# Herstellung von Chitosan und einige Anwendungen

## Dissertation

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| | |
|---|---|
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Herrn Prof. Dr. Martin G. Peter danke ich für die Überlassung des Themas, sein stetes Interesse an dieser Arbeit und für seine Diskussionsbereitschaft und Hilfestellung

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Dla mojej ukochanej Joasi, dzięki której ta praca powstała

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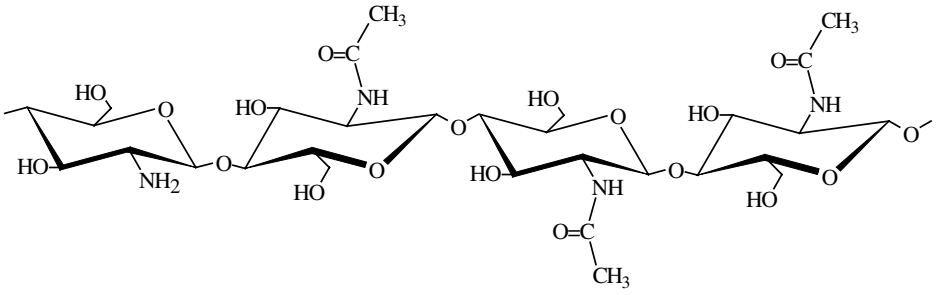
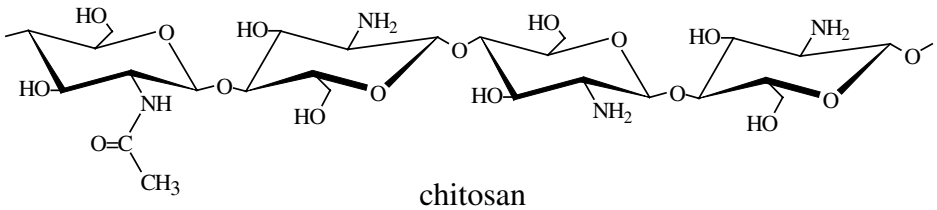
## Introduction

### 1. Structure and reactivity of chitin and chitosan

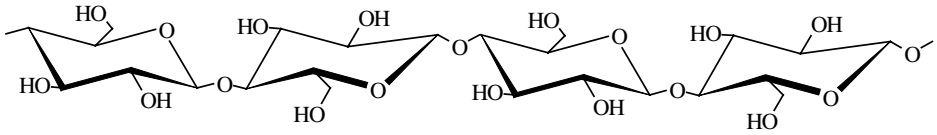
Chitosan is a polysaccharide obtained by the deacetylation of chitin, which is one of the most abundant organic materials, second only after cellulose in the amount produced annually by biosynthesis[1]. Chitin occurs in animals, particularly in crustacean, molluscs and insects, where it is a major constituent of the exoskeleton, and in the certain fungi creating the cell walls as the principal fibrillar polymer (Table 1). Branconnot first discovered it in 1811 as an alkali-resistant fraction originating from higher fungi. In 1823, Odier isolated an insoluble residue, which he named chitin (Greek, χιτων, tunic or covering)[2]. Chitin is a copolymer - poly[β-(1-4)-2-acetamido-2-deoxy-D-glucopyranose] containing a low percentage of 2-amino-2-deoxy-β-glucopyranose residual units. Its idealized structure is shown in Scheme 1, where some structural similarity to cellulose is visible, except that the C(2)-hydroxyl group of this biopolymer is transposed by an acetamide group.



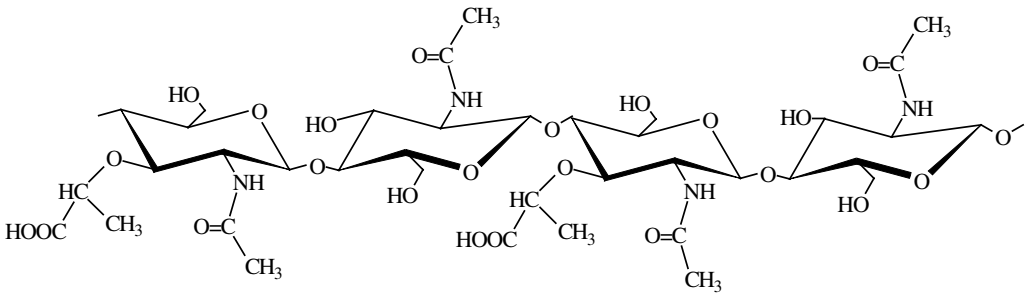
chitin



chitosan



cellulose



murein

Scheme 1. Structure of chitin and chitosan together with structurally similar polysaccharides cellulose and murein

Table 1. Occurrence of chitin in living organism[2]

| Organism | Structure | Chitin | | Other components | |
|---|---|---|---|---|---|
| | | % Organic fractions | Crystal type | Inorganic | Organic |
| FUNGI | | | | | |
| *Ascomyceta* | Cell walls and structural membranes | Traces – 45 | | - | Polysaccharides such as glucan or mannans |
| *Basidiomyceta* | of mycelia stalks and spores | | | | |
| *Phycomyceta* | | | | | |
| *Imperfectii* | | | | | |
| ALGAE | | | | | |
| *Chlorophyceae* | Cell wall components | + | – | - | Cellulose, alginian |
| PROTOZOA | | | | | |
| *Rhizopoda* | Cyst wall, shell | + | – | Silica, iron | Proteins and lipids |
| *Ciliata* | Cyst wall | + | – | | Proteins |
| *Cindaria* | | 3.2-30.3 | α | - | Proteins, tanned, |
| *Hydrozoa* | Perisarc, coenosteum, | + | α | $CaCO_3$ | - |
| *Anthozoa* | Skeleton | + | – | $CaCO_3$ | Proteins |
| *Scyphozoa* | Podocyst | + | – | - | Proteins |
| BRYOZOA | Ecocyst | 1.6-6.4 | – | $CaCO_3$ | Proteins |
| PHORONIDA | Tubes | 13.5 | – | - | Proteins |
| BRACHIPODA | | | | | |
| *Articulata* | Stalk cuticle | 3.8 | | - | Collagen |
| *Inarticulata* | Stalk cuticle, shell | 29.0 | β, γ | $CaCO_3$ | - |
| ANNELIDA | Chaetae | 20.0-38.0 | β | - | Quinone-tanned proteins |
| *Polychaeta* | Jaws | 0.28 | – | unidentified | |
| MOLLUSCA | | | | | |
| *Polyplacophora* | Shell plates, mantle bristles redula | 12.0 | – | $CaCO_3$, iron | Proteins |
| *Gastropoda* | Shell, redula, jaws, stomacal plates | 3.0-36.8 | α | $CaCO_3$, silica | Tanned proteins, conchiolin |
| *Cephlopoda* | Calcified shell, pen, jaws, redula | 3.5-19.5 | α,β,γ | $CaCO_3$ | Conchiolin, tanned protein |
| *Lamellibranchia* | shells | 0.1-17.3 | α,β,γ | $CaCO_3$ | Proteins, conchiolin |
| ARTHOPODA | | | | | |
| *Crustacea* | Calcified cuticles, intersegments | 48.0-85.0 | α | $CaCO_3$ | Arthopodins + sclerotins |
| *Insecta* | membranes | 20.0-60.0 | α | - | Arthopodins + sclerotins |
| | Hardened cuticle | | | | |
| POGONOPHORA | Tubes | 33.0 | β | - | Proteins |

