linkage of $(GlcN)_n$ substrate. In fact, the chitosanase can hardly produce GlcN monomer from any $(GlcN)_n$ substrate [2]. The release of UMB from $(GlcN)_3$-UMB results from the terminal attack of the chitosanase. Thus, the k_{cat} value for UMB release from $(GlcN)_3$-UMB is much lower than that for $(GlcN)_6$ hydrolysis. We think that the latter explanation is more appropriate in this case.

We concluded that a novel substrate, $(GlcN)_3$-UMB, is convenient for kinetic analysis for chitosanases, especially in the lower substrate concentration range of the substrate. Further kinetic study of the *Streptomyces* sp. N174 chitosanase is now under progress in our laboratory using the novel substrate.

Acknowledgement

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A rust fungus turns chitin into chitosan upon plant tissue colonization to evade recognition by the host

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Summary
Biotrophic fungal pathogens have to carefully avoid recognition by the host tissue they invade in order to prevent the elicitation of induced resistance reactions. Rust fungi have evolved an elaborate way of penetrating plant tissues through natural openings of leaf surfaces - stomates - circumventing the need of physically wounding the host tissue. Using wheat germ agglutinin as a specific probe for chitin and a rabbit antiserum directed against fully de-N-acetylated chitosan, we have shown that germ tubes and appressoria of the wheat stem rust fungus growing on the surface of the host leaf contain chitin in their cell walls, while the cell walls of substomatal vesicles and infection hyphae produced inside the host leaf contain chitosan. We speculate that this change in surface properties protects the fungus from attack by host plant chitinases and thus, prevents the production of elicitor active chitin oligomers.

Introduction
The wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*, is an obligately biotrophic pathogen causing rust disease on wheat leaves. Unlike necrotrophic fungi, biotrophs such as rust fungi must not kill their host tissue. Instead, they rely on living host cells for their nutrition. These fungi, therefore, have to colonize their host tissues with utmost caution, taking care not to reveal their presence to the plant cells in order to avoid triggering of active resistance mechanisms.

One strategy rust fungi have evolved for cautious host tissue colonization is the penetration into the leaf tissue of their host plants via the natural openings of the stomates [1, 2]. The germ tubes emanating from rust uredospores tightly adhere to the cuticular surface of plant leaves. The growing tip of the germ tube appears to be delicately sensitive to the surface topography of the leaf. By sensing the grooves brought about by the anticlinal walls separating epidermal cells, the germ tube is able to orient itself on the leaf surface. Growth perpendicular to the grooves leads to the rapid homing in on a stoma. When the tip of the rust germ tube recognizes the stoma, growth halts and an appressorium develops at the tip of the germ tube. The production of a slim penetration peg at the underside of the appressorium allows the fungus to grow through the narrow stomatal opening. A substomatal vesicle is formed inside the leaf, and intercellular infection hyphae then start colonizing the host tissue. Upon contact with a host cell wall, a haustorium mother cell forms at the tip of the infection hypha, a delicate haustorial neck starts penetrating the host cell wall, and a fungal haustorium is built in the periplasmic space to draw nutrients from the host cell. The evolution of this elaborate series of

infection structures allows the rust fungus to gain access to its nutrient source with a minimum of host cell wounding or physical disturbance.

Plant cells, however, have developed strategies to recognize penetrating pathogens, even such cautious ones as the rust fungi [3, 4]. The molecular recognition of typical fungal surface components, termed elicitors, can trigger the rapid induction of a diverse range of active disease resistance mechanisms. Most plants possess apoplastic chitinases which will partially hydrolyze the cell walls of penetrating fungal hyphae, and the so produced chitin oligomers possess elicitor activity in most plants [5]. Nanomolar concentrations of chitin oligomers trigger the induction of an oxidative burst - the very rapid production of toxic hydrogen peroxide - by plants cells, the biosynthesis of phytoalexins - low molecular weight antimicrobial compounds -, or lignin - as a structural reinforcement of plant cell walls. Moreover, chitin oligomers often induce host chitinase genes - leading to an autocatalytic cycle of enzymic chitin hydrolysis, recognition of chitin oligomers, and induction of the chitinase genes.

Fungal cell walls are generally assumed to contain chitin covalently linked to an amorphous matrix made up of ß-1,3-1,6-glucans and proteoheteroglycans [6]. Instead of or in addition to chitin, some fungi may contain chitosan, the partially or fully de-N-acetylated counterpart of chitin. Histochemical data indicate that the cell walls of pathogenic fungi are build of several distinct layers, but little is known on the chemical composition of these layers. In particular, the cell walls of rust fungi seem to be highly complex, and their architecture seems to vary between the different infection structures [7, 8]. Major changes in cell wall composition appear to occur at the transition from appressorium to substomatal vesicle, and again at the transition from the haustorial mother cell to the haustorium. Clearly, these are critical moments in the colonization of host tissue by a rust fungus: first the crossing of the epidermis from the outside into the inside of the leaf, then the crossing of the host cell wall from the intercellular space of the leaf into the periplasmic space of the cell.

Using lectins with different sugar specificities, a change of surface properties of the fungal cell wall at the first of these transitions is visible [9]. While germ tubes and appressoria, both growing on the leaf surface, stain positively with the chitin-specific lectin wheat germ agglutinin (WGA), the substomatal vesicle and the infection hyphae growing inside the leaf do not bind WGA, indicating the lack of chitin or its masking in these fungal structures. We have now used WGA and a rabbit antiserum raised against fully de-N-acetylated chitosan in order to reveal the presence of chitin and chitosan in the cell walls of the series of infection structures differentiated by the stem rust fungus during host tissue colonization.

Materials and Methods

Uredospores of the wheat stem rust fungus *Puccinia graminis* (Pers.) f. sp. *tritici* (Eriks. & E. Henn) (race 32) were propagated on fully susceptible, adult wheat plants *(Triticum compactum* cv. Little Club) growing in automatically regulated growth chambers in pre-fertilized soil. Differentiation of infection structures was induced by a mild heat shock (2 h, 30 °C) starting 2 h after seeding uredospores into polystyrene petri dishes and adding distilled water [10].

A polyclonal anti-chitosan-antiserum was raised in rabbits by Dr. H. Deising, Halle, Germany. Fully de-N-acetylated chitosan (= polyglucosamin) with an average degree of polymerisation of 850, kindly provided by Dr. A. Domard, Lyon, France, was used as an immunogen.

The specificity of the antiserum was tested by covalently binding glycol-chitin and glycol-chitosan (0.1, 1, 10, and 100 $\mu g\ ml^{-1}$) via free amino groups to Immobilon-AV-membrane. Binding of the antiserum to the immobilised antigens was visualised using alkaline phosphatase-conjugated goat-anti-rabbit secondary antibodies.

Rust germlings with or without infection structures were blocked with BSA prior to incubation with the antiserum and/or FITC-conjugated wheat germ agglutinin. Bound antibodies were visualised with Texas Red-conjugated goat-anti-rabbit secondary antibodies. Following extensive washing, germlings were mounted in distilled water and analysed with an Olympus epifluorescence photomicroscope equipped with excitation filter U-MNB 470-490 and barrier filter BA 515.

Results and Discussion

An antiserum was obtained from the rabbits immunised with fully de-N-acetylated chitosan. The antibodies specifically recognised chitosan but showed only weak binding to chitin. The antiserum was active in weak dilutions only, indicating a rather low antibody titer had been achieved. Preliminary evidence suggest that the epitope recognised by the antibodies is partially N-acetylated. It may thus be speculated that the rare N-acetyl-glucosamine residues present even in the 'fully' de-N-acetylated chitosan may be the only immunogenic part of the polymers, while the vast majority of glucosamine residues do not induce an immune response. We are currently analysing the epitope in more detail. Moreover, we are in the process of screening for monoclonal or recombinant antibodies to chitosan.

A mild heat shock induced the synchronous differentiation of the typical infection structures of the wheat stem rust rust fungus, *Puccinia graminis* f. sp. *tritici,* in vitro. Appressorium formation started during the heat shock period and was terminated within three to four hours. Substomatal vesicles appeared shortly after the end of the heat shock period and were fully formed within four hours. Infection hyphae started to grow around six hours later. No haustorial mother cells and haustoria were formed under these conditions.

The germlings were probed for the presence of chitin in their cell walls using wheat germ agglutinin, and for the presence of chitosan using the anti-chitosan antiserum. WGA revealed the presence of chitin on the surface of germ tubes and appressoria, but not on substomatal vesicles and infection hypae. In contrast, chitosan was present on the surface of substomatal vesicles and infection hypae, but not on germ tubes and appressoria. Thus, the cell walls of fungal structures naturally formed on the outer surface of the host leaf contained chitin, while chitin was replaced by chitosan in the cell walls of those infection structures formed naturally inside the host leaf.

We interprete this change in surface properties observed upon ingress into the host tissue as an adaptation of the rust fungus to its biotrophic life style. As long as the fungus grows on the outer surface of the host leaf, a strong and resisting cell wall is required. Chitin is a superior polysaccharide to be used in the construction of such a cell wall. In particular, the build up of turgor pressure in appressoria [11, 12] can be expected to make chitin an indispensable component of the cell wall of this infection structure. However, once inside the protected space of the host leaf, the strength of a chitinous cell wall becomes less important. On the contrary, the presence of plant chitinases turns chitin into a hazard for the fungus. The enzymatic degradation of the chitin may not only weaken the fungal cell wall to the point of rupture [13], it may also generate elicitor-active chitin

oligomers which upon recognition by the host cells can induce active disease resistance mechanisms [5]. The incorporation of chitosan instead of chitin most likely leads to the building of a physically less robust cell wall in the intercellular infection structures. However, this drawback can be assumed to be more than balanced by the achieved resistance to plant chitinases.

Interestingly, the growing tips of infection hyphae of the wheat stem rust fungus always stained positively for chitin while the rest of these hyphae contained chitosan rather than chitin in the cell wall. Obviously, chitin is incorporated into the growing cell wall at the apex, and the chitin is then de-N-acetylated to form chitosan in the subapical region of the hyphae. Most likely, the complex and highly sophisticated mechanism of tip growth of fungal hyphae, involving the initial deposition of an elastic cell wall at the growing apex which is then gradually rigidified as the apex moves on [14], requires the deposition and possibly cross-linking of chitin. The de-N-acetylation of chitin into chitosan appears to be possible only in a subapical region not involved in tip growth.

Conversion of chitin into chitosan in fungal cell walls is achieved by the enzyme chitin deacetylase [15]. In the bean rust fungus, *Uromyces viciae-fabae,* chitin deacetylase became detectable after appressorium formation [16], and a labeling pattern identical to that described here for the wheat stem rust fungus was obtained upon staining with WGA and the anti-chitosan antiserum [9, 17]. A similar change from chitin to chitosan was observed in the cell walls of *Colletotrichum* species upon penetration of host leaves [17, 18], and evidence was obtained for the involvement of a chitin deacetylase [19, 20, 21]. These results may indicate that chitin deacetylase could be an important pathogenicity factor for biotrophic plant pathogenic fungi.

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Antibiotic kanosamine is an inhibitor of chitin biosynthesis in fungi

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Summary
Antibiotic kanosamine is an inhibitor of cell-wall biosynthesis in fungi. The product of its intracellular metabolism, kanosamine-6-phosphate is an inhibitor of glucosamine-6-phosphate synthase (GlcN-6-P synthase), the enzyme catalysing formation of glucosamine-6-phosphate from fructose-6-phosphate and glutamine. Antibiotic is transported into the cells by the active transport pathway and phosphorylated. Inhibition of the first committed step in the pathway leading to formation of UDP-GlcNAc causes inhibition of chitin synthesis, deep cell morphological changes and fungicidal effect.

Introduction

Kanosamine, 3-amino-3-deoxy-D-glucose, naturally occurring carbohydrate is known as an inhibitor of bacterial cell wall synthesis. It was previously suggested that the product of intracellular metabolism may be an inhibitor of any enzyme catalysing one of the initial reactions in hexosamine biosynthetic pathway [1].

L-glutamine: D-fructose-6-phosphate amidotransferase (GlcN-6-P synthase) EC 2.6.1.16 is an enzyme catalysing the first committed step in a pathway leading to the eventual formation of UDP-GlcNAc. This sugar nucleotide provides N-acetyl-D-glucosamine for the biosynthesis of aminosugar-containing macromolecules: chitin and mannoproteins in fungi, peptidoglycan and teichoic acid in bacteria and glycoproteins in mammals.

Inhibition of chitin biosynthesis, an important constituent of the fungal cell wall, results in inhibition of septum formation, cell agglutination and fungicidal effect. So far, two enzymes of the chitin biosynthetic pathway have been proposed as possible targets for antifungals. Chitin synthase (EC 2.4.1.16, UDP-2-acetamido-2-deoxy-D-glucose: chitin acetylamino-deoxyglucosyltransferase) is inhibited by polioxins and nikkomycins, structural analogues of the enzyme substrate, UDP-GlcNAc [2, 3]. GlcN-6-P synthase is inhibited by anticapsin and some other glutamine analogues [4, 5]. Peptides containing a rationally designed glutamine analogue, FMDP, show antifungal activity *in vitro* and *in vivo* [6, 7]. Any other inhibitors of GlcN-6-P synthase demonstrating antifungal activity has not been known so far.

In the present paper we demonstrate results of our studies on mechanism of antifungal action of kanosamine, its transport, intracellular metabolism and influence of kanosamine-6-phosphate on GlcN-6-P synthase activity.

Materials and Methods

Yeast Strains and Culture Conditions

Candida albicans ATCC 10261, *Saccharomyces cerevisiae* ATCC 9763 and clinical isolates of *Candida humicola* and *Candida glabrata* were grown in Sabouraud medium and in YNB medium at 30 °C with shaking at 160 rpm.

Materials

Kanamycin, hexokinase, silica gel plates and microcristalline cellulose were from Sigma Chemicals Co., ion exchange resins were from Serva Feinbiochemica

Kanosamine isolation

Kanamycin was hydrolysed with 6M HCl at 60° C for at least 2 hours. The resulting hydrolysate was analysed by TLC on silica gel plates, using solvent system 1 (nBuOH: AcOH: H_20, 4:1:1, v /v /v). Compounds containing amino groups were detected using the ninhydrin reagent. After evaporation the solid residue was redissolved in solvent system 2 (nBuOH: AcOH: H_20, 4:2:1, v /v /v) and the resulting solution was chromatographed on a cellulose column developed with the same solvent system. Fractions which showed a single ninhydrin-positive spot (R_f= 0,28; TLC analysis on silica gel plates in solvent system 1) were collected and evaporated. The solid residue was dissolved in water and passed through the Dowex 50 WX 8 (H^+) column. Pure kanosamine hydrochloride was eluted with 40 mM HCl. Solution was lyophilised to give very hygroscopic white powder. NMR spectrum was consistent with the expected structure. Resulta at the TLC analysis and the melting point were in agreement with literature data [8].