This correspondence in structure is reflected in the identical roles played by these two polymers in nature, both behaving as a structural and defensive material[2]. Chitin is also related structurally to murein, which is a main structural polymer creating the cell walls of bacteria. Chitin possesses a highly ordered structure with an excess of crystalline regions and appears in three polymorphic forms: α, β and γ–chitin, which differ in the arrangement of the chains within their crystalline regions. In α-chitin, the chains are anti-parallel, in β–chitin, they are parallel, and in γ–chitin two of three chains are parallel and the third is anti-parallel. The chains associate with one another by very strong hydrogen bonding between the amide groups and carbonyl groups of the adjuncted chain. The hydrogen linkages account for the great insolubility of chains in water and for the formation of fibrils. Compared with the ordinary α-chitin, β–chitin is distinguished by its loose packing of molecules because of their arrangement in a parallel fashion. Therefore, β–polymorph shows a higher susceptibility to lysozyme [E.C. 3.2.1.17] than α-chitin and may be useful for developing materials with desirable biodegradability[3].

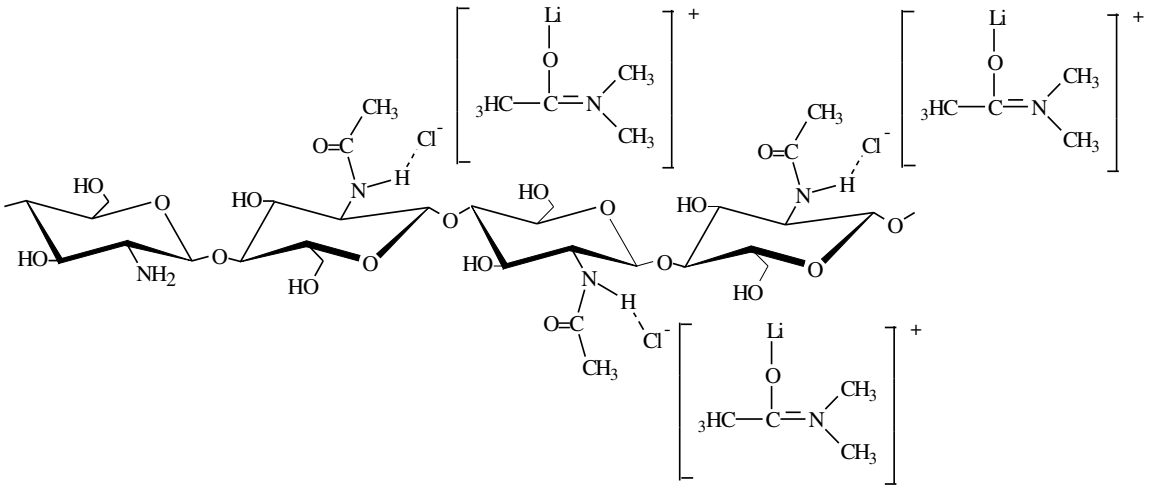
Chitosan is chemically defined as a copolymer of two residues: 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-glucopyranose, as shown in Scheme 1. The proportion of glucosamine is higher than N-acetylglucosamine, producing much better solubility in an aqueous solution of organic and numerous inorganic acids. Chitosan was first reported by Rouget in 1859, who obtained the acid soluble fraction of chitin during boiling in a concentrated potassium hydroxide solution[1]. Previously, it was used in the Orient for the treatment of skin abrasions and in America for the healing of machete gashes[1]. This copolymer is a principal derivative obtained by industrial means from chitinous wastes. It occurs naturally in some fungi but its content is much less than that of chitin[4,5]. The U.S. Food and Drug Agency has defined chitosan as "*a cationic carbohydrate polymer intended for use as a precipitating agent of proteinaceous material from food processing plants. It is chemically derived by deacetylation of the naturally occurring chitin in crab and shrimp shells. It may be used in an amount not to exceed that necessary to accomplish its intended effect. Chitosan when fed as a component of feed to livestock shall be present at no more than 0.1% of the feed. Proteinaceous material coagulated with chitosan must have safety and efficacy data approved before it can be registered or offered for sale*" [6].

The difference between chitin and chitosan lies in the degree of deacetylation (DD), usually defined as the ratio of the number of glucosamine to the total amount of N-acetylglucosamine and glucosamine, being the most important parameter determined for chitosan and chitin. The quality and properties of chitosan products such as purity, viscosity, molecular weight, polymorphous structure, and DD may vary widely because many factors can affect on the characteristics of the final product during the manufacturing process. It ought to be remembered that not only the total amount of deacetylated amino groups but also the distribution pattern of the minor component substitution: D-glucosamine contents in chitin, N-acetyl-D-glucosamine contents in chitosan, influence the properties of the biopolymer.

The degree of deacetylation is one of the more important chemical parameters distinguishing chitosan and chitin, being a statistic interpretation of the product obtained after the process of deacetylation and characterizing the macromolecular composition of chains of biopolymers. The value of DD affects the properties of this natural polymer such as its solubility in aqueous acid solution, extent of swelling in water, susceptibility to biodegradation, bioactivity, biocompatibility, etc. The methods most widely used for DD determination are as follows: $^{1}$H - NMR[7,8,9,10,11], $^{13}$C - NMR (solid state or in a form of gel)[10,12,13,14], IR spectroscopy (in form of pellets with KBr or as films)[15,16,17,18,19,20,21,22], titration methods (acid-base titration, potentiometric, colloid, metachromatic, etc.)[10,22,23,24,25,26], dyes absorption[10,22], UV spectroscopy[10,22,27,28], hydrolytic technique[11,29,30], gas chromatography (GC)[10,20], circular dichroism[10], periodate oxidation[10], residual salicylaldehyde analysis[10,18], reaction with 2,4-dinitrofluorobenzene[10,31], etc..

The average molecular weight of native chitin is larger than one million, while commercial chitosan products fall between 100,000 to 1,200,000. The molecular weight of chitin and chitosan can be determined by the methods such as chromatography[32,33], light scattering[33,34], and viscometry[10,33,35,36,37]. For determination of the viscosity of chitosan, a solution solvent system containing urea, acetic acid, and sodium chloride is usually used. In a case of chitin, the mixture of N,N-dimethylacetamide (DMAc) containing lithium chloride as a solvent is applied. The mechanism of dissolution of chitin in DMAc-LiCl solvent is shown in Scheme 2. The solubility of chitin is not dependent on its molecular

weight, but it related to the degree of N-acetylation described by the number of N-acetyl amino groups. Chitosan is insoluble in DMAc-LiCl. This phenomenon illustrates the importance of the $C(2)NHCOCH_3$ groups during dissolution. However, the insolubility of chitin in a pure DMAc solvent leads to the conclusion that LiCl plays an important role. This fact can be explained by the coordination of $Li^+$ ions with the carbonyl oxygens of four amide groups while $Cl^-$ ions hydrogen bond to –NH- group[38,39]. Nevertheless, chitin contains amide groups, which are a potential site for the creation of complex with $Li^+$ ions. Thus, the strong interaction of $Li^+$ ions to carbonyl oxygens on the polysaccharides chain is possible[39]. Summarizing, the precise determination of the effect of the structure of the complex on the increase of the solubility of chitin is not known. α–Chitin is better soluble in a DMAc-LiCl solvent system than β–chitin, which has a slower dissolution course and the resultant viscosity of solution is much more viscous[40].



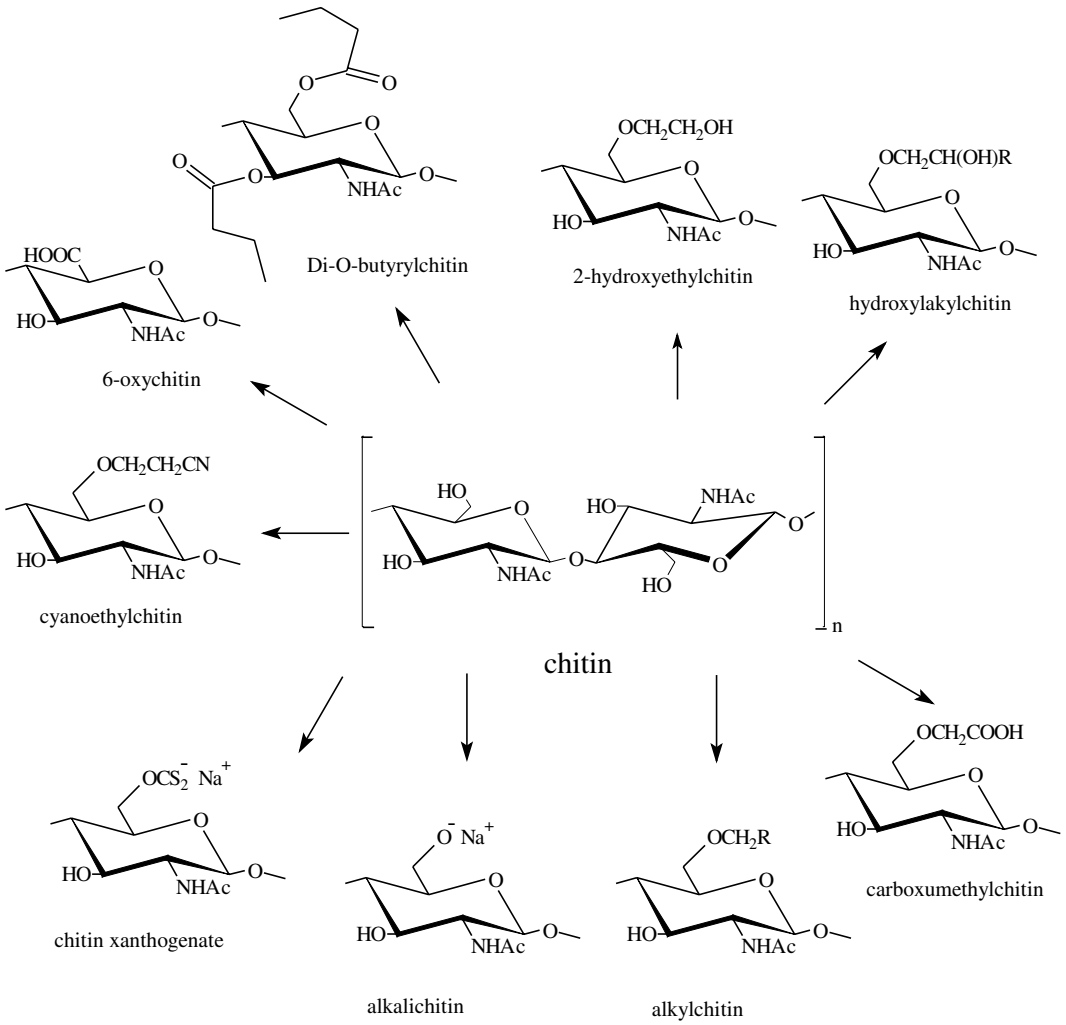
Scheme 2. Proposed, idealized mechanism of solution of chitin in DMAc-LiCl[41]

Chitin is soluble in concentrated inorganic acids such as HCl, $H_2SO_4$, and $H_3PO_4$ and its β polymorph form is dissolved in a concentrated formic acid[41]. However, during dissolution in HCOOH, a decrease in the average molecular weight of polymer was found. A number of organic carboxylic acids, such as: dichloroacetic acid or trichloroacetic acid are used as solvents[41].

The viscosity of chitosan solution is related to many factors, such as: DD[20], average molecular weight[20, 42], concentration[42], ionic strength[42], pH[10] and temperature[10]. The increase in DD with the decrease of molecular weight strongly reduces the viscosity of chitosan solution. Augmentation of the temperature of chitosan solution usually decreases its viscosity, and pH change may yield a different result depending on the type of acid used as a solvent. An increase of chitosan ionization as well as ionic strength would reduce polymer solution viscosity. In media containing low ionic strength, chitosan adopts an extended conformation because of electrostatic repulsion between chain segments. In media of high ionic strength, the biopolymer chains become more flexible and adopt a random coil conformation. The chain flexibility also depends on the temperature, illustrating the decrease of viscosity with an increase in this parameter. Application of urea for the determination of average molecular weight of chitosan is useful because of the capacity of urea to break hydrogen bonds between chitosan chains. Chitosan chains take in the presence of urea the conformation of random coil[41].

Chitosan is insoluble in water, alkali and most organic solvents, but soluble in most aqueous solutions of organic acids such as formic, acetic, lactic, citric, etc., when the pH of this solution is lower than 6.3. Moreover, when the pH of an aqueous solution is raised above 6.5, polysaccharide is precipitated from the solution in the form of a gelatinous-like flock[43]. Some diluted inorganic acids such as nitric acid, hydrochloric acid, perchloric acid and phosphoric acid can be helpful for the preparation of chitosan solutions, but only by prolonged agitation and frequent warming. The preparation of water-soluble chitosan was reported[44], produced from alkali chitin dispersion (homogenous deacetylation of chitin) and dissolved in the absence of acids in contrast to chitosan salts soluble in water.

Polycationic behaviour, caused by the existence of free, protonated amino groups, makes chitosan capable of forming complexes with derivatives having negative charges such as polymers, proteins, dyes, etc. In addition, it can selectively bind with cholesterol, fat, tumor cells, or DNA and RNA.