Phosphorylation of kanosamine in vitro and isolation of Kanosamine-6-phosphate

Kanosamine was phosphorylated in 0.1 M TRIS HCl buffer pH 8.0 containing 60 mM $MgCl_2$, 0.3 mM EDTA and 5.7 mg of ATP for each 1 mg of aminosugar. After adjusting pH to 8.0 with 0.5 M NaOH hexokinase (EC 2.7.1.1.) from baker yeast was added (30 units/ ml). Solution was incubated at 30 °C overnight with stirring. The course of reaction was defined by TLC analysis (solvent system 1, solvent system 3 [i-PrOH: H_2O; 1:1; v /v]). Compound was detected by use of ninhydrin, Hanes (specific for sugar phosphates), Elson - Morgan (specific for aminosugars), aniline phthalate (specific for reducing sugars) reagents and alcalic solution of $KMnO_4$ (specific for polioles)[9]. NMR spectrum was consistent with the expected structure. The product was purified on columns of Dowex 1 X 8 (OH^-) and Dowex 50 WX 8 (H^+) and characterised by TLC analysis (solvent systems 1 and 3).

Transport studies

C. albicans cells, grown exponentially in YNB medium at 30 °C were harvested, washed with saline and resuspended in 50 mM potassium phosphate buffer containing 1% glucose to the final cell density corresponding to 1.0 mg dry wt cells/ ml. Suspension was preincubated at 30 °C for 10 min and kanosamine, dissolved in a minimal aliquot of potassium phosphate buffer was added to a final concentration of 5 mg/ ml (23 mM). At that moment and after 5, 10, 15, 30, 60, 90 minutes aliquots of 100 µl were taken and diluted 1: 100. The samples were immediately filtered through Whatman GF/ C glass fibre filters under suction and filtrates were collected for the determination of kanosamine concentration. The kanosamine concentration level was measured by the TNBS method. 0.625 ml aliquots of the solution containing 0.8 mg/ ml of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in 4% $Na_2BH_4O_7$*10 H_2O were added to 0.5 ml samples of filtrates. The resulting mixtures were incubated at 37 °C for 30 minutes and absorption at λ = 420 nm was measured.

Metabolism of kanosamine in cell free extract

Kanosamine was added to *Candida albicans* ATCC 10261 cell free extract, prepared as described previously [10], to the final concentration 5 mg/ ml. The mixture was incubated at 30 °C and course of reaction was followed by TLC analysis (solvent system 1).

Kanosamine intracellular metabolism

Candida albicans ATCC 10261 cells were grown on YNB liquid medium in the presence or absence of 1 mg/ ml kanosamine. After 18 hours of incubation at 30 °C cells were broken by the small scale glass beads procedure. Crude extracts were analysed by TLC (solvent system 1, aniline phthalate spray reagent). Activity of GlcN-6-P synthase was measured in crude extracts as described below.

Antifungal activity of kanosamine

MIC was determined by a microdilution method in liquid YNB medium. Cell wells containing serially diluted kanosamine and control wells, without the antifungal agent were inoculated with 10^5 cells of an overnight culture in YNB medium and incubated for 24 hours at 30 °C. MIC was defined as the lowest antifungal agent concentration preventing visible growth.

Light and fluorescence microscopy

Samples of the *Candida albicans* cells suspensions grown in the presence or absence of kanosamine were taken for inspection by visible light and fluorescence microscopy. For fluorescence microscopy cells were stained with Calcofluor White. Samples of cell suspension (100 µl) were combined with 100 µl aliquots of the Calcofluor White in 25 mM potassium phosphate buffer, pH 6.9 (300 µg/ ml), and the whole mixture was incubated for 5 min. Than cells were harvested, washed with the same buffer, resuspended and suspensions were examined by fluorescence microscopy using the Olympus Microscope. The excitation was at $\lambda = 365$ nm and the bypass was at $\lambda = 420$ nm.

GlcN-6-P synthase purification. Kinetic studies on enzyme inhibition.

Candida albicans GlcN-6-P synthase overproduced by *Saccharomyces cerevisiae* YRS C-65 was purified to homogenity as described previously[11]. Enzyme activity was determined by the modified Elson – Morgan procedure. Enzymatic reactions were carried out in 25 mM phosphate buffer pH 6.9, containing 1 mM EDTA and 1 mM DTE. Fru-6-P and glutamine concentrations were either fixed (7.5 mM and 10 mM respectively, for general activity and IC 50 determination) or variable (0.5–7.5 mM and 0.625–10 mM respectively, for K_i determinations). Inhibitory constants were determined from the secondary plots of k_{app} vs. inhibitor concentration, derived from Lineweaver – Burk plots.

Results and Discussion

Preparation of kanosamine and kanosamine-6-phosphate

Kanosamine, as a free aminosugar, was isolated many years ago from the culture broth of *Bacillus aminoglucosidicus* [8]. The method of chemical synthesis of this aminosugar is also known [12]. However we have decided to isolate kanosamine from the acid hydrolysate of kanamycin. For this purpose we have elaborated a purification procedure which is a modified version of the method known from literature [13].

The NMR spectrum of isolated compound was consistent with the expected structure. TLC analysis and melting point were in agreement with values reported for kanosamine hydrochloride (Rf =0,28; solvent system 1, m.p. 120 °C (decomp.)) [8]. The whole procedure afforded 110 mg of pure kanosamine hydrochloride from 300 mg of kanamycin.

The literature data suggested that kanosamine may be phosphorylated at 6-OH with yeast hexokinase [14]. We have elaborated the conditions of enzymatic phosphorylation allowing an almost qualitative conversion of kanosamine into kanosamine-6-phosphate and procedure for purification of the phosphorylated product (yield 90 %). An NMR spectrum of the isolated compound was consistent with expected structure. TLC analysis: R_f =0.1 (solvent system 1), R_f =0.55 (solvent system 3).

Antifungal activity of kanosamine

The previous literature data on chemical and biological properties of kanosamine confirmed the antibacterial activity of this compound. We have found that kanosamine inhibits growth of some fungi, but the minimal inhibitory concentration values determined by the microplate broth dilution method in YNB medium are very high (table 1). Similar values were found when the experiment was performed in Sabouraud medium.

Test organism	MIC (mg/ ml)
Candida albicans ATCC 10261	10
Candida humicola	10
Candida glabrata	>20
Saccharomyces cerevisiae	5

Table 1. Antifungal activity of kanosamine

The microscopic examination of cells treated with kanosamine revealed morphological changes characteristic for compounds inhibiting chitin biosynthesis. This alterations included cell agglutination, visible destruction of cell integrity and inhibition of septum formation (fig 1). Similar morphological changes were previously described as a result of action of FMDP-peptides, containing an inhibitor of GlcN-6-P synthase [15] and nikkomycins and polioxins - inhibitors of chitin synthase [16, 17]

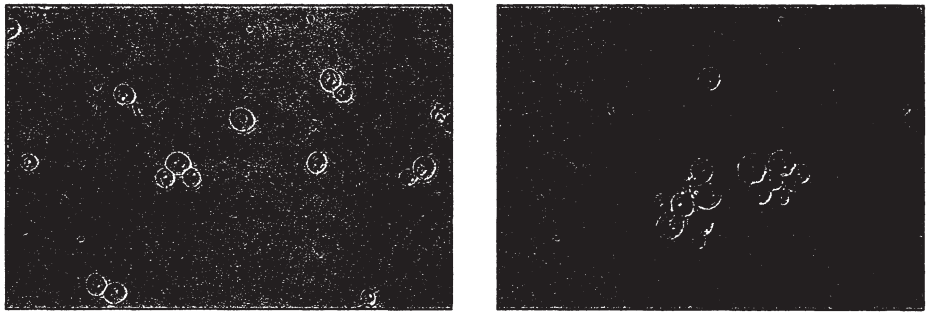


Fig 1. *C. albicans* cells. (a) untreated (b) treated with 10 mM kanosamine

Inhibition of chitin synthesis have been also confirmed by fluorescence microscopy. Staining of fungal cells with Calcofluor White reveals upon illumination the chitin-rich domains in fungal cell walls, especially in septum and bud scars regions [18], that can be easily seen in fig. 2a. Cells treated with kanosamine, shown in fig. 2b, contained less Calcofluor- White-stained regions

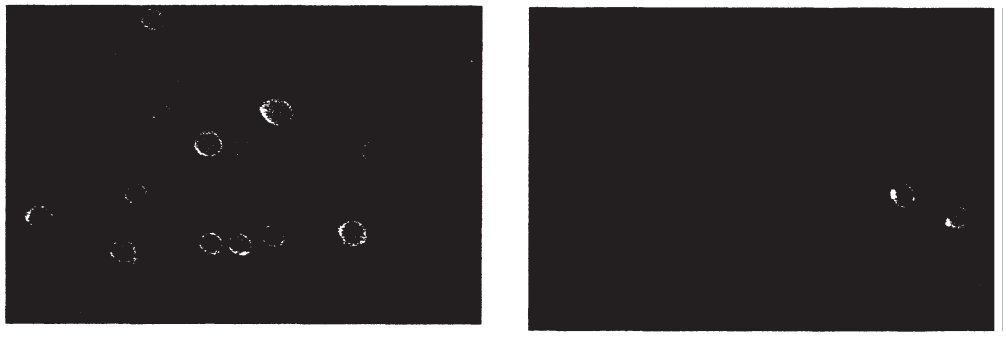


Fig. 2 Fluorescent microscopy. (a) control cells (b) cells treated with kanosamine

Uptake of kanosamine by C. albicans cells

Uptake of low-molecular weight compounds is usually measured radiochemically. Since the radioactively labelled kanosamine was not available we have elaborated a chemical method allowing the colorimetric determination of kanosamine concentration.

Kanosamine reacted with 2,4,6-trinitrobenzene sulphate under conditions described in Materials and Methods giving a yellow product. Since the absorbance of the resulting solution measured at λ= 420 nm showed a linear dependence on kanosamine concentration in the 5 - 20 ng/ ml range, this method could be successfully applied for the determination of kanosamine concentration. Fig. 3 shows that kanosamine was accumulated by *C. albicans* cells suspended in phosphate buffer containing 1% glucose. The initial uptake rate was 8.6 ± 0.3 nM/ min/ mg dry weight. The uptake was totally inhibited in the presence of 1 mM NaN_3 (not shown) what suggested the active transport against the concentration gradient. We have not identified the permease transporting kanosamine but the glucose transporter is the most likely candidate.

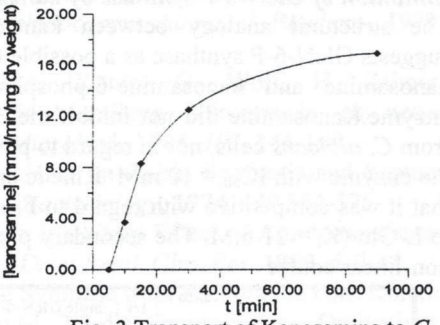
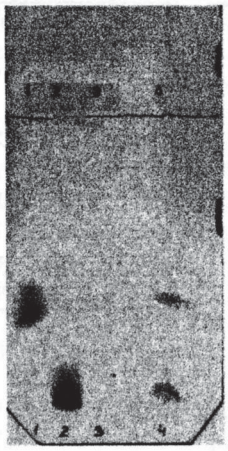


Fig. 3 Transport of Kanosamine to *C. albicans* cells

Intracellular metabolism of kanosamine

The TLC was used for the semi-quantitative analysis of kanosamine metabolism in *C. albicans* cells. Spots corresponding to kanosamine and kanosamine-6-phosphate could be detected in crude extract prepared from *C. albicans* cells treated with kanosamine, 5 mg/ ml (fig. 4). Kanosamine was phosphorylated by C. albicans cell free extract as shown in fig. 5, although the rate of this process is not very high.



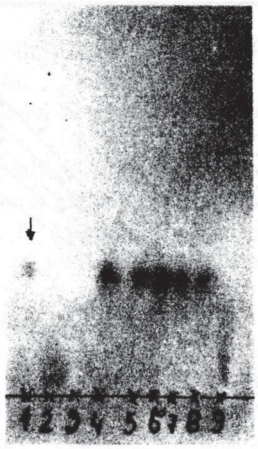


Fig. 4 (left) Intracellular metabolism of kanosamine. 1 - kanosamine, 2- kanosamine-6-phosphate, 3- control crude extract, 4 - crude extract from cells grown in the presence of kanosamine

Fig. 5 (right) Phosphorylation of kanosamine in cell-free extract. 1- kanosamine, 2 -kanosamine-6-P, 3- cell free extract from *C. albicans* ATCC 10261, 4- reaction mixture t=0, 5- t=1h, 6 t=3h, 7- t=5h, 8- t=9h, 9 - t=18h

Activity of GlcN-6-P synthase measured in control cell-free extract was substantially higher (0.38 u/min/ mg protein) than in the crude extract from cells grown for 15 hoursin the presence of kanosamine, 5 mg/ml (0.25 u/min/mg proteins).

Inhibition of GlcN-6-P synthase by kanosamine-6-phosphate

The structural analogy between kanosamine-6-phosphate and glucosamine-6-phosphate suggests GlcN-6-P synthase as a possible target for the former in *C. albicans* cells. Therefore, kanosamine and kanosamine-6-phosphate were tested as potential inhibitors of the enzyme.Kanosamine did not inhibit the enzyme activity, neither in crude extracts prepared from *C. albicans* cells, nor in regard to pure enzyme, while kanosamine-6-phosphate inhibited the enzyme with IC_{50} = 10 mM. Kinetic analysis of the inhibition ,shown in figure 6, revealed that it was competitive with regard to Fru-6-P (K_i = 5.9 mM) and uncompetitive with regard to L-Gln (K_i = 21 mM. The secondary plots (k_{app} vs. inhibitor concentration) shows unusual, non-linear course

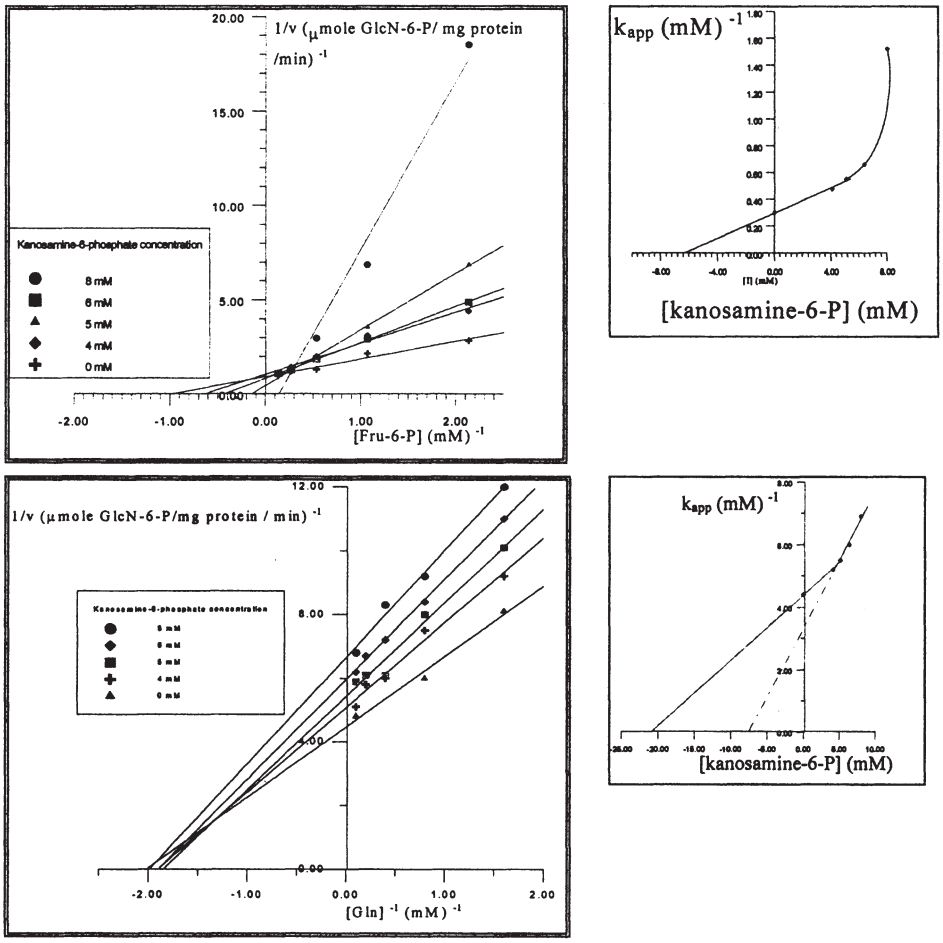


Fig. 6 Inhibitionof GcN-6-P synthase by kanosamine -6-phosphate

Results of our studies indicate that kanosamine is an inhibitor of chitin biosynthesis in fungi.

Acknowledgements
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PCR Amplification of Chitin Deacetylase Genes

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Summary

Chitosan is the product of chitin deacetylation. It is an important compound which is used in over 1000 technical applications. With the longterm goal of replacing current production processes for chitosan, which rely on chemical chitin deacetylation, we are searching for new chitin deacetylase enzymes in cultivated microorganisms or total microbial communities. To this end, we are developing a PCR based technique to amplify chitin deacetylase genes.

Introduction

The degradation of chitin via the chitosan pathway has been postulated [1], but until now chitin deacetylase enzymes have not been found in bacteria. They are, however, quite common in fungi, where their function is a reduction of the cell wall rigidity during growth, spore formation, or autolysis. Only two fungal chitin deacetylase genes have been sequenced so far, namely from *Mucor rouxii* [2] and *Saccharomyces cerevisiae*. To circumvent the probem of unculturability of > 99,9 % of marine bacteria, we are developing a PCR strategy which allows to directly amplify chitin deacetylase (*cda*) genes from DNA extracted from pure cultures or ultimately microbial communities, e.g. from marine sediment. This strategy is currently tested with *Aspergillus nidulans,* known to have an interesting chitin deacetylase enzyme, which is however not sequenced [3].

Primer design

Standard linear sequence alignment of the *Mucor rouxii* chitin deacetylase sequence revealed a low degree of homology to putative chitin deacetylase genes from Eubacteria, which does not allow the design of conserved degenerate primers. Alternatively, functional sequence domains, defined by the Protein Domain Database ProDom (**www.toulouse.inra.fr/prodom.htm**) were analysed irrespective of their location within the gene, thus relating sequence information to function and taking into account the mosaic pattern of enzyme evolution. In such a way, a highly conserved region, named *cda-conserved,* was identified within a putative cellulose binding domain of the *Mucor rouxii* chitin deacetylase

gene (Fig. 1), which is present in all homologous genes known so far (*cda* homologues in *Bacillus stearothermophilus* and *Saccharomyces cerevisia, nodB* from Rhizobium) and can also be found in some xylanases and acetyl xylan esterases. This region was selected as target site for degenerate primers.

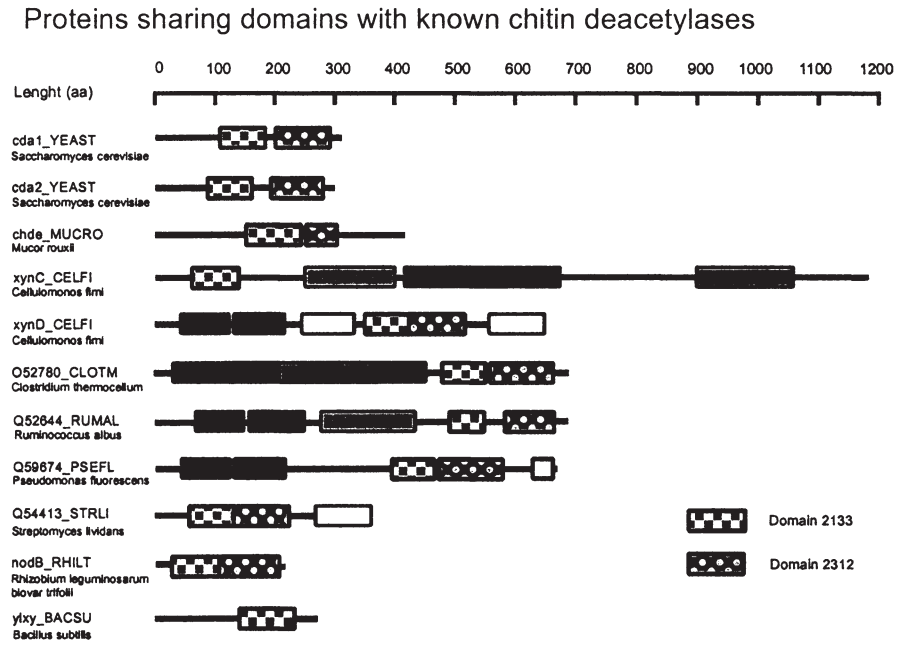

Fig. 1 Localization of functional domains within chitin deacetylases and related enzymes

PCR strategy

Amplification of sequences flanking the the conserved sequence domain *cda-conserved* requires a special PCR strategy. The principle of bubble PCR (vectorette PCR) is shown in Fig. 2. It is based on restriction digested genomic DNA, to which a specially designed bubble anchor containing non complementary regions is ligated. PCR is performed using one specific, degenerate primer targeting the conserved region of *cda*, and a second primer targeting the complement of the non complementary region of the bubble. The beauty of bubble PCR lies in the specific amplification of the first single stranded copy of the desired PCR product. The practical application of this approach requires extensive optimization of PCR conditions to exclude amplification between bubbles and unspecific primer binding.

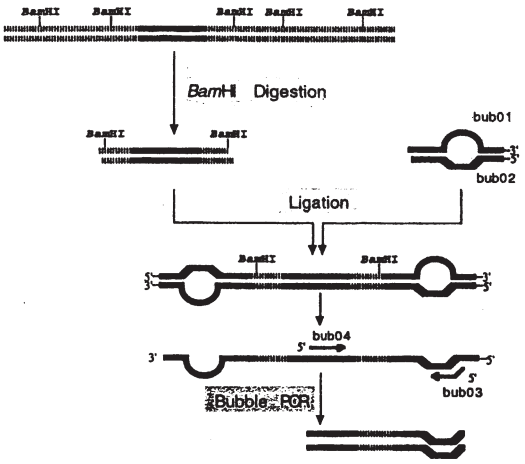

Fig. 2 PCR amplification strategy for determining the nucleotide sequence of unknown flanking regions adjacent to the known conserved sequence domain in *cda* genes. Genomic DNA is schematically shown with solid black lines, representing known conserved regions of *cda*, and short bar regions representing unknown sequence flanking *cda*. Use of „bubble anchor" (bub01 (phosphorylated) and bub02 (non-phosphorylated) as well as primers complementary to the known sequence within *cda* (bub04), and the same strand as one of the bubbles (bub03) allows for PCR amplification of unique flanking regions. Modified after [4].

Amplification of putative *cda* genes from microorganisms

Aspergillus nidulans is known to deacetylate chitin using a chitin deacetylase enzyme with unique characteristics: It is thermostable between 30 and 100°C with an optimum at 50°C, and functions in a pH range between 4.0 and 7.5 with an optimum at pH 7.0. Most importantly for a technical process, *Aspergillus* chitin deacetylase is activated by low concentrations of the endproduct acetate [3]. A clone bank of expressed sequence tags of *Aspergillus nidulans* [5] exhibited a region of strong homology to *cda* enzymes (Fig. 3). The bubble PCR technique was therefore applied to genomic *A. nidulans* DNA to amplify the putative *cda*-gene (Fig. 4).

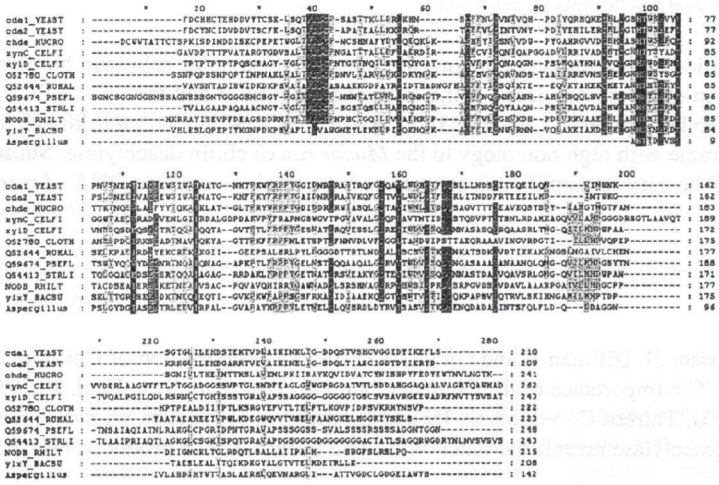

Fig. 3 Manual alignment of chitin deacetylases, related enzymes and a cDNA clone of *Aspergillus nidulans.* Visualised by GeneDoc [6].

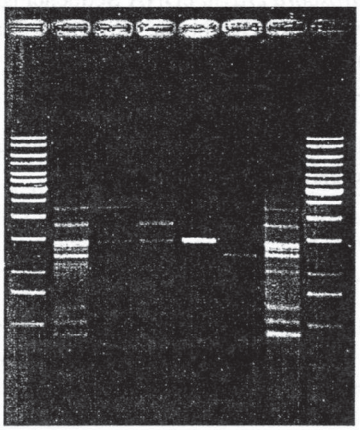

Fig. 4 PCR amplification of putative *cda* fragments from *A. nidulans.* A pure culture of *A.n.* was grown over night on potato-dextrose-broth. DNA was extracted using the modified protocol of Chow and Käfer [7], digested with BamHI and ligated to bubble-anchors.
Lane 1 and 8, standard size marker; lane 2 and 7, Bubble PCR; Lane 3 to 6, Bands were excised from lane 2 of the gel and reamplified using the same primers as for bubble PCR. Controls with single forward primer and single bubble primer were negative and are not shown here.

Outlook

The amplification of regions flanking the conserved putative cellulose binding domain commonly found in chitin deacetylases, using a degenerate primer and the bubble PCR strategy, will in the next step be applied to a *Bacillus stearothermophilus* strain which is known to possess an open reading frame with high homology to the *Mucor rouxii* chitin deacetylase. Subsequently, this technique will be used to amplify *cda* sequences from total community DNA of marine habitats.

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Amplification of antifungal effect of GlcN-6-P synthase and chitin synthase inhibitors

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Summary
The possibility of enhancement of antifungal efficacy of glucosamine-6-phosphate (GlcN-6-P) synthase and chitin synthase inhibitors by synergism has been studied. Synergistic effects have been observed for combinations of GlcN-6-P synthase inhibitors, N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) or 2-amino-2-deoxy-D-glucitol-6-P (ADGP), with membrane permeabilising polyene macrolides or with the chitin synthase inhibitor nikkomycin X. On the other hand, combination of nikkomycin and an antifungal oligopeptide Lys-Nva-FMDP has demonstrated an antagonistic effect. Possible mechanisms of the observed phenomena have been discussed.

Introduction
Chitin is an important constituent of cell wall of most human pathogenic fungi. Since this polysaccharide is absent from mammalian cells, the enzymes involved in chitin biosynthesis are considered promising targets in antifungal chemotherapy. Two classes of oligopeptide agents: FMDP-peptides, containing inhibitors of glucosamine-6-phosphate (GlcN-6-P) synthase and nikkomycins - inhibitors of chitin synthase show relatively high antifugal *in vitro* and *in vivo* activity [1,2,3]. Unfortunately, these antifungals are rapidly degraded by serum peptidases [4,5] and, on the other hand, fungi can easily develop a phenotypic resistance to them, due to the fact that peptide permeases are not essential for fungal growth [6,7]. Several structural modifications of nikkomycins have not improved their antifungal chemotherapeutic properties [8,9]. On the other hand, N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) and 2-amino-2-deoxy-D-glucitol-6-P (ADGP) have been found to be strong inhibitors of fungal GlcN-6-P synthase [10,11] but poor antifungal agents, due to their slow uptake into the cells [12, A. Janiak, unpublished data].

One of the possibilities of enhancement of effectiveness of antimicrobial compounds is combination therapy. The phenomenon of synergism often enables a reduction of effective doses of chemotherapeutic agents. The only example of this approach in antifungal chemotherapy that has been applied in clinical practice is combination of 5-fluorocytosine and amphotericin B [13]. A combined action of chitin synthesis inhibitors with other antifungal agents has not been extensively studied so far. The synergistic *in vitro* interactions of nikkomycin with azole antifungals against *Candida albicans* have been demonstrated [14] and confirmed for other medically important fungi, also *in vivo* [15,16]. Synergism has been also shown for combination of nikkomycin and papulacandin B, the inhibitor of $\beta(1\rightarrow3)$glucan synthase [17].

In the present communication we report the results of our recent studies on antifungal *in vitro* activity of combinations of GlcN-6-P synthase and chitin synthase inhibitors.