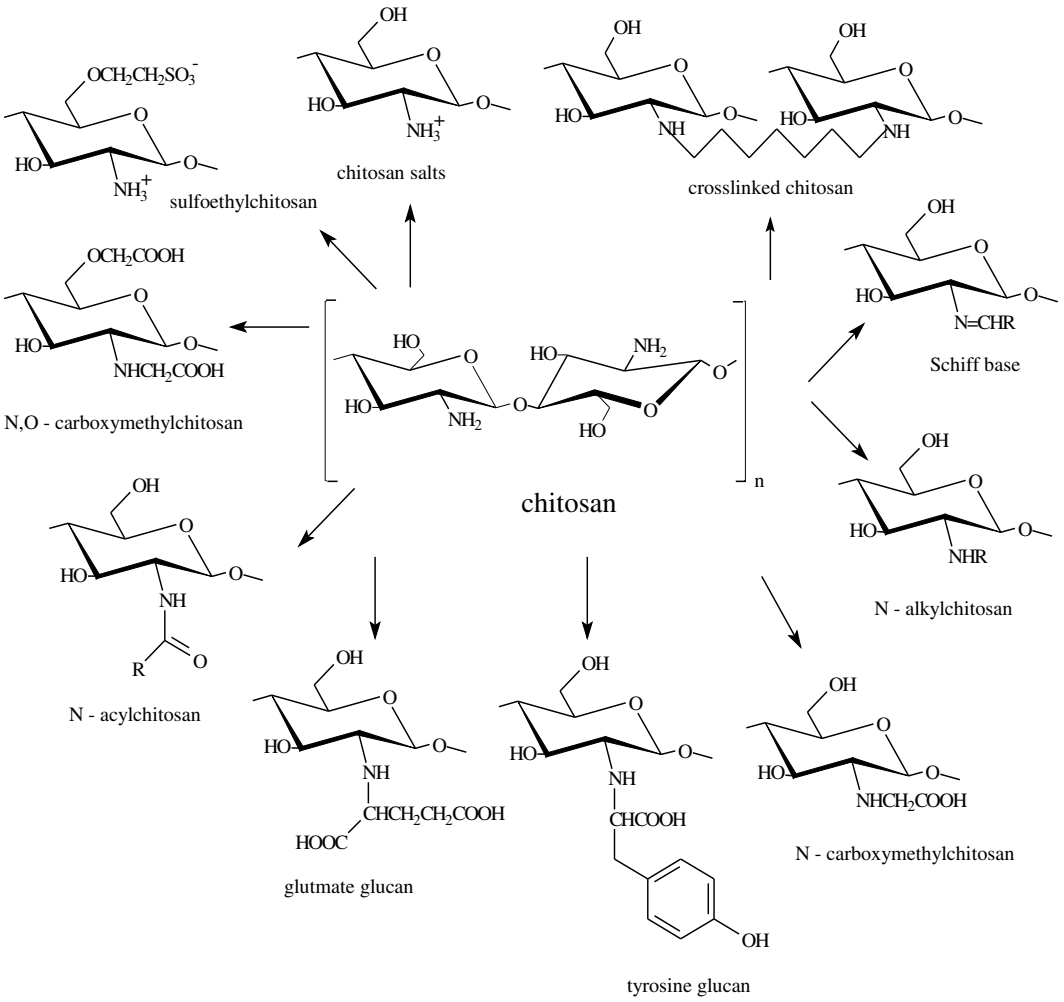

Scheme 3. Reactions of chitin[51]

Chitosan forms also chelate with metal ion required the involvement of -OH and -O$^-$ groups on the D-glucosamine residues as ligands, or else two or more amino groups from one chain binding to the same metal ion. The free amino groups of chitosan are considered much more effective for complexing metal ions than acetyl groups in chitin. Nevertheless, the increase of free amino groups did not increase the ability to form derivatives because of the dependence on the crystallinity, affinity to water, distribution of monomers (GlcN or GlcNAc)[45]. The capacity for metal adsorption could be enhanced by crosslinking[46], controlled N-acetylation[1] or bonding with other polymers like glucan[47]. Chitosan and its derivatives have shown biological activity such as antibacterial, antifungal and antiviral properties[48]. The construction of definite molecular, supermolecular, and chemical structure (an alteration of charge, etc) may enhance their susceptibility to degradation, wound healing, the induction of organism defense reactions to the action of pathogens[49-50] and the release of suitable amount of oligomers owing partly to their bioactivity[50].

Chemical modification of chitin and chitosan produced under mild conditions to protect the glycosidic and acetamido linkage leads to chitin-like products with higher solubility, which showed higher biodegradability, bioactivity, reactivity, etc. The above-mentioned may be treated with 2-chloroethanol to obtain 2-hydroxyethylchitin (glycochitin)[51]. A useful, biodegradable diester of chitin is Di-O-butyrylochitin[52], applied for the preparation of microsphere forms, and strong transparent film and fibres[53] or chitin fibres were obtained by means of alkaline hydrolysis of di-butyrylochitin precursor filament[109]. The DMAc-LiCl system was used for the formation of fibres[54], by exposure to the films[55] or as a homogenous system for synthesis of chitin derivatives[41]. Several possible reactions of chitin are shown in Scheme 3.

The most common derivative of chitosan is N-carboxymethylchitosan (CMCh), obtained by the reaction with glyoxylic acid. This in used for recovery and separation of metal ions from various

wastes [51,56]. Its potential to crosslink of CMCh with epichlohydrin could increase the ability to create chaletes with insoluble and amorphous products. Several other reactions are shown in Scheme 4.




Scheme 4. Reactions of chitosan[51]

The presence of free amine groups enables the cross-linking process, which increases the reactivity of chitosan and its linkage with other derivatives. Controlled N-acylation with acetic anhydride yields water-soluble, partially re-N-acetylated chitin[51]. A similar reaction carried out with higher carboxylic acid anhydrides produces N-acyl-, N-arylidiene- or N-alkylidiene transparent gel-like dispersions[57]. The Schiff reaction with aldehydes or keton creates the corresponding imines – converted to N-alkyl derivatives by a hydrogenation process.

## 2. Preparation of chitin and chitosan

Various procedures have been reported and developed over the years for the preparation of chitin. Isolation of chitin from crustacean shell or armour wastes consists in principle of three steps:

⇒ Protein removal – deproteinization,
⇒ Inorganic materials (principally calcium carbonate and/or calcium phosphate) removal – demineralization,
⇒ Elimination of caretonoid pigments – decolouration[58,60,75].

In the case of procedures to acquire chitin from insects, it is necessary to implement the process of deproteinization and/or decolouration without the removal of inorganic compounds. It is also necessary to add phenylthiourea to inhibit tyrosinase activity and to prevent darkening[59].

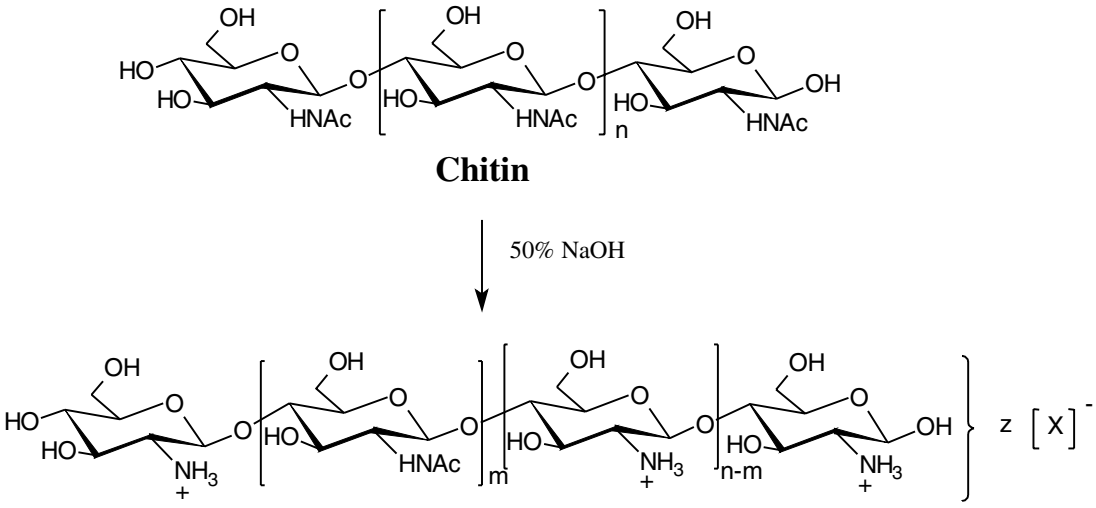
The two former steps also can be conducted in reverse order, i.e., demineralization, followed by deproteinization. Otherwise, in the most instances deproteinization is carried out prior to demineralization[60]. For the removal of protein an aqueous solution of sodium hydroxide at an elevated temperature (at 50-100°C, for 0.5-6 h, concentration of 0,25M–2,5M – depending on the preparation method)[32,61] is most preferred, but $Na_2CO_3$, KOH, $K_2CO_3$, $Ca(OH)_2$, $Na_2SO_3$ solutions were also

reported[60,62]. The use of proteolytic enzymes (proteinase such as: papain, trypsin, chymotrypsin) has been noted and examined in a number of references[63, 64, 65, 66,67]. While these treatments do not modify chitin, complete replacement of protein was not achieved. Demineralization is conventionally accomplished by treatment with dilute hydrochloric acid at room temperature for 0,5 – 24 h with concentration of 0.275M-2.0M to dissolve the calcium carbonate as calcium chloride[32,61,68-69]. As exceptions to above formic acid, nitric acid or sulfuric acid as demineralization agents with vigorous agitation at temperatures 0-100 °C for 2 – 4 h were reported[70,71,72]. A temperature no higher than room temperature is favoured to minimize the depolymerization of biopolymer. As a non-degradative method of extraction of minerals treatment of EDTA at pH 9.0 – 10.0 was carried out[40,64,73]. The amount of residual inorganic contaminants is usually called the ash content.

The amour of crustaceans contains colouring compounds (caretonoids), principally astacene, astaxanthin, canthaxanthin, lutein and β-carotene[74-75]. These compounds do not appear to be bonded with other compounds such as protein or inorganic materials. However, the deproteinization or demineralization process does not extract above enumerated compounds (except treatment of high-concentrated alkali – during the deacetylation process)[76]. Agitation with ethyl alcohol or acetone or a mixture thereof with diethyl ether at temperatures of 20-60°C for 0,25–12h can be helpful for the removal of colouring derivatives[32,67-77]. Optionally, bleaching with $KMnO_4$, NaOCl or $H_2O_2$ destroys the pigments too[58,60].

An important aspect is the particle size of crustacean's wastes. A decrease in the size of the amours usually increases the deproteinization as well as the decolouration yield[78].

The most attractive approach to the production of chitin from crustacean shell is a new method using lactic bacterial fermentation. During this process the ground shell wastes are inoculated with a lactic bacteria culture producing lactic acid and mixed with a carbohydrate origin (e.g. glucose). Acidification occurs to lower the pH and dissolve calcium carbonate. At the same time, the enzymes existing in the shellfish viscera hydrolyze the residual proteins. After separation, solid chitin is purified by standard treatment using sodium hydroxide and hydrochloric acid; however with much lower consumption of chemicals[79].




Scheme 5. Deacetylation of chitin[76]

The main process for the production of chitosan is N-deacetylation of chitin. Amide linkages are more difficult to cleave under basic conditions than an ester group, thus it is necessary to apply vigorous conditions to remove N-acetamido groups. However, acetamide groups adjacent to *trans* hydroxyl groups are much more resistant to the N-deacetylation than *cis*-related analogues. Chitin possess *2,3-trans* arrangements of substituents in its monosaccharide units and is remarkably stable to most reagents, including aqueous alkali[34].

Generally, chitosan is produced by treatment of purified chitin with high-concentrated alkali (KOH or NaOH with a concentration of 40 – 50 wt%) usually at temperatures of 100 °C or higher (Scheme 5).

The physico-chemical properties of produced chitosan depend on various factors:

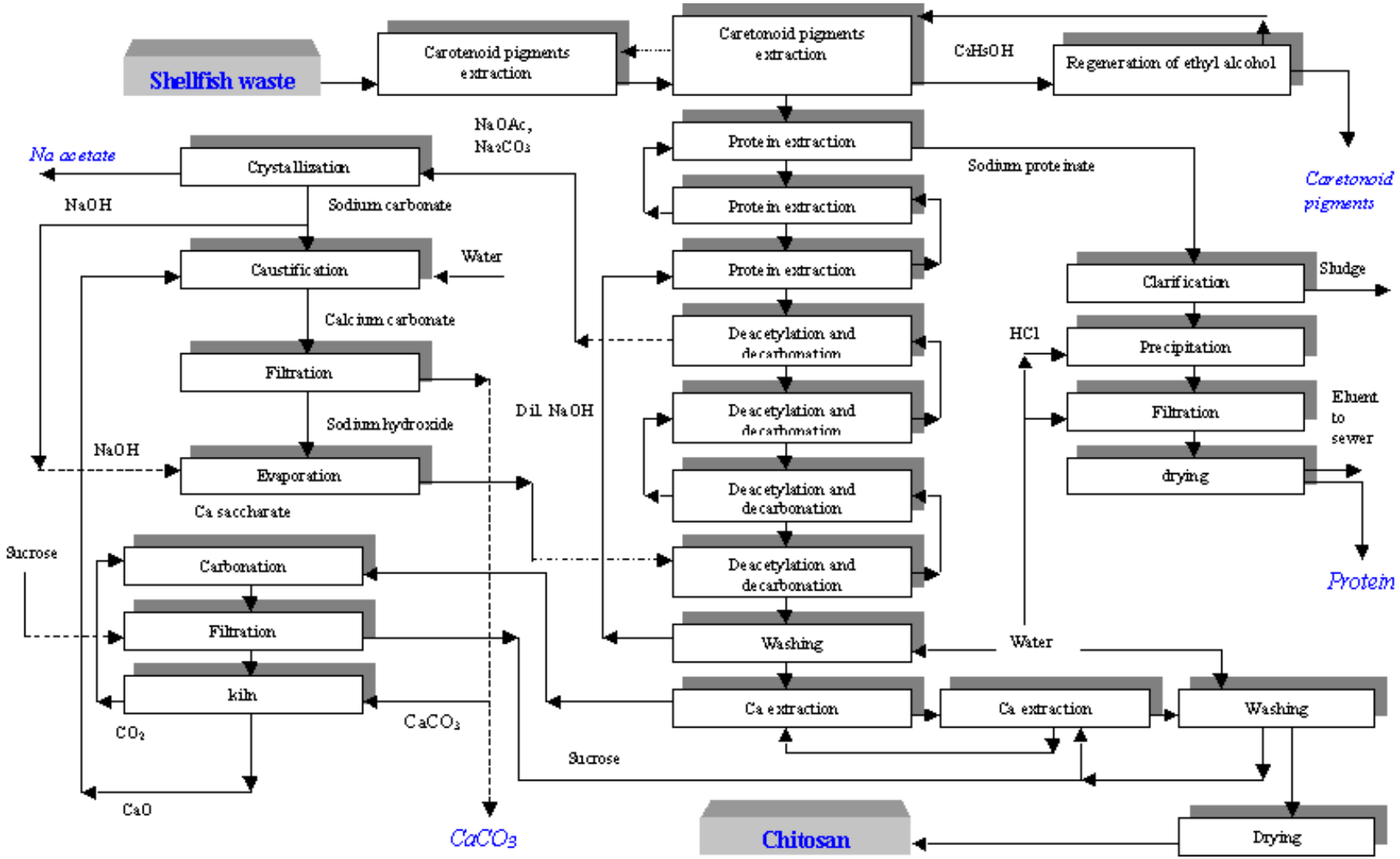
- ⇒ *Concentration of deacetylation agent:* Reduction of the alkali concentration increases the time required to obtain soluble polymer with a less viscous product [58,60,17,80],
- ⇒ *Time of deacetylation:* Degree of deacetylation rises rapidly for 1-2 h while longer time of deacetylation causes a lower improvement in DD. A strong depolymerization of polymer chains during this time was observed. In summary, the prolongation of deacetylation increases DD with a reduction in average molecular weight[58,60,76-77,81-82],
- ⇒ *Ratio of chitin to alkali:* The low ratio of chitin to alkali increases the solubility of deacetylated chitosan with a decrease in deacetylation time. This affects the homogeneity of deacetylation media[58,83].
- ⇒ *Temperature of deacetylation*: A relatively high temperature and strong alkali leads to improvement of solubility at shorter reaction time[57,59,71,77,84].
- ⇒ *Atmosphere*: Free access of oxygen to chitin during deacetylation has a substantial degrading effect on the chitosan. Chitin deacetylation in an atmosphere of nitrogen yields chitosan with a higher average molecular weight than that prepared according to standard conditions, while differences in nitrogen and ash compositions were found[32,58,60,83]. The degradation effect of air becomes more pronounced with the reduction in the deacetylation time[58],
- ⇒ *Type of the source:* Chitin from the crustaceans is much more difficult to deacetylate than any other[76,85,86,87]. This can be a result of the different molecular structure of chitins as well as accompanying compounds such as minerals. β–Chitin is more reactive than is the case of α–polymorph type[60],
- ⇒ *Particle size:* The lower particle size distribution of deacetylating chitin yields chitosans with lower average molecular weight. The deacetylation yield depends on the extent of swelling of chitin particles. Chitin with smaller particles requires a shorter swelling time, resulting in a higher deacetylation rate[32,58,76,84],
- ⇒ *Type of deacetylation:* The deacetylation process may be provided by two systems:
  - homogeneous from alkalichitin dispersion, yielding an amorphous product with an increase of random position of N-acetylated and free glucosamine units,
  - heterogeneous, distinguished by block copolymers structure of polymer and an increase in crystallinity for high-deacetylated chitosan[88].