Materials and Methods

Micro-organisms.C. albicans ATCC 10261, *C. albicans* ATCC 26278, *S. cerevisiae* ATCC 9763 and clinical isolates of *C. glabrata, C. kruzei, C. parapsilosis, C. famata, C. humicola* were stored on Sabouraud Agar slants.

Antifungal agents. FMDP, Lys-Nva-FMDP, ADGP and MF-AME were synthesised at the Technical University of Gdańsk. Nikkomycin X was a gift from Professor Hans Zahner, University of Tubingen, Germany. Amphotericin B was from Sigma.

Susceptibility testing. Antifungal activity was determined by the serial dilution method in microtiter plates. The tests were performed in YNB minimal medium containing 2% glucose. Inoculum size was 10^4 cells ml^{-1}. Plates were incubated at 37 °C for 24 h and the results were read visually. MIC was definied as the lowest drug concentration preventing visible growth.

Determination of the combined effect. Serial twofold dilutions of agents tested were prepared in microtiter plates so that each well contained a unique combination of two antifungals. The concentrations were chosen to encompass the MIC for the strains to be tested. Plates were inoculated with 10^3 cells ml^{-1} from overnight cultures. After 24 h incubation at 37 °C, plates were examined and fractional inhibitory concentration (FIC) indexes were calculated as follows: FIC index = (MIC of agent 1 in combination)/(MIC of agent 1 alone) + (MIC of agent 2 in combination)/(MIC of agent 2 alone). The FIC index for each row of dilutions was calculated and the greatest deviation from 1.0 was used to characterise the interaction. agents.

Other methods. GlcN-6-P synthase activity was assayed *in situ* [18].

Results and Discussion

The antifungal agents studied in this work differ markedly as far as their chemical structures and modes of action are concerned. N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) and 2-amino-2-deoxy-D-glucitol-6-P (ADGP) are inhibitors of GlcN-6-P synthase, an enzyme catylysing the first committed step in the chitin biosynthetic pathway. Data shown in Table 1 indicate that their antifungal activity is very low, with MIC values in the mg/ml range, despite the fact that the inhibitory potency of these compounds in regard to their intracellular target is much higher. They inhibit 50 % of activity of the pure GlcN-6-P synthase from *C. albicans* at 1.1 μg ml^{-1} and 57.5 μg ml^{-1}, respectively. The reason for such a difference is a slow uptake of both compounds into fungal cells. FMDP is transported by amino acid permeases, preferably by the glutamine/asparagine transporter but its affinity to these proteins is low (S. Milewski, unpublished data). On the other hand, ADGP can cross the cell membrane only by free diffusion but its ionic structure is a reason for the very low rate of this process. The antifungal activity of both compounds can be therefore improved only if a more efficient way of their penetration into the cells is found. In the case of FMDP this goal has been achieved by incorporation of this compound into an oligopeptide structure. Oligopeptides containing FMDP are quickly transported by rather non-specific peptide permeases, cleaved intracellularly by peptidases and the released FMDP can efficiently inhibit GlcN-6-P synthase [19]. Lys-Nva-FMDP used in this study is one of the best optimised antifungal agents of this type, what is reflected by low MIC values shown in table 1. Any method of amplification of antifungal activity of ADGP has not been proposed so far. Nikkomycin, the inhibitor of chitin synthase demonstrated generally good antifungal activity,

although the MIC values are in most cases slightly higher than those found for Lys-Nva-FMDP. Amphotericin B (AMB) is a well-known antifungal agents used routinely in clinics for the treatment of disseminated mycoses. This antibiotic binds to the ergosterol and form chanels in the fungal cell membrane [20]. MF-AME is a structural derivative of AMB developed at the Technical University of Gdańsk, demonstrating much better chemotherapeutic index than the original antibiotic [21].

Table 1. Antifungal *in vitro* activity of antifungal agents, expressed as MIC ($\mu g\ ml^{-1}$) values

	AMB	MF-AME	NIKK	FMDP	ADGP	LNF
C. albicans 26278	0.78	1.56	1.56	625	5000	0.39
C. albicans 10261	0.78	1.56	0.78	625	5000	0.78
S. cerevisiae 9763	0.39	1.56	12.5	625	2500	6.25
C. glabrata	0.39	0.78	12.5	1250	2500	1.56
C. kruzei	0.78	0.78	400	2500	5000	800
C. parapsilosis	0.39	1.56	1.56	2500	2500	6.25
C. famata	0.39	0.78	3.12	>5000	5000	0.78
C. humicola	0.78	1.56	12.5	2500	5000	1.56

We used the checkerboard serial dilution microplate method to study the antifungal effect of combinations of GlcN-6-P synthase inhibitors with nikkomycin and FMDP or ADGP with AMB or MF-AME. The results of these experiments have been presented as isobolograms. Two representative graphs are shown in Fig. 1 a-b. The values of fractional inhibitory concentrations (FIC) calculated for each experiment as described in the M&M section, have been summarised in table 2. FIC indexes are generally acceptable quantitative

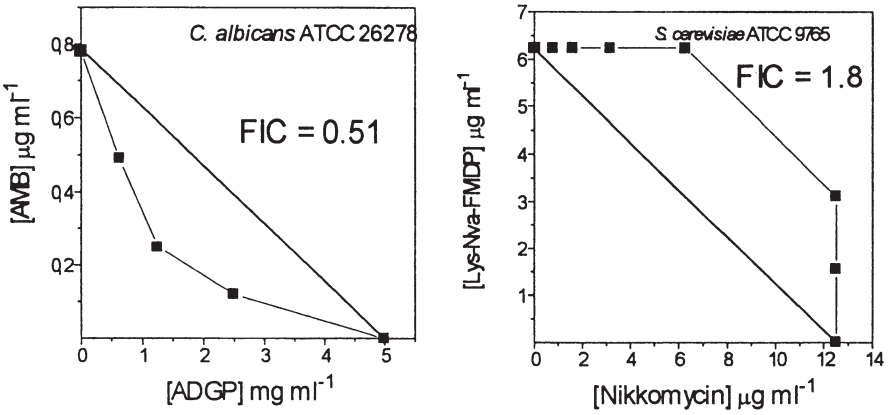

Figure 1. Isobolograms representing effects of combined action of (a) ADGP with AMB and (b) Lys-Nva-FMDP with nikkomycin

measures of extent of interaction between two agents used in combination. The effect of interaction is considered synergistic when FIC < 1, additive for FIC = 1 and antagonistic when FIC > 1. Data shown in table 2 indicate that synergism was observed for combinations of ADGP with AMB or MF-AME against all the fungal strains tested, with FIC indexes ranging between 0.21 and 0.84. The much weaker synergistic effect, with numerous examples of an additive effect or even antagonism was noted for the combination of FMDP and AMB or MF-AME. Relative strong synergism was observed in most cases for combinations of ADGP or FMDP with nikkomycin. On the other hand, the combined action of nikkomycin and Lys-Nva-FMDP resulted in strong antagonism.

Table 2. FIC indexes found for the combinations of antifungal agents

	AMB - ADGP	**MF-AME - ADGP**	**AMB - FMDP**	**MF-AME - FMDP**	**FMDP - NIKK**	**ADGP - NIKK**	**LNF - NIKK**
C. albicans 26278	0.51	0.21	1.0	0.92	0.99	1.0	1.6
C. albicans 10261	0.42	0.32	0.95	0.86	0.80	0.32	1.5
S. cerevisiae 9763	0.34	0.39	0.92	0.92	0.49	0.1	1.8
C. glabrata	0.78	0.84	0.83	0.94	0.76	0.49	2.1
C. kruzei	0.69	0.58	1.0	0.78	0.64	0.76	1.8
C. parapsilosis	0.56	0.47	0.95	1.2	0.79	0.51	2.4
C. famata	0.73	0.58	-	-	-	1.0	2.0
C. humicola	0.72	0.64	0.81	0.90	0.88	0.77	1.2

There are three generally accepted basic mechanisms of synergistic activity of chemotherapeutic agents: (a) increase by one agent of the permeability of the cell wall and cell membrane by the second agent; (b) inhibition of enzymes able to degrade the second agent and (c) double blocking of by the two components of successive steps in the metabolic sequence [22]. There is little doubt that the strong synergism observed for the combination of ADGP with AMB or MF-AME can be best explained by the mechanism (a). ADGP alone very poorly penetrates the cell membrane and even the small perturbations in the membrane structure caused by the action of AMB/MF-AME should facilitate the penetration. This assumption was confirmed by the experiment in which the GlcN-6-P synthase activity was measured under *in situ* conditions. *C. albicans* cells were treated for 60 min with antifungal agents, processed as described in M&M and GlcN-6-P activity was determined. The results of this experiment are shown in Fig. 2a. ADGP alone at 5 mg ml^{-1} inhibited the enzyme activity by 78%, while at the 10-fold lower concentration the inhibition was negligible. The enzyme inhibitory effect of ADGP, 0.5 mg ml^{-1}, was enhanced in the presence of AMB, 0.1 μg ml^{-1}.

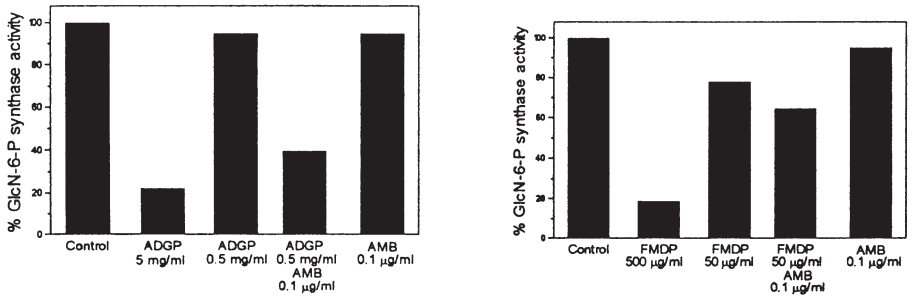

Figure 2. Inhibition of *C. albicans* GlcN-6-P synthase activity *in situ* by (a) ADGP and (b) FMDP alone and in combinations with AMB

On the other hand, the enhancement of GlcN-6-P synthase inhibitory potency of FMDP by AMB was much lower (Fig. 2b) thus confirming the tendency noted from comparison of FIC indexes. The weaker amplification of the FMDP effect by AMB/MF-AME provides another evidence confirming that membrane alterations do not always facilitate the transport of poorly permeable substances. It was previously shown that inhibition of ergosterol biosynthesis by azole antifungals has no effect on the rate of nikkomycin transport into the *C. albicans* cells [14]. It seems reasonable to assume that membrane alterations may result in better uptake of compounds penetrating by free diffusion. If the agents are taken up by the active transport systems, their effective accumulation inside the cells is always possible, even if the rate of this process is low because of the low affinity to the respective transporter.

GlcN-6-P synthase and chitin synthase catalyse respectively the first and the last step in the chitin biosynthesis pathway. The synergistic antifungal effect observed for the combinations of ADGP or FMDP with nikkomycin may be therefore explained by the mechanism (c). These compounds use the different ways of entry into the cells (free diffusion, the amino acid transport system and the oligopeptide transport system, respectively). Therefore, they do not compete with each other for transporter binding sites. Such a competition may be a serious obstacle, preventing the possibility of synergism. The antagonistic effect of combination of Lys-Nva-FMDP and nikkomycin may serve as a good example. Both compounds are oligopeptides and are transported into fungal cells by the di-tripeptide permease and the oligopeptide permease [5,6].

We are fully aware of the fact that the possibility of practical application of the drug combinations tested by us is rather unlikely. The antifungal activity of FMDP and ADGP is very low, even after amplification caused by AMB/MF-AME or nikkomycin. Nevertheless, we have shown that such amplification is possible.

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β-*N*-Acetylhexosaminidases: two enzyme families, two mechanisms

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Summary

Recently, three novel β-*N*-acetylglucosaminidases have been cloned and shown to differ from family 20 β-*N*-acetylhexosaminidases in substrate specificity and amino acid sequence. However, they display similarity with β-glucosidases of family 3 in a defined region, assumed to constitute the active site. These novel enzymes may therefore employ a double displacement mechanism involving a covalent glycosyl-enzyme intermediate. Such a mechanism holds for most retaining glycosidases, but apparently not for family 20 β-*N*-acetylhexosaminidases, where strong evidence has been provided for a mechanism involving substrate assistance from the 2-acetamido group and which proceeds through an oxazoline intermediate. In contrast, a covalent intermediate on a novel β-*N*-acetylglucosaminidase from *Vibrio furnisii* (EXO2_VIBFU) was detected by mass spectrometry using the newly synthesized slow substrate 2-acetamido-2-deoxy-5-fluoro-α-L-idosyl fluoride, providing firm evidence for a double displacement mechanism. Furthermore, peptide mapping allowed the identification of the catalytic nucleophile. Analysis using the basic local alignment search tool (BLAST) of current sequence databases revealed fifteen open reading frames that are highly similar and characterized by a putative *N*-acetyl binding motif. These enzymes form a separate branch of unique β-*N*-acetylglucosaminidases within family 3, distinguishable from family 20 β-*N*-acetylhexosaminidases by substrate specificity, amino acid sequence, and catalytic mechanism.

Introduction

Enzymes that catalyze the hydrolysis of terminal amino sugar linkages are ubiquitous and they have been isolated and cloned from many different organisms. The term β-*N*-acetylhexosaminidase (E.C. 3.2.1.52) is commonly used for enzymes hydrolyzing β-linked *N*-acetylhexosamine residues from the nonreducing end of oligosaccharides or glycoconjugates. These enzymes previously have been referred to also as β-*N*-acetylglucosaminidases and chitobiases. Until recently, however, all cloned β-*N*-acetylhexosaminidases were found to be sequentially related and relaxed in their substrate specificity for D-gluco/D-galacto configuration. A number of biological functions have been identified for exo-acting β-*N*-acetylhexosaminidases (for refs. see [10]), which include lysosomal degradation of glycolipids and glycoproteins, as well as the maintenance of bacterial and fungal cell walls during growth. There has been a renewed interest in β-*N*-acetylhexosaminidases, largely owing to their role in lysosomal storage diseases in humans and as potential targets for new antibiotics [11-13].

β-*N*-Acetyl-hexosaminidases have been grouped exclusively into family 20 of glycosyl hydrolases, which so far comprise over 66 families that have been classified on the basis of amino acid sequence similarity [14]. This classification system has been useful in allowing structure and mechanism to be predicted for all members of a family. The structure of a bacterial chitobiase has been determined and used for modeling human β-*N*-acetylhexosaminidase, in order to provide insights into the structural basis of human hexosaminidase-related diseases [5]. Crystal structure data as well as kinetic studies [6-7], have provided evidence for a mechanism involving substrate assistance from the 2-acetamido group involving an oxazoline or oxazolinium ion intermediate (Figure 1a).