In general, as was explained above, the alkaline deacetylation of chitin proceeds rapidly until the polymer is deacetylated above 75-85%; after that time a further treatment has only a very limited effect on the extent of deacetylation.

Gradual deacetylation is preferred to obtain a high-deacetylated product with a low reduction in average molecular weight. This process involves the procedures shown below, which can be repeated several times:

- ⇒ washing with water between successive deacetylation stages,
- ⇒ dissolution/reprecipitation of chitosan between successive deacetylation treatments.

Two explanations of this phenomenon have been proposed. The first one is based on the observed effect of sodium hydroxide concentration on the swelling of cellulose, suggesting that during washing the concentration of sodium hydroxide within the chitin/chitosan particles gradually rises to the maximum swelling concentration. This augmentation of the swelling facilitates diffusion of alkali in to the crystal regions. The second explanation suggests that chitin forms a complex with the alkaline medium. The chitin-medium complex possess a much smaller constant rate of the deacetylation step than that for the deacetylation step of uncomplexed chitin. Washing and drying are considered to rupture the above-mentioned complex, thereby converting the remaining N-acetylated glucosamine residues back, yielding much more reactive forms for the subsequent deacetylation treatment[79]. It can be concluded that in order to obtain a highly deacetylated product it is necessary to carry out the deacetylation procedure at temperature 100°C for 1h during multiple treatments. This can be more effective than a single treatment in a similar procedure for the total time[58,89].




Scheme 6. Preparation of chitosan[34,99]

The treatment of chitin with agents containing concentrated solutions of sodium hydroxide in the presence of an organic solvent such as 2-propanol, 2-methyl-2-propanol or acetone constitutes an alternative method of chitin deacetylation. However, the yield of deacetylation (low DD, higher $\bar{M}_V$) is lower then the value of this parameter obtained with aqueous sodium hydroxide alone[57,90]. The application of enzymes such as fungal and bacterial deacetylases during this process seems to be an attractive technique, since it is necessary to take longer time to gain the soluble product [91,92,93,94].

The commercial production of chitin and chitosan is additionally limited by the economic calculations for the cost of reagents and the possibility to the recycle them, and costs of the non-marketable wastes and their elimination charges.

Scheme 6 shows the typical industrial process of chitosan production offering nearly complete conversion of shell wastes into marketable commodities and recovery of proteins, sodium acetate, carotenoid pigments and lime or calcium carbonate as by-products[34].

Sodium hydroxide is a deacetylation agent with concentrations from 30 wt% to 50 wt% at temperatures of 120 °C-150 °C.

Before the deacetylation process, protein extraction was carried out with diluted sodium hydroxide obtained after washing of deacetylated and decarbonated chitinous. Simultaneously with the deacetylation of chitin, the mineral residues are converted to calcium hydroxide and then to calcium carbonate.

Chitin and chitosan are presently industrially recovered from crustacean wastes in the United States of America, Japan, India, and to a lesser extent in Russia, Norway and other country in Europe e.g. France, Germany, Poland. Two essential facts require further attentions:

⇒ chitinous organisms, mainly crabs, shrimps, prawns, and krills are very abundant all over the world and limited part of these resources are being exploited by the marine food industry producing canned or frozen meat, proteins, and shell wastes,

⇒ production of chitin is carried out usually as a secondary activity related to the marine food industry.

The preparation of chitin and chitosan from *Insecta* does not involve the above-described limitations, except for the last one. Economic restrictions make it necessary to connect this production with other applications. In this way, insect chitin and chitosan can be manufactured, similar to crustaceans biopolymers, as co-products.

Chitin can also be obtained from sources other than marine wastes: large quantities of fungi grown currently in fermentation systems producing organic acids such as citric acid, antibiotics, and enzymes, constitute a potential source of chitin[34].

The richest source of crustacean chitin is krill[95]. Estimates on the amounts of these shrimp-like crustacean that are distributed in all oceans over the world ranged from 200 - 800 million tons. Economically most important is the Antarctic krill, *Euphausia superba*, with individual specimens measuring ca. 5-7 cm in length. It is generally accepted that an ecological balance is maintained at annual catches up to 50 million tons. The total allowable catch (TAC) is only 3% of this figure, or 1.5 million tons per year. Actually, this is by far not reached: data from 1992 show that only 23% of TAC is taken out of the Antarctic Sea. From the total harvest of 350,000 tons per year, a share of 200,000 tons is taken by Russian fleets, with a decreasing tendency, and 70,000 tons by Japan, with an increasing tendency[76]. Thus, concerns about the depletion of krill in the Antarctic Sea lack any rationale. Krill is a rich source not only for chitin but also for proteins and lipids, especially organic pigments (dye) of the carotenoid group and unsaturated fatty acids. The ability to recover the above valuable components of crustacean wastes, especially carotenoid pigments and chitin, plays an important position in utilization of marine food wastes concerning the environmental protection.

Another rich source of chitosan are the deep-water shrimp, *Pandalus borealis*, which are caught in large quantities and may yield ca. 700 tons of chitosan per year[76,96]. These figures are very low when compared with the quantities of starch or cellulose that are utilized, in the order of $>>10^8$ tons annually. However, a comparison of chitosan with polyglucose glycans is misleading in many respects, since the former is an ideal material for high value applications and chitosan is much too precious to be a competitor to cellulose or starch.

Tables 2 and 3 show the chemical composition of various crustacean wastes produced from Antarctic krill *Euphausia superba*, from North Shrimp *Pandalus borealis,* as well as from the insect larval of *Calliphora erythrocephala.*

The Protan Lab. Inc. (USA) offers the premium industrial grade of chitosan with approximate prices ranged from 16 to 25 USD/kg, whereas the industrial grade is available from 15 to 20 USD/kg[97]. The Silver Sea Marine Products (India) offers industrial grade chitosan at a price higher than 30 USD/kg[98], and the Japanese companies at prices not lower than 30-40 USD/kg[99], whereas the Sea Fisheries Institute, Gdynia (Poland) offers krill chitosan from small-scale production at prices not lower than 70-100 USD/kg[100].

Table 2. Chemical composition of various crustaceans wastes[58]

| Source | Chemical composition (%) | | | | | |
|---|---|---|---|---|---|---|
| | Protein | Lipid | Ash | Chitin | Carotenoid pigment | Water |
| *Pandalus borealis* * | 23,2 | 14.3 | 33.4 | 28,9 | 0.2 | ---- |
| *Euphausia superba* * | 47.4 | 28.9 | 15.6 | 3.8 | --- | ---- |
| *Euphausia superba* | 10.0 | 6.1 | 3.3 | 0.8 | --- | 78.9 |

* dry basis

Table 3. Chemical composition of insect's larves of *Calliphora erythrocephala* [101]

| Source | Chemical composition (%) | |
|---|---|---|
| | Protein, lipids, carotenoid pigments | Chitin |
| *Calliphora erythrocephala* * | 74.3 | 25.7 |

* dry basis

The potential markets for chitin and chitosan are Japan, USA, UK, France and Germany. The market in the year 2,000 is projected at 2 billion USD. Japan is considerably advanced in the technology and commercialization of chitosan – the market in this country absorbs about 600-700 tons per year[102].

## 3. Application of chitosan

Commercial chitosan is available as flakes[103], powder[45], as well as processed products like films or membranes[104,105,106], beads or microcapsules[107-108], fibres[109,110,111] and as well as a new form of this biopolymer: microcrystalline chitosan (MCCh)[112-113]. The application of chitosan and its derivatives is presented in Table 4.

### 3.1. Commercial chitosan

Commercial chitosan is usually offered as flakes or powder. The products of various companies differ in purity, granulation, colour, DD, average molecular weight, and solubility. Specific properties of the commercial chitosan flakes distributed by Protan Lab. Inc. are shown in Table 5. This product may contain insoluble, highly N-acetylated fractions, which derive from the core of the particles due to the heterogeneous deacetylation.

N-acetylglucosamines in the acid-soluble fractions are randomly distributed, whereas the insoluble fractions contain relatively long blocks of N-acetylated units.

### 3.2. Chitosan beads

Chitosan beads are usually prepared by dropping high-viscous chitosan salt solutions (acetate, malate, citrate, chloride, etc) in a basic solution with slow agitation[114-115]. The diameters of drop as well as the stream of solution control the diameter of the beads.

Table 4. Application of chitosan

| Application | Example | Possible apply to chitosan forms: |
|---|---|---|
| ***Water treatment*** | Removal of metal ion[116] | *Beads, membranes, powders, MCCh* |
| | Flocculant/Coagulant | *Salts* |
| | Protein[61] | *Beads, membranes, salts* |
| | Dyes[117] | *Beads, powders, MCCh, fibres* |
| | Amino acids[118] | *Beads, salt, films* |
| | Filtration[119] | *Membranes* |
| ***Pulp and paper*** | Surface treatment[178] | *Solution of salts,* |
| | Photographic paper[120] | *Solution of salts forming films* |
| | Carboniess Copy paper[121] | *Solution of salts forming films* |
| ***Medical*** | Bandages, Sponges[122,123,124] | *Salts, MCCh, fibres, gel-like dispersions* |
| | Blood cholesterol control[125-126] | *Salts, MCCh* |
| | Tumor inhibition[127-128] | *Salts, solution* |
| | Membranes[129] | *Membranes, films* |
| | Dental/Plague Inhibition[130] | *Powders, salts, MCCh* |
| | Skin burns/Artificial skin[131] | *MCCh, salts, gel-like dispersions* |
| | Contact lens[132] | *Salts* |
| | Control release drugs[133-134] | *Microcapsules, MCCh, gel-like dispersions* |
| | Bone Disease Treatment[135] | *Microcapsules, implants* |
| ***Cosmetics*** | Make-up powder[136] | *Powder, MCCh* |
| | Nail polish[137] | *Gel-like dispersions* |
| | Moisturizers | *Gel-like dispersions, solution* |
| | Fixtures[137] | *Powder, gel-like dispersion* |
| | Bath Lotion[137-138] | *Gel-like dispersions, salts* |
| | Face, Hand and Body creams[137-138] | *Gel-like dispersions, salts* |
| | Toothpaste[138] | |
| | Foam enhancing[138] | *Gel-like dispersions* |
| | | *Gel-like dispersions* |
| ***Biotechnology*** | Enzyme immobilization[104, 118,139,140,141,161] | *Membranes, microcapsules, beads, powder* |
| | Protein separation[142] | *Membranes, beads, films* |
| | Chromatography[160] | *Beads* |
| | Cell recovery[143] | *Beads,* |
| | Cell immobilization[144] | *Beads, membranes, films* |
| | Electrodes and sensors[145-146] | *Membranes, films* |
| ***Agriculture*** | Seed coating[49,147] | *Salts, MCCh* |
| | Leaf coating[49-50] | *Salts, MCCh* |
| | Hydroponic/fertilizer[148] | *Salts, MCCh* |
| | Controlled agrochemical release[149] | *Microcapsules, MCCh* |
| ***Food*** | Removal of dyes, solid, acids[150,159] | *Salts, beads, films* |
| | Preservatives[151] | |
| | Colour stabilization[152] | *Salts forming films* |
| | | *Salts* |
| ***Textile*** | Sanitary fibrous materials[110-111] | *Fibres, solution, gel-like dispersions,* |
| | Surgical threads[110-111] | *MCCh* |
| | Textile material[110-111,163,164,165] | *Fibres, solution, gel-like dispersions, MCCh* |
| | | *solution, gel-like dispersions, MCCh* |
| ***Membranes*** | Reverse osmosis[34] | *Membranes, films* |
| | Permeability control[153] | *Membranes, films* |
| | Solvent separation[105,154] | *Membranes, films* |

The chemical and mechanical behaviour of beads (solubility, mechanical resistance, and sorption behaviour) are enhanced by crosslinking with bifunctional compounds[155], such as glutarldehyde[108,144,156,157], glyoxal[157], terephthaloyl chloride[158], hexamethylene diisocyanate[158], etc.

The beads may be applied to the recovery of liquid wastes, heavy metal ions[116,159], as chromatographic support[160] or for an immobilization process[161]. The interesting forms of chitosan beads manifesting a magnetic behaviour[162] are prepared by the addition of iron oxide (magnetite) before bead formation. These are more simply recovered from fermentation baths than standard ones and may be used again by means of multiple treatments.