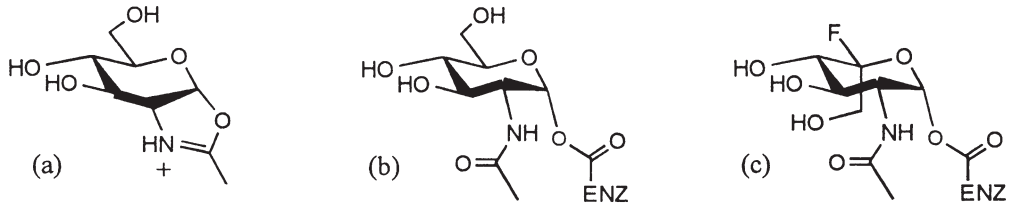

Figure 1. Presumed reaction intermediates for family 20 β-*N*-acetylhexosaminidases (a) and family 3 β-*N*-acetylglucosaminidases (b). Neighboring group participation leads to an oxazoline/ oxazolinium ion intermediate (a) whereas nucleophilic attack leads to a covalent glycosyl-enzyme intermediate (b). The nucleophile can be identified using a slow substrate to trap an intermediate (c).

Recently, however, three novel β-*N*-acetylglucosaminidases have been cloned and found to show no sequence similarities with members of family 20 [1-3]. Instead, they display similarity with glucosidases of family 3. These novel enzymes may therefore employ a double displacement mechanism such as that found for most retaining glycosidases. In these enzymes a pair of carboxyl groups in the active site are involved in bond cleavage. The first carboxyl group functions as a nucleophile, attacking the sugar anomeric centre and displacing the aglycone leaving group to result in the formation of a covalent glycosyl-enzyme intermediate (Figure 1b). The other carboxylic acid functions as an acid/base catalyst. In the first step it functions as an acid, accelerating the departure of the aglycon by protonation of the glycosidic oxygen. In the next step, it functions as a general base catalyst, to promote the attack of water at the anomeric centre of the glycosyl-enzyme intermediate, yielding the product and free enzyme. Fluorinated substrate analogues have been used to trap covalent intermediates of several glycosidases [15]. Both reaction steps, glycosylation as well as deglycosylation, are slowed down due to the inductive effect of the fluorine substituent which destabilizes the electron deficient oxocarbenium ion-like transition state. However, introduction of a very good leaving group accelerates the glycosylation step and the covalent intermediate accumulates. Using suitable inhibitors or slow substrates the intermediate can be identified by mass spectrometry. Furthermore, peptide mapping may allow the identification of the active site nucleophile.

Here we show the trapping of a covalent glycosyl-enzyme intermediate on β-*N*-acetyl-glucosaminidase from *Vibrio furnisii* (EXO2_VIBFU), using a newly synthesized slow substrate, and the identification of the catalytic nucleophile of a family 3 β-*N*-acetyl-glucosaminidase. These results show indubitably that this β-*N*-acetylglucosaminidase operates

by a mechanism different from that of the classical β-*N*-acetylhexosaminidases of family 20. Sequence alignments reveal that β-*N*-acetylglucosaminidases form a branch within family 3, characterized by a putative *N*-acetyl binding motif [8]. Whereas family 20 enzymes have a relaxed specificity for D-gluco/D-galacto-configuration, family 3 enzymes are more likely to be real β-*N*-acetylglucosaminidases (E.C. 3.2.1.30).

Materials and Methods

The gene coding for *V. furnissii* β-*N*-acetylglucosaminidase (EXO2_VIBFU) was a generous gift from Dr. Saul Roseman, Dept. of Biology and the McCollum-Pratt Institute, The John Hopkins University, Baltimore, MD). Cloning and expression of EXO2_VIBFU has been described previously [1,8]. The synthesis of the novel mechanism based inhibitor, 2-acetamido-2-deoxy-5-fluoro-α-L-idopyranosyl fluoride (5FIdNAc) has been described [8]. Mass spectra were recorded on a PE-Sciex API 300 triple-quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an Ionspray ion source. Sequences were retrieved from the GenBank sequence databank. Sequence similarity searches were performed using BLASTP 2.0.9 at http://www.ncbi.nlm.nih.gov/. The phylogenetic tree was constructed by the neighbour-joining method using the computer program 'TreeView' by Roderic D. M. Page, Institute of Biomedical and Life Sciences, University of Glasgow, Scotland, UK.

Results and Discussion

Sequence similarity and phylogenetic relationship: β-*N*-Acetylhexosaminidases have been classified into family 20 of the glycosyl hydrolases on the basis of amino acid sequence similarities and hydrophobic cluster analysis of their catalytic domains [14]. This family represents only β-*N*-acetylhexosaminidases (E.C. 3.2.1.52), with an oligosaccharide lacto-*N*-biosylhydrolase (E.C.3.2.1.140) as the only exception. However, three novel β-*N*-acetylglucosaminidases from *Vibrio furnisii* (EXO2_VIBFU; [1]), *Alteromonas sp.* (HEXA_ALTSO; [2]) and *Streptomyces thermoviolaceus* (NAGA_ STRTH, [3]) do not bear any significant similarity to the above group. Instead they display significant similarity with glycosyl hydrolases of family 3. This family comprises primarily β-glucosidases, but also contains β-xylosidases, cellodextrinases, and exo-β-glucanases. BLAST analysis [9] of non redundant databases revealed fifteen sequences, largely from genomic data, that are closely related to the three novel β-*N*-acetylglucosaminidases. They appear to form a separate branch within family 3 of the glycosyl hydrolases (Fig. 2). Two open reading frames from the genome of *Escherichia coli* (YCFO_ECOLI) and *Haemophilus influenza* (YCFO_HAEIN) show 57 and 50 % identity, respectively, towards EXO2_VIBFU. These genes code for small proteins with a molecular mass of approximately 37 kDa. Other open reading frames extracted from bacterial genomic databases have some 30 % identity; these being from *Rickettsia prowazekii*, (HYPO_RICKPR), *Zymomonas mobilis* (HYPO_ZYMMO), *Borrelia burgdorferi* (HYP2_BORBU), plus two mycobacterial genes from *Mycobacterium tuberculosis* (HYPO_MYCTU) and *Mycobacterium leprae* (HYPO_MYCLE), gene products of which have the size of 37-40 kDa. However, genes from *Borrelia burgdorferi* (HYP1_BORBU), *Bacillus subtilis* (YBBD_BACSU), *Synechocystic sp.* (HYPO_SYNSP), *Streptomyces coelicolor* (HYPO_STRCO), and *Thermotoga maritima* (HYPO_THERMA) are about twice this size, much like HEXA_ALTSO and NAGA_STRTH. Interestingly, most of the enzymes of family 3 that are not β-*N*-acetylglucosaminidases are large proteins. The recently solved structure of the *Hordeum vulgare* β-glucan exohydrolase (EXO1_HORVU; [23]) revealed that this enzyme

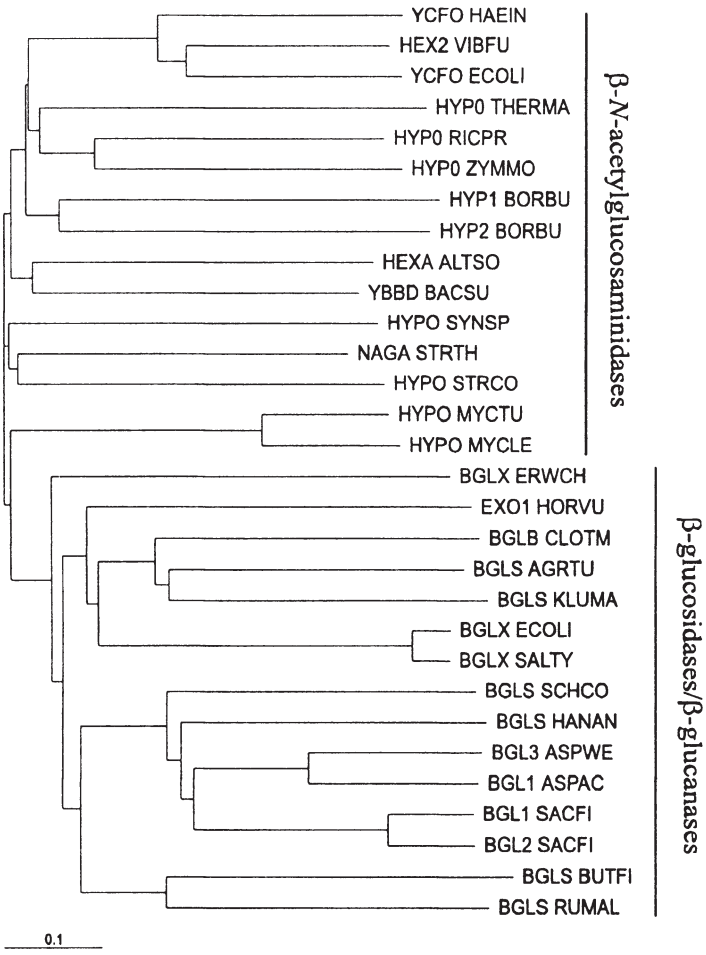

Figure 2. Phylogenetic tree of 30 selected glycosyl hydrolases of family 3. This family mainly consists of β-glucosidases and *exo*-β-glucanases. However a separate branch consisting of fifteen β-*N*-acetylglucosaminidases has been identified. Amino acid sequences were retrieved from the database of carbohydrate active enzymes [14] or from genomic data. The accession numbers of the Swiss-Prot database for the sequences are as follows: YCFO_HAEIN, P44955; EXO2_ VIBFU, P96157; YCFO, P75949; HYPO_THERMA, [16]; HYPO_ RICPR, [17]; HYPO_ ZYMMO, [18]; HYP1_ BORBU and HYP2_ BORBU [19]; HEXA_ALTSO, P48823; YBBD_ BACSU, P40406; HYPO_SYNSP, [20]; NAGA_ STRTH, O82840; HYPO_ STRCO, [21]; HYPO_MYCTU and HYPO_ MYCLE, [22]; BGLX_ ERWCH, Q46684; EXO1_HORVU, [23]; BGLB_CLOTM, P14002; BGLS_ AGRTU, P27034; BGLS_ KLUMA, P07337; BGLX_ECOLI, P33363; BGLX_SALTY, Q56078; BGLS_ SCHCO, P29091; BGLS_ HANAN, P06835; BGL3_ ASPWE, P29090; BGL1_ASPAC, P48825; BGL1_ SACFI, P2506; BGL2_SACFI, P22507; BGLS_ BUTFI, P16084; BGLS_ RUMAL, P15885.

has a two domain structure in which the active site is a cleft formed by the interface of the two domains. However, the *N*-terminal $(\beta/\alpha)_8$-barrel domain represents the only domain in the smaller bacterial β-*N*-acetylglucosaminidases, and should therefore be sufficient to contain the whole active site.

A highly conserved stretch of sequence has been identified that shows 50 to 100 % identity for the putative β-*N*-acetylglucosaminidases. This region, with the pattern (K-H-[FI]-P-G-[HL]-G-x(4)-D-[ST]-H), has been proposed to be involved in binding of the 2-acetamido group of the substrate and suggested as a unique identifier for family 3 β-*N*-acetyl-glucosaminidases [8]. As more and more sequence data become available this pattern should be useful for assignment of genes coding for novel β-*N*-acetylglucosaminidases of family 3. However, structural data is required to confirm the involvement of this stretch of amino acids in *N*-acetyl group binding.

Differences in specificity for D-gluco/D-galacto-configuration: Some cloned family 20 β-*N*-acetylhexosaminidases have also been characterized in terms of their substrate specificity. In addition to hydrolyzing 4-nitrophenyl β-*N*-acetylglucosaminide (pNP-GlcNAc), all of these family 20 enzymes efficiently hydrolyze 4-nitrophenyl β-*N*-acetylgalactosaminide (pNP-GalNAc) [for refs see 10,13]. Activity ratios (pNP-GlcNAc/ pNP-GalNAc), most commonly encountered for the hydrolysis of these substrates range from 1:1 to 5:1 (Table 1). In contrast, the three cloned family 3 enzymes have been shown to display a great preference for pNP-GlcNAc (Table 1). This is in accordance with the finding that the family 3 *Aspergillus wentii* β-glucosidase (BGL3_ASPWE; cf. Fig. 2), shows great preference for pNP-Glc, with a relative hydrolysis rate ($k_{cat,\ pNP\text{-}GlcNAc}$ /$k_{cat,\ pNP\text{-}GalNAc}$) of 10^6 [24]. Moreover, two ORFs from genomes of *E. coli* and *B. subtilis* were assigned on the basis of molecular weight and other biochemical characteristics to β-*N*-acetylglucosaminidases in earlier studies [25-26]. These enzymes were found to have no activity on pNP-GalNAc, but it should be kept in mind that these data have been obtained with enzymes purified from cell homogenate, thus the apparent

Table 1. Activity ratio for β-GlcNAc'ase/β-GalNAc'ase for selected enzymes assigned to family 3 or family 20 of glycosyl hydrolases [14].

Family	Organism	Protein, Swiss-Prot code	Reference	Relative activity
20	*Homo sapiens*	HexB, P07686	[28]	8.1
20	*Sus scrofa*	HexB, Q29548	[27]	1.8
20	*Entamoeba histolytica*	Hex1, P49009	[29]	3.7
20	*Bombyx mori*	HexC, P49010	[30]	1.0
20	*Candida albicans*	Hex1, P43077	[31]	1.3
20	*Trichoderma harzianum*	Hex1, P78738	[32]	1.3
20	*Dictyostelium discoideum*	HexA, P13723	[33]	5.6
20	*Serratia marcescens*	Chb, Q54468	[34]	3.3
20	*Vibrio furnissii*	Exo1, P96155	[35]	2.1
3	*Vibrio furnissii*	Exo2, P96157	[1]	36
3	*Alteromonas sp.*	HexA, P48823	[2]	55
3	*Streptomyces thermoviolaceus*	NAGA, O82840	[3]	38.5
3	*Escherichia coli*	YCFO, P75949	[25]	∞
3	*Bacillus subtilis*	YCFO, P40406	[26]	∞

absence of activity should be considered with caution. Activity ratios (pNP-GlcNAc/ pNP-GalNAc) of >35:1 for substrate hydrolysis appear to be typical for family 3 β-*N*-acetyl-glucosaminidases. Although available data are limited and in no case has a detailed kinetic analysis been carried out, there appears to be significant difference in substrate specificity between the two enzyme families.

Interestingly, a unique role for these novel β-*N*-acetylglucosaminidases in the cell wall metabolism of gram negative (*E. coli*) as well as gram positive (*B. subtilis*) bacteria has been proposed [25-26]. These cytosolic enzymes might function in the recycling of cell wall material. Support for this possible role has been obtained by the discovery that GlcNAc-MurNAc is a better substrate than pNP-GlcNAc, and therefore also functions as a potent inhibitor of pNP-GlcNAc hydrolysis [25-26]. β-*N*-Acetylglucosaminidases of family 3 are expected to be quite common for organisms possessing a chitin or peptidoglycan framework i.e. bacteria, fungi, arthropods, and they might play a role in the maintenance of the cell wall or *exo*-skeleton.

Kinetic evidence for two mechanisms: Kinetic evidence has been provided for an anchimeric assistance mechanism involving an oxazoline intermediate for a family 20 enzyme from jack bean. The stable analoque β-N-acetylglucosamine-thiazoline has been synthesized and shown to be a potent competitive inhibitor (K_i = 280 nM) for the family 20 enzyme [6]. However, a K_i value of greater than 30 mM has been estimated for EXO2_VIBFU (D.J. Vocadlo & S.G. Withers, unpublished results), consistent with the notion that a different mechanism is followed by this enzyme.

Trapping of a covalent glycosyl-enzyme intermediate: The newly synthesized 2-acetamido-2-deoxy-5-fluoro-α-L-idosyl fluoride (5FIdNAcF) is a slow substrate for enzymes from both families (D.J. Vocadlo & S.G. Withers, unpublished results), but only in the case of the family 3 enzyme EXO2_ VIBFU was a glycosyl-intermediate identified by mass spectrometry (Fig. 2). The mass difference observed by ESMS between the native (37253 Da) and inhibited enzyme (37475 Da) is 222 Da, a value that is consistent with the addition of a single 5FIdNAc label (222 Da). The observation of a covalent intermediate provides strong evidence for a double displacement mechanism involving an enzymic nucleophile.

Identification of the Labeled Active Site Peptide by ESMS: Peptic hydrolysis of 5FIdNAc-labeled EXO2_VIBFU resulted in a mixture of peptides which was separated by reverse-phase HPLC and detected using ESMS. The peptide bearing the label was identified in a second run by using the tandem mass spectrometer in the neutral loss mode. In this technique the ions are subjected to limited fragmentation by collisions with an inert gas (N_2) in a collision cell. Since the ester linkage between the 5FIdNAc inhibitor and the enzyme is the most labile linkage present, it readily undergoes homolytic cleavage. This cleavage results in the loss of a neutral sugar residue, leaving the peptide moiety with its original charge, but with a known decrease in mass (222 Da). The peptide bearing the label is therefore identified by scanning for a mass decrease of 222 Da between the first and the third quadrupoles of the triple quadrupole instrument. Isolation of the peptide by HPLC and subsequent tandem MS sequencing permits the identification of Asp-241, within the sequence IVFS**D**DLSM, as the catalytic nucleophile. This aspartate residue is conserved among all known members of family 3 and is located at the end of β-sheet 7 of the $(\beta/\alpha)_8$-barrel, within the consensus pattern [LIVM](2)-[KR]-x-[EQK]-x(4)-G-[LIVMFT]-[LIVT]-[LIVMF]-[ST]-**D**-x(2)-[SGADNI] that defines family 3 enzymes.

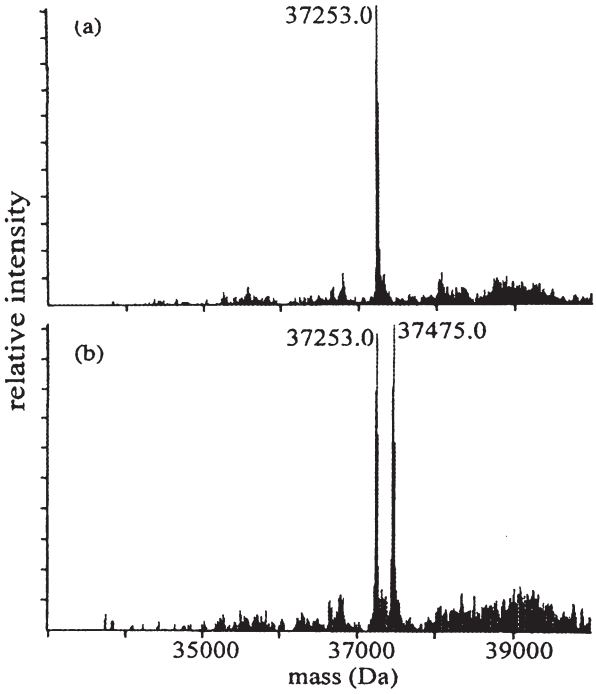

Figure 3. Electrospray ionization mass spectrometry (ESMS) of (a) unlabeled EXO2_VIBFU (37253 Da) and (b) labeled and unlabeled enzyme species in approximately equimolar amounts after incubating with 5FIdNAcF (10.08 mM). The mass shift of 222 corresponds to covalently bound substrate analog.

In summary, our findings and previous data unequivocally show that family 20 β-*N*-acetylhexosaminidases and family 3 β-*N*-acetylglucosaminidases are two distinct enzyme families. They differ in their amino acid sequence, in their specificity towards D-GalNAc and their behaviour with inhibitors. It is concluded that they operate by two different mechanisms.

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Purification and characterisation of chitin deacetylase from *Absidia orchidis*

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Summary

The method of purification of chitin deacetylase from mycelial extracts of the fungus *Absidia orchidis* and further characterisation of this enzyme are presented. The crude enzyme extract was applied to a gel chromatography Phenyl Sepharose ®HP column and then loaded onto a Q Sepharose HP column. Specific activity of purified enzyme was 12.31 U/mg and final purification degree was 147. The apparent molecular mass of the enzyme estimated by SDS-Page was around 75 kDa. When O – hydroxyethylated chitin (glycol chitin) was used as a substrate, the optimum pH for enzyme activity was 5,5 and the optimum temperature was 50°C.

Introduction

Chitin is a troublesome waste, but the deacetylated form of chitin – chitosan has a broad variety of reported applications. At present, chitosan is produced by the thermochemical deacetylation of chitin. An alternative procedure based on the enzymatic deacetylation of chitin could be an improved method for producing chitosan with well-controlled properties. The aim of the researches was purification of chitin deacetylase from *Absidia orchidis* and further characterisation of this enzyme.

Materials and Methods

1. *Strain, substrates and chemicals*

- *Absidia orchidis*, obtained from AR-Poznań (Poland),
- Glycol chitosan, purchased from Sigma,
- Glycol chitin, synthesized from glycol chitosan by the method of Araki and Ito [1],
- All chromatography media (HiPrep Sephacryl S-200, Q Sepharose HP), purchased from Pharmacia Biotech,
- Molecular weight markers, purchased from BIO-RAD.

All other chemicals were of the highest purity commercially available.

2. Culture medium

Absidia orchidis was grown in a complex medium containing: 4.0g glucose, 1.0g peptone, 0.1g yeast extract, 0.5g $(NH_4)_2SO_4$, 0.1g K_2HPO_4, 0.1 NaCl, 0.5g $MgSO_4 \cdot 7H_2O$, 0.01g $CaCl_2$, 100 cm^3 H_2O.

3. Culture conditions

Bath culture was performed at bioreactor BIOFLO III (Braun, Germany). The culture medium (4.5 dm^3) was added to the bioreactor, sterilised (121°C, 20 min.) and inoculated with 500 cm^3 of 2-day old shaken culture.

The fungi were grown with following cultivation conditions:

- Temperature 26 °C,
- pH 5.5,
- Aeration 7 dm^3/min,
- Stirring 200 rpm,
- Time of cultivation 48 h.

4. Crude enzyme extract preparation

Crude enzyme preparation consisted of following steps:

- Biomass separation (centrifugation at 6 000 rpm, 20 min),
- Biomass homogenisation with Tris-HCl buffer, pH 7,3,
- Storage of homogenised biomass 3 days at –18°C and one day at 4°C
- Centrifugation and filtration of supernatant,
- Addition of ammonium sulphate to supernatant (80% saturation),
- Centrifugation and dissolution of precipitate in a buffer solution.

5. Enzyme purification

A two-stage chromatography was used to purify the enzyme solution:

- Gel chromatography on Sephacryl S-100 High Resolution column (equilibration and elution with 0.1M NaCl in 60 mM Na2HPO4 – buffer A),
- Ion exchange chromatography on HiLoad Q Sepharose 26/10 column (equilibration with 60 mM Na2HPO4 and elution with a linear gradient of 0.1M NaCl in 60 mM Na2HPO4).

6. Molecular weight determination

Molecular weigh of chitin deacetylase was determinated using polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were stained with Coomassie blue R-250 and molecular mass was measured by comparison to following molecular mass markers: myosin (200 kDa), β galactosidase (116,25 kDa), phosphorylase b (97,40 kDa), serum albumin (66,20 kDa), ovalalbumin (45,00 kDa), carbonic anhydrase (31,00 kDa), trypsin inhibitor (14,4 kDa) and aprotinin (6,5 kDa).

7. Enzyme essays

Chitin deacetylase activity was measured using a water soluble chitin derivative (glycol chitin) as the substrate. Standard enzyme essays were done in buffer A with glycol chitin and the reaction was initiated by the addition of enzyme solution to reaction mixture. Incubation time was 60 min at 50°C. Acetic acid released by the action of chitin deacetylase on glycol chitin was determined using gas chromatography.

8. Effect on pH

Glycol chitin and enzyme extract was added to 0.1M NaCl in 60 mM Na_2HPO_4 buffer. The pH was adjusted using 1 M HCl solution. The reaction and enzyme activity assay were carried out as described for the standard enzyme assay.

9. Effect on temperature

The influence of temperature on the chitin deacetylase activity was investigated in the range 20 – 70°C according to the standard assays.

The temperature stability was determined by preincubating the enzyme extracts for 0-24 h. After 1h, 2h, 3h, 4h, 5h and 24h preincubated enzyme extract was added to the reaction mixture containing 0.1M NaCl in 60 mM Na_2HPO_4 buffer and glycol chitin

Results

1. Enzyme purification

Figure 1 shows the chromatography diagram for gel chromatography on Sephacryl S-100 High Resolution 26/60 column.

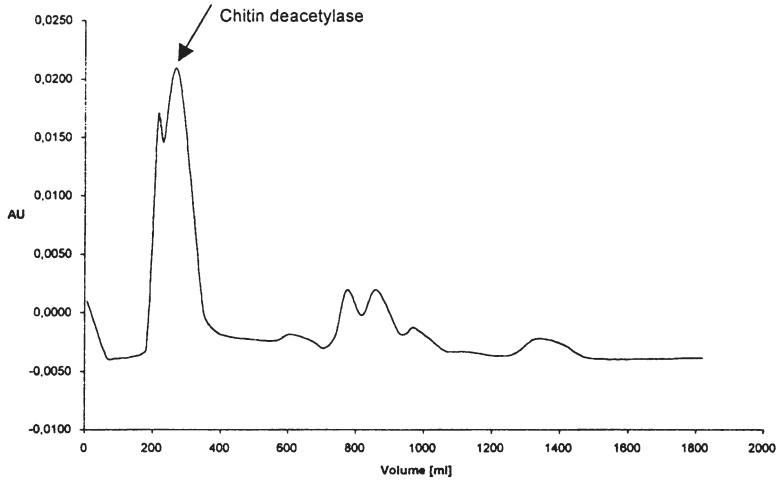

Fig.1. Purification of chitin deacetylase on Sephacryl S-100 High Resolution 26/60 column.

A sample (200-350 ml) of partially purified chitin deacetylase was collected. The specific activity of the sample was 5.97 U/mg. The sample was used in the second step of purification.

Figure 2 shows the chromatography diagram for ion exchange chromatography on HiLoad Q Sepharose 26/10 column.

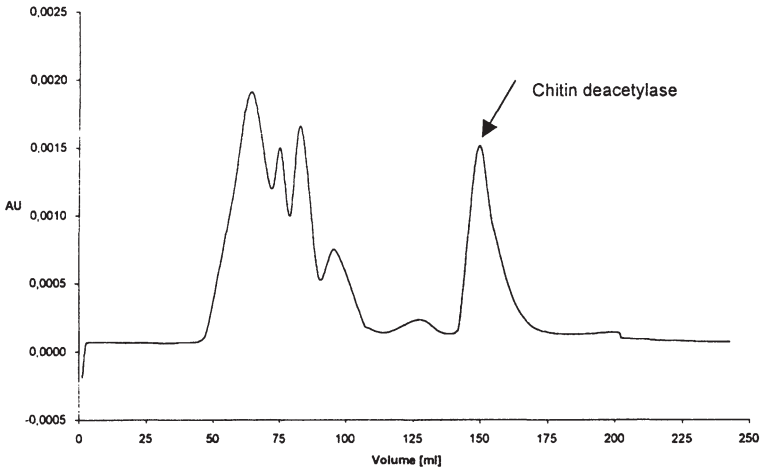

Fig.2. Purification of chitin deacetylase on HiLoad Q Sepharose 26/10 column.

The fraction (145-175 ml) containing chitin deacetylase activity were collected and used as purified enzyme extract. The specific activity of the enzyme was 12.31 U/mg. The total purification degree was 147.

2. Molecular weight determination

The molecular mass of chitin deacetylase was determined to be around 75 kDa.

3. Effect on pH

Figure 4 shows pH effect on enzyme activity.

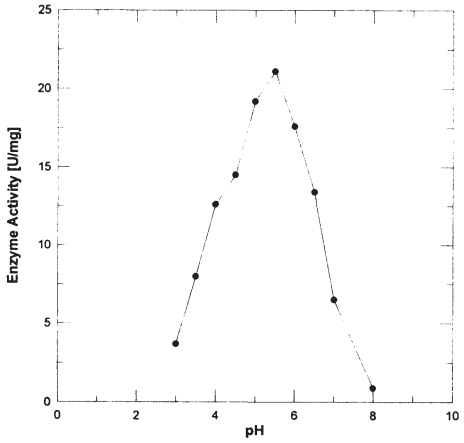

Fig. 3. Effect on pH

When O–hydroxyethylated chitin (glycol chitin) was used as a substrate, the optimum pH for enzyme activity was 5,5.

4. Effect on temperature

Figure 4 shows temperature effect on enzyme activity.

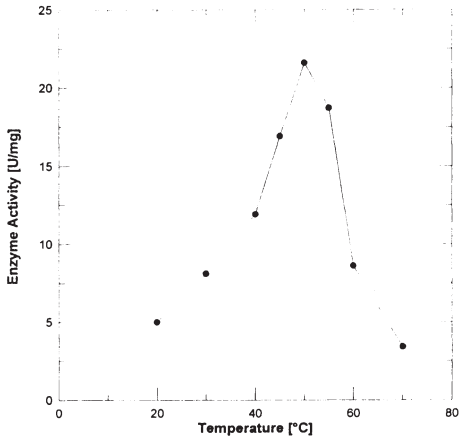

Fig. 4. Effect on temperature

When O–hydroxyethylated chitin (glycol chitin) was used as a substrate, the optimum temperature was 50°C.

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Effect of aluminum ion on hydrolysis reaction of carboxymethyl- and dihydroxypropyl-chitin with lysozyme

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Summary

Hydrolysis reaction of two kinds of water-soluble chitin derivatives, that is, carboxymethyl (CM)- and dihydroxypropyl (DHP)-chitin with lysozyme was carried out. The optimum pHs of lysozyme were 5.0 and 3.8 for CM- and DHP-chitin, respectively. There were large differences in the reactivities between both substrates, that is, in the case of DHP-chitin the reactivity was 7.9-10% of that for CM-chitin, as was found from the relationship between the initial rate of hydrolysis reaction and the initial substrate concentration. From the results of Lineweaver-Burk plot at various temperatures, the enzymatic reaction of both substrates at any temperatures obeyed Michaelis-Menten mechanism, and K_m and V_{max} were estimated. Moreover, the apparent activation energies were estimated and the influence of aluminum ion as the inhibitor on the enzymatic reaction was investigated for both substrates.

Introduction

Chitin, widely found in nature, particularly in marine invertebrates, insects, fungi and algae, is a biopolymer composed of β-1,4 linked 2-acetamide-2-deoxy-*D*-glucose (*N*-acetylglucosamine, (GlcNAc)) residues. For many years, effective utilization of chitin has been studied by a lot of workers. The utilization were generally classified in two manners, that is, a case in which chitin is used as a large molecular chain of polysaccharide, and a case where it is utilized as hydrolyzates such as monosaccharide and oligosaccharides. As an example in the former, Gonzalez-Davila *et al.* [1] reported that Cd(Ⅱ) and Pb(Ⅱ) in seawater were adsorbed to chitin. And chitin was used as a support of immobilization of α-chymotrypsin by Heras and Acosta [2]. In the latter case, for example, Roby *et al.* [3] studied chitin oligosaccharides as elicitors of chitinase activity in melon plants. Akiyama *et al.* [4] synthesized partially *N*-deacetylated chitin oligomers and found that they have strong elicitors for phytoalexin induction in *Pisum sativum* and *Phaseolus vulgaris*.

In terms of the utility of chitin, we have devoted our attention to an enzymatic preparation of higher molecular-weight chitin oligosaccharides. The final goal of this research is establishment of a preparation system of physiologically active chitooligosaccharides by a method of enzymatic reactions, such as hydrolysis and transglycosilation with chitinase or lysozyme. So, as a basic research, the kinetics of hydrolysis reaction of the water-soluble chitin derivatives, carboxymethyl (CM)-chitin and dihydroxypropyl (DHP)-chitin by lysozyme was investigated.

Materials and Methods

Substrate

CM-chitin was synthesized according to the reaction **Scheme 1** by the modified method of Tokura *et al.* [5]. 5 g of chitin powder from crab shell was suspended in 40 m*l* of 50 wt% sodium hydroxide solution including 0.2 wt% sodium dodecyl sulfate at room temperature and stood for 3 hours *in vacuo*. And the slurry was kept in a freezer at −30℃ overnight. The alkali-chitin prepared above was suspended in 100 m*l* of isopropyl alcohol at room temperature, and to it monochloroacetic acid was added gradually with stirring until the reaction mixture was neutralized. The product was filtrated using a glass filter and washed with 2 *l* of ethanol. The residue was dried in an oven controlled at 50℃ overnight. The reactant was dissolved in 400 m*l* of water and the insoluble matter was removed by centrifugation. The supernatant was extracted with 2 *l* of acetone to precipitate the crude CM-chitin sodium salt and it was collected by filtration. The product was dried at 50℃ overnight and dissolved in 150 m*l* of distilled water. Then the solution was dialyzed carefully using a cellulose dialyzer tubing (M.W. cutoff; 8000, diameter; 32 mm) against distilled water. And the insoluble matter was removed by centrifugation, then the supernatant was concentrated and freeze-dried to obtain the CM-chitin sodium salt. The CM-chitin sodium salt was dissolved in 150 m*l* of distilled water, whose pH was controlled at about 1.0, and it was stirred for 1 hour. This solution was added into 1 *l* of acetone and the precipitate was collected by filtration. The product was washed by a mixture of distilled water and acetone (volume ratio, 1:20) and followed by washing with acetone carefully. The final product was obtained by drying *in vacuo*.

DHP-chitin was synthesized according to the reaction **Scheme 2**. 5 g of the chitin powder was suspended in 50 m*l* of 42 wt% NaOH aqueous solution and stood for 4 hours *in vacuo*. The suspension was filtrated and an alkali-chitin cake was obtained. The alkali-chitin cake and 60 g of crushed ice were mixed in a beaker and stirred until obtaining a highly viscous homogeneous solution. This solution was diluted by 175 g of cooled 20 wt% NaOH aqueous solution and stirred until obtaining a homogeneous solution. And 24 m*l* of glycerol α-monochlorohydrin was gradually added into the solution obtained by the stirring, then the reaction mixture was stood at room temperature overnight. 10 m*l* of acetic anhydride was added into the product in an ice bath and then 50 m*l* of acetic acid was slowly added. The product was dialyzed carefully using the cellulose dialyzer tubing against running water for 3 days and then against distilled water for 10-14 days. The insoluble matter was removed by centrifugation and the supernatant was concentrated. The final product was obtained by freeze-drying.

Enzymatic reaction

The degree of reaction was followed by the modified Schales method [6,7]. That is, the degree of reaction was obtained by analysis of reducing sugar of GlcNAc formed in the hydrolysis reaction. The analysis of the reducing sugar was conducted using potassium ferricyanide, that is, the enzyme activity was evaluated by measuring the change in absorbance at 418.5 nm due to the reduction of Fe(Ⅲ) to Fe(Ⅱ) with aldehyde group in the reducing sugar.

pH dependencies, effects of temperature, and influences of aluminum ion were investigated for the two kinds of substrate using lysozyme as the enzyme.

Results and Discussion

pH dependencies

Figure 1 shows pH dependencies of the hydrolysis reaction for both substrates. The relative activity in the figure was defined as the largest activity obtained in the optimum pH of each substrate to be 100%. The optimum pHs of lysozyme are 5.0 for CM-chitin and 3.8 for DHP-chitin. When glycol chitin was used as the substrate, the optimum pH was 4.2 [7]. The optimum pH of lysozyme was slightly influenced by kind of substrate. It can be concluded that this variation of the optimum pH was influenced by pK_a of substituent in water-soluble chitin derivatives (pK_a= 4.74 and 16 for acetic acid and alcohol, respectively). In short, even if the initial pH of substrate solution was constant, the pH could be changed by liberation of proton from the substituent of the water-soluble chitin derivatives. So in the reaction process, it is assumed that the practical pH of solution is slightly lowered. **Figure 2** shows the relationships between the initial rate of hydrolysis reaction of lysozyme and the initial concentration of substrate at 310 K. Solid lines in the figure were the calculated ones by Michaelis-Menten equation as described later. It is clear that there is a remarkable difference in reactivity between CM-chitin and DHP-chitin, that is, in the case of DHP-chitin as the substrate the reactivity was decreased to 7.9-10% of that for the case of CM-chitin. This difference may be due to the correlation between the structure factor of the substrate and the active site of lysozyme. That is, it may be assumed that lysozyme molecule would be hard to approach the substrate by steric hindrance of substituents. And it can be reasoned that substrate specificity of lysozyme is not relatively strict. Consequently, lysozyme can catalyze the hydrolysis reaction of not only bacterium cell wall (combination of *N*-acetylmuramic acid with *N*-acetylglucosamine) as a true substrate but also chitin (consisting of only *N*-acetylglucosamine) or water-soluble chitin derivatives. In regard to water-soluble chitin derivatives, any substrates were hydrolyzed, but they cannot be substrate analogue. After all, catalytic site Glu^{35} and Asp^{52} of lysozyme recognize not whole structure but β-1,4-glycosidic linkages. **Figures 3 and 4** show Lineweaver-Burk plot of the hydrolysis reaction at various temperatures based on Eq. (1) for CM-chitin and DHP-chitin, respectively.

$$1/v_0 = (K_m/V_{max})/[S]_0 + 1/V_{max} \quad (1)$$

From the figures it is found that the reaction kinetics of these enzymatic reactions obeyed Michaelis-Menten mechanism. The maximum velocities, V_{max}, and the Michaelis constants, K_m, were estimated from the Lineweaver-Burk plots at various temperatures and listed in **Table 1**. For both substrates, K_m was not influenced by temperature so much. V_{max} was increased with temperature. It was 3.9-6.8% of that for the case of DHP-chitin, which indicates that the efficiency of the hydrolysis reaction of DHP-chitin with lysozyme was much lower than CM-chitin. From the Arrhenius plots of hydrolysis reaction of CM- and DHP-chitins with lysozyme, apparent activation energies were estimated to be 60.6 and 64.8 kJ/mol for CM- and DHP-chitins, respectively. **Figures 5 and 6** show Lineweaver-Burk plots showing the effects of aluminum ion on the hydrolysis reaction of both substrates by lysozyme. In the case of CM-chitin, two linear lines when aluminum ion exists or not in the media intersect in a point on $1/[S]_0$ axis in Fig. 5. This indicates that aluminum ion acts as a competitive inhibitor. While in the case of DHP-chitin, from the intersection of two lines in Fig. 6, inhibition mechanism of aluminum ion is a mixed-type inhibition. The inhibition constants of aluminum ion for both substrates were estimated according to each inhibition mechanism to be as follows, $K_{i(CM)} = 3.37 \times 10^{-2}$ mol/dm^3 and $K_{i(DHP)} = 1.51 \times 10^{-2}$ mol/dm^3.

Table 1 Values of K_m and V_{max} in hydrolysis reaction of CM-chitin and DHP-chitin with lysozyme at various temperatures

Temperature [K]	CM-chitin		DHP-chitin	
	K_m [kg/m^3]	V_{max} [kg/(m^3 · s)]	K_m [kg/m^3]	V_{max} [kg/(m^3 · s)]
303	3.25	4.66×10^{-5}	1.27	2.04×10^{-6}
310	2.46	4.85×10^{-5}	1.35	3.25×10^{-6}
313	3.26	6.99×10^{-5}	1.39	4.75×10^{-6}
316	2.57	9.90×10^{-5}	1.28	5.64×10^{-6}
319	1.45	1.57×10^{-4}	1.18	6.07×10^{-6}

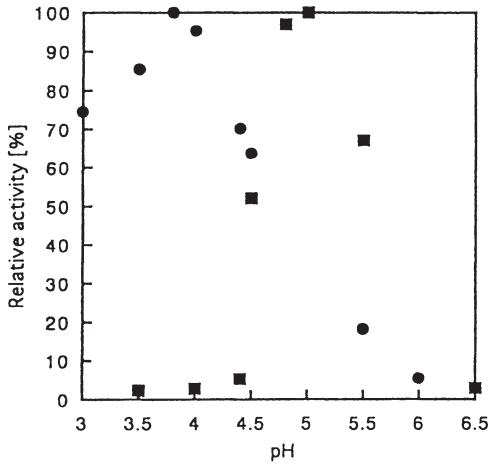

FIG. 1. pH Dependences of lysozyme activity for CM-chitin (■) and DHP-chitin (●) as substrate. Reaction temperature; 310K, Reaction time; 3600s (CM-chitin), 7200s (DHP-chitin).

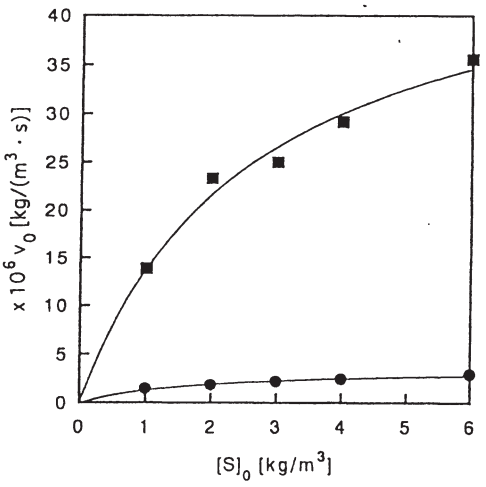

FIG. 2. Relationship between initial rate of hydrolysis Reaction and initial concentration of substrate. Substrate; (■)CM-chitin and (●)DHP-chitin, Reaction temperature; 310K.

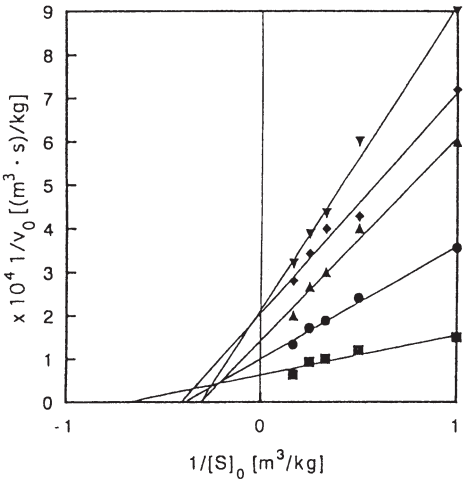

FIG. 3. Lineweaver-Burk plot of hydrolysis reaction of CM-chitin with lysozyme at various temperatures, (▼)303K, (◆)310K, (▲)313K, (●)316K, and (■)319K.

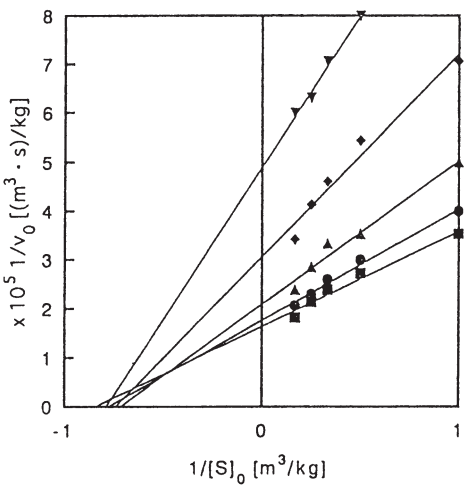

FIG. 4. Lineweaver-Burk plot of hydrolysis reaction of DHP-chitin with lysozyme at various temperatures, (▼)303K, (◆)310K, (▲)313K, (●)316K, and (■)319K.

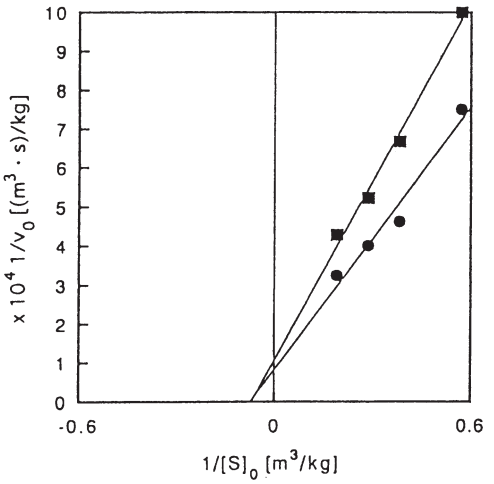

FIG. 5. Influence of aluminum ion on hydrolysis reaction of CM-chitin with lysozyme. Reaction temperature; 310K, pH 5.0. Symbols: ■, Al^{3+} added; ●, Al^{3+} none.

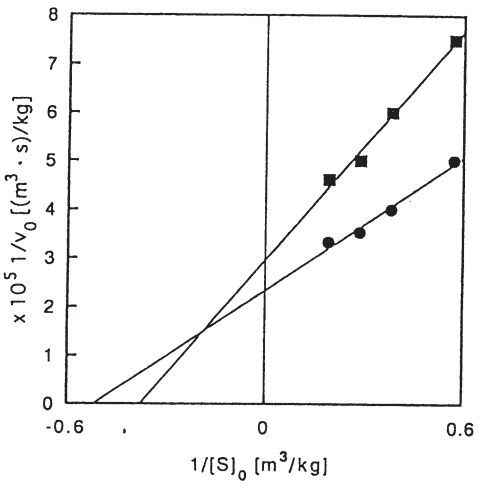

FIG. 6. Influence of aluminum ion on hydrolysis reaction of DHP-chitin with lysozyme. Reaction temperature; 310K. pH 3.8. Symbols: ■, Al^{3+} added; ●, Al^{3+} none.

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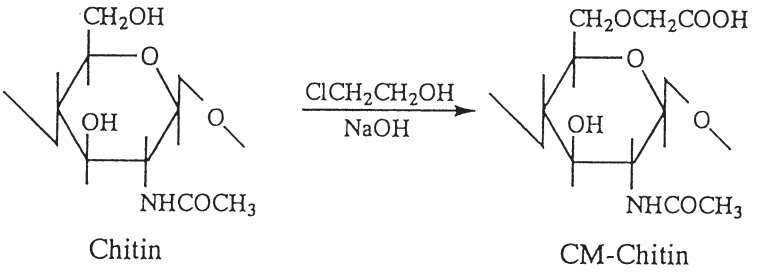
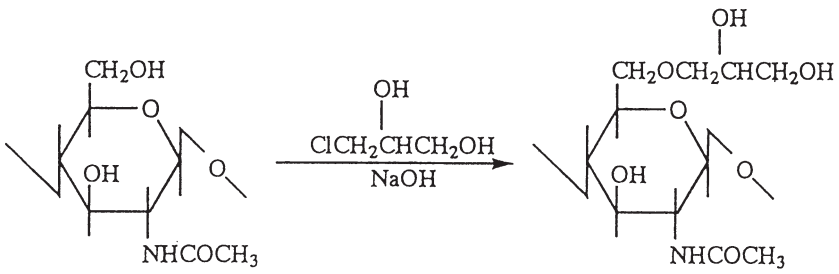


Chitin　　CM-Chitin

Scheme 1　Reaction pathway to prepare CM-chitin from chitin



Chitin　　DHP-Chitin

Scheme 2　Reaction pathway to prepare DHP-chitin from chitin

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Structure and Function Relationship of Human *N*-Acetyl-*D*-Glucosamine 2-Epimerase (Renin Binding Protein). -Identification of Active Site Residue-

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Summary

Renin binding protein (RnBP) is a proteinous renin inhibitor firstly isolated from the porcine kidney. Recently the protein was identified as the enzyme of *N*-acetyl-*D*-glucosamine (GlcNAc) 2-epimerase [EC 5. 3. 1. 8]. The recombinant human GlcNAc 2-epimerase was specifically inhibited by $ZnCl_2$ and SH-reagents such as *N*-ethylmaleimide, 5, 5'-dithiobis-2-nitrobenzoate, or iodoacetic acid, indicating that the most probable reactive site is cysteine residue. To identify the active site residue(s), we have constructed ten cysteine residue mutants (C41S, C66S, C104S, C125S, C210S, C239S, C302S, C380S, C386S, and C390S) for human GlcNAc 2-epimerase and expressed in *Escherichia coli* cells. The relative specific activity of the C41S, C66S, C125S, C210S, C239S, C302S, C386S, and C390S are the same to that of wild type enzyme. The specific activity of the C104S mutant is about 20% to that of wild-type enzyme. The C380S mutant did not show any GlcNAc 2-epimerase activity. These results indicate that Cys380 is essential for the enzymatic activity of human GlcNAc 2-epimerase.

Introduction

Renin [EC 3.4.23.15] is a key enzyme of renin-angiotensin-aldosteron cascade. The enzyme is mainly synthesized in the kidney and released into the circulation by several stimuli and controls blood pressure. Renin binding protein (RnBP) is a endogenous renin inhibitor firstly isolated from porcine kidney as a complex of renin so called high molecular weight renin [1]. The purified porcine kidney RnBP exists as a dimer [2] and dissociates monomers in the presence of sulfhydryl-oxidizing and -alkylating reagents [3]. The primary structures of several animal RnBPs are deduced from the nucleotide sequence of cDNAs and showed that RnBPs have conserved leucine-zipper motif which has essential role for the formation of RnBP homodimer and RnBP-renin heterodimer [4-8]. Co-expression experiments of human renin and RnBP in the mouse pituitary AtT-20 cells indicate that

RnBP regulates active renin secretion from the transformants [9]. Moreover, the isolation of human [10] and rat [11] RnBP genes showed that both genes spans about 10 kilobase pairs (kb) and consists of 11 exons separated 10 introns. On the other hand, the cDNA cloning of porcine kidney *N*-acyl-*D*-glucosamine 2-epimerase [EC 5.3.1.8] [12] and the expression and characterization of human RnBP [13] showed that *N*-acyl-*D*-glucosamine 2-epimerase was identical with RnBP. The enzyme catalyses the inter conversion between *N*-acetyl-*D*-glucosamine (GlcNAc) and *N*-acetyl-*D*-mannosamine (ManNAc), and ATP is necessary as an effector [12-14]. However the catalytic residue(s) of the enzyme has not been identified although the high homology of animal RnBPs.

In the present study, conserved ten cysteine residues of human RnBP were replaced to serine residues so that we could identify the cysteine residue that is essential for catalytic activity. The results indicate that cysteine 380 is the active site residue.

Materials and Methods

Materials-----Restriction enzymes, DNA ligation kit Ver. 2.0, site-directed mutagenesis system (Mutan^R-Super Express Km), and *Escherichia coli* JM109 and MV1184 competent cells were obtained from Takara Shuzo. The wild-type recombinant human (rh) RnBP and rabbit anti-rhRnBP antiserum were prepared by the method of Takahashi *et al.* [14].

Construction of Mutant Plasmids-----The oligonucleotide-directed dual amber (ODA) method [15] using Mutan^R Super Expression system was used for the construction of ten cysteine-serine mutants of human GlcNAc 2-epimerase (RnBP). The 1.2 kb *Eco RI* fragment of pUHRB6 [13], wild-type rhRnBP expression vector, was ligated into the same site of pKF18K to construct pKHRB6. PCR was carried out in a 50 µl reaction mixture comprising 5 pmole selected primer, 5 pmole mutagenized primer, 10 ng of pUKHRB6 as a template, 5 µl of 10 x LA PCR buffer, 8 ul of 2.5 mM each of dNTP mixture, and 2.5 units of LA Taq™ DNA polymerase. The PCR was performed over 30 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 3 min). The amplified DNA was used to transform *E. coli* MV1184 cells and the transformants were selected on a LB plate containing 50 µg/ml of kanamycin. Mutations were confirmed by sequence analysis with a Applied Biosystems 373S-1B DNA sequencer. The mutant cDNAs were subcloned into the newly developed *E. coli* expression vector, named pUK223-3 [16].

Western Blotting-----Western blotting was carried out by the method of Towbin *et al.* [17]. Samples were loaded onto 5-20% gradient of polyacrylamide gel (PAGEL 520, ATTO) and electrophoresed according to Laemmli [18] at 20 mA of constant current. After the electrophoresis, proteins were transferred onto nitrocellulose membrane. The membrane was immersed in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20 (Buffer A) containing 5% skim milk, then incubated for 2 h at room temperature with rabbit anti rhRnBP antiserum (1: 1000 dilution with Buffer A containing 2 mg protein/ml of *E. coli* extract). After the incubation, membrane was washed three times with Buffer A, incubated for 1 h at room temperature with alkalinephosphatase conjugated anti-rabbit IgG (Fc) (1 : 5000 dilution with

Buffer A, Promega), and washed three times with Buffer A. The membrane allowed to react with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for the color development.

Quantitative Western Blotting-----The purified rhRnBP (4 ng to 4,000 ng) or *E. coli* extracts that were expressed mutants or wild-type rhRnBP were resolved on SDS-PAGE and color developed as described above. The RnBP bands were cut out and put into microtubes. The membranes were washed extensively with Buffer A, then the membranes were incubated for 30 min at 25 °C with 0.5 ml of 5 mg/ml of *p*-nitorophenyl phosphate in 0.1 M Tris-HCl, pH 9.5 containing 5 mM $MgCl_2$. The reaction product, *p*-nitorophnol, was quantified by measuring the absorbance at 405 nm.

Analytical Procedures-----Protein was determined by the method of Bradford [19] using bovine serum albumin as the standard. GlcNAc 2-epimerase activity was measured by the rate of formation of GlcNAc from the substrate, ManNAc. The reaction mixture at 100 µl contained 0.1 M Tris-HCl, pH 7.5 containing 10 mM $MgCl_2$, 50 mM ManNAc, 5 mM ATP, and 20 µl of enzyme solution was incubated for 30 min at 37 °C. The reaction was terminated by 5 min boiling and the reaction product was quantified by high performance liquid chromatography with pulsed amperometric detection using DIONEX Bio-CL gradient pump and DIONEX Carbopac PA-1 column equipped with a DIONEX Model PAD 2 detector.

Results and Discussion

Effects of Metals on Human GlcNAc 2-Epimerase Activity -----Table I shows the effects of metal ions on rhRnBP, GlcNAc 2-epimerase activity. The reaction mixture contained 0.1 M Tris-HCl, pH 7.5, 50 mM ManNAc, and 5 mM ATP in the presence or absence of metal ions. 10 mM EDTA inhibits about 57% of human GlcNAc 2-epimerase activity. On the other hand, $MgCl_2$, $MnCl_2$, and $CaCl_2$ enhance the GlcNAc 2-epimerase activity. On the contrary, $ZnCl_2$ greatly inhibits GlcNAc 2-epimerase activity.

Table I. Effects of Metals on Human GlcNAc 2-Epimerase Activity.

Reagents	Final conc. (mM)	Relative activity (%)
None	----	100
EDTA	10	43
$MgCl_2$	10	164
$MnCl_2$	10	158
$CaCl_2$	10	152
$ZnCl_2$	10	0

Effects of SH-Reagents-----The effects of sulfhydryl-oxidizing or alkylating reagents on rhRnBP as a GlcNAc 2-epimerase were also investigated. *N*-ethylmaleimide (1 mM), monoiodoacetic acid (10 mM), or 5, 5'-dithiobis (2-nitrobenzoic acid) (0.1 mM) inhibited more than 95% of GlcNAc 2-epimerase activity. The molecular weights of normal rhRnBP and SH-reagent treated RnBP were estimated to be 82,000 and 44,000 by gel filtration, respectively. Our previous studies showed that porcine and rat RnBPs exist as dimers. The dimers dissociate monomers in the presence of SH-oxidizing or -alkylating reagents. These results imply that the dimerization are essential for the functional activity of human GlcNAc 2-epimerase. Moreover, the active site residues seems to be cysteine residue(s) in the RnBP molecules. Thus, we constructed 10 cysteine mutants for the identification of active site residue of human RnBP.

Expression of Mutant RnBPs in E. coli Cells-----Each cysteine residue in human RnBP was individually changed to serine residue by site-directed mutagenesis using pUKHRB6, that contained the full length of human RnBP cDNA. Mutagenesis was confirmed by DNA sequencing. The *E. coli* expression vector, pUK223-3, and *E. coli* JM109 cells were used for the expression of wild-type or mutant rhRnBPs. An overnight culture (1 ml) of JM109 cells harboring different mutant plasmid was used to inoculate 50 ml of 2 x YT (1.6% polypepton, 1.0% yeast extract, 0.5% NaCl, pH 7.0) containing 0.1 mg/ml of ampicillin. The culture was incubated at 30 °C for 5 h, then expression of recombinant protein was induced with 1 mM isopropyl-ß-D-thiogalactopyranoside. Cells were harvested 3 h after induction. The cells were sonicated with 5 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 10 μM leupeptin then centrifuged at 20,000 x g for 30 min. The supernatant was used for the Western blotting or assay of GlcNAc 2-epimerase. When the extracts from *E. coli* cells harboring different plasmids, only single protein bands corresponding the human RnBP were visible on the immunoblot. The molecular weights (45,000) of mutated RnBPs expressed in *E. coli* JM109 cells were identical to that of the wild-type enzyme (data not shown).

Table II. Relative Specific Activity of Mutant RnBPs

RnBPs	Relative specific activity (%)	RnBPs	Relative specific activity (%)
Wild-type	100	C239S	141
C41S	63	C302S	70
C66S	107	C380S	0
C104S	26	C386S	93
C125S	96	C390S	75
C210S	120		

As the levels of the synthesized recombinant proteins differed to each other, mutant RnBP concentrations were normalized by quantitative Western blotting as described under **"Materials and Methods"**. The relative specific activities of mutant RnBPs are summarized in Table II. The activities of C41S, C66S, C125S, C210S, C239S, C302S, C386S, and C390S are nearly the same to that of rhRnBP. The specific activity of C104S mutant is 26% to that of wild-type enzyme. The C380S mutant had no enzyme activity. These results clearly show that cysteine 380 is essential for the enzymatic activity of human GlcNAc 2-epimerase, RnBP.

Acknowledgement

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