Table 5. Typical properties of commercial chitosan produced by PROTAN Laboratory Inc.[97]

| | |
|---|---|
| Grain size | < 3 mm |
| Apparent specific weight | 0.15 ± 0.05 |
| Moisture | 10 % |
| Alkali soluble and ash | 5.0 % |
| Solution colour | Clear |
| Average molecular weight | 100,000 – 300,000 Da |
| Amine content | 7-10 % |
| Viscosity: | |
| (1 wt% in 1 wt% acetic sol.) | 2,000 – 3,000 cP |
| (0.5 wt% in 0.5 wt% acetic sol.) | 200 – 500 cP |

### 3.3. Chitosan fibres and textile containing chitosan

Chitosan fibres are prepared according to several methods[163,164,165], but most frequently an acetic acid solution of chitosan for the spinning process is used. The mechanical behaviour of fibres is increased by crosslinks with the bifunctional compounds, similar as for films and beads. The prepared fibres are distinguished by a better ability to utilize most commercial conventional chitosan, continuous or staple forms, controlled biodegradability and bioactivity, and are prepared by means of relatively simple technology. However, fibres containing chitosan are usually friable[110]. The application of special additives both to the spinning solution and coagulation bath enables one to modify, in a controlled way, the supermolecular structure of chitosan and consequently to modify the biodegradability and bioactivity of fibres[167].

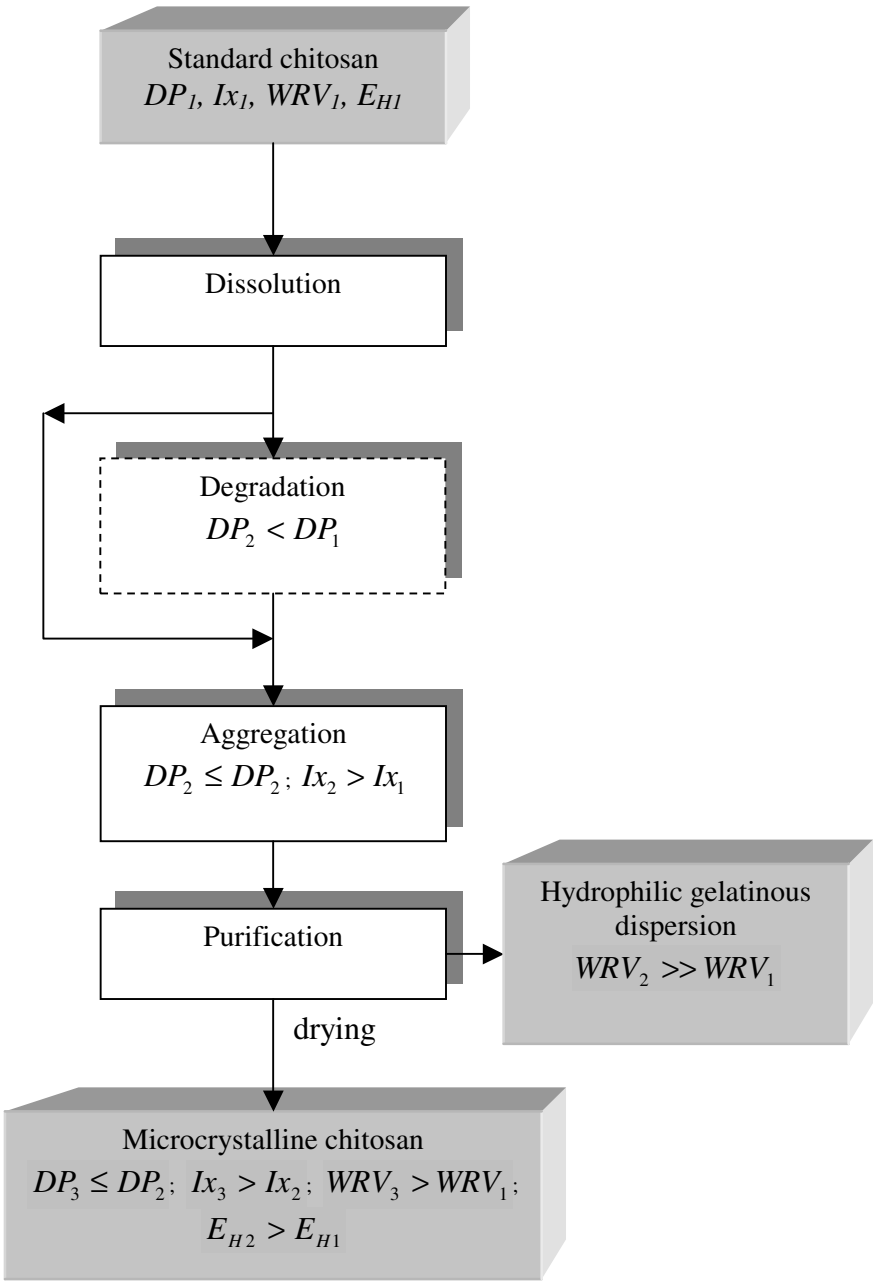
The possibility to coat textile fibres (natural or man-made) with chitosan by means of impregnation improves the mechanical properties of such treated textiles; their staining behaviour, water resistance, electroconductivity, and adds bioactive as well as bacteriostatic behaviour[111]. The impregnation of textiles may be processed by spraying, foularding or the introduction of chitosan (in the form of xanthate salts or solid micrioparticles) to viscose[167].

The use of MCCh gel-like dispersion causes advantages such as reduction in the textile coating procedure, absence of chitosan regeneration processes, and deficiency of liquid wastes after impregnation using chitosan solution[111]. It was reported that the adhesion of chitosan to wool fibre is weaker than to cellulose fibres. This phenomenon depends mainly on the ionic interaction between carboxyl groups of wool and amine groups of chitosan as well as on hydrogen linkages. However, chitosan dissolution in the dyebath has been found during the process of dyeing. The reaction of hydroxyl groups of chitosan with the reactive dyestuff in the alkaline medium yields a stable colouration to the impregnated textile[166]. The increase in dyeing intensity of the fabric after impregnation with MCCh gel-like dispersion was noted.

### 3.4. Microcrystalline chitosan (MCCh)

A new physical-chemical type of chitosan is its microcrystalline form, existing as gelatinous water dispersion or a powder[112,113,167]. It is prepared by the precipitation of polymer from an acidic aqueous solution of acids into a sodium hydroxide solution as a coagulation agent (Scheme 7.). The gelatinous dispersion, possessed a much higher WRV coefficient than original chitosan after air-drying to form a suitable form of powder having higher crystallinity, is a consequence of this process. There is some, in principle, similarity to the production of microcrystalline cellulose (MCC) from diluted viscose. However, the formation of MCC by means of coagulation of cellulose xanthate and regeneration of cellulose is only to some extent comparable to the phenomena, which takes place during MCCh aggregation. The process of MCCh preparation by coagulation in basic conditions involves a number of phenomena, such as neutralization, coagulation, and aggregation of polymer chains. These above-mentioned facts create the specific behaviour of the product and are mainly responsible for its molecular and supermolecular structure differing from the raw substrate.

It may be assumed that the properties of MCCh depend on several parameters of the preparation process, such as properties, origin, conditions of the degradation process, conditions of aggregation procedures, parameters of drying, as well as the presence of electrolytes[111].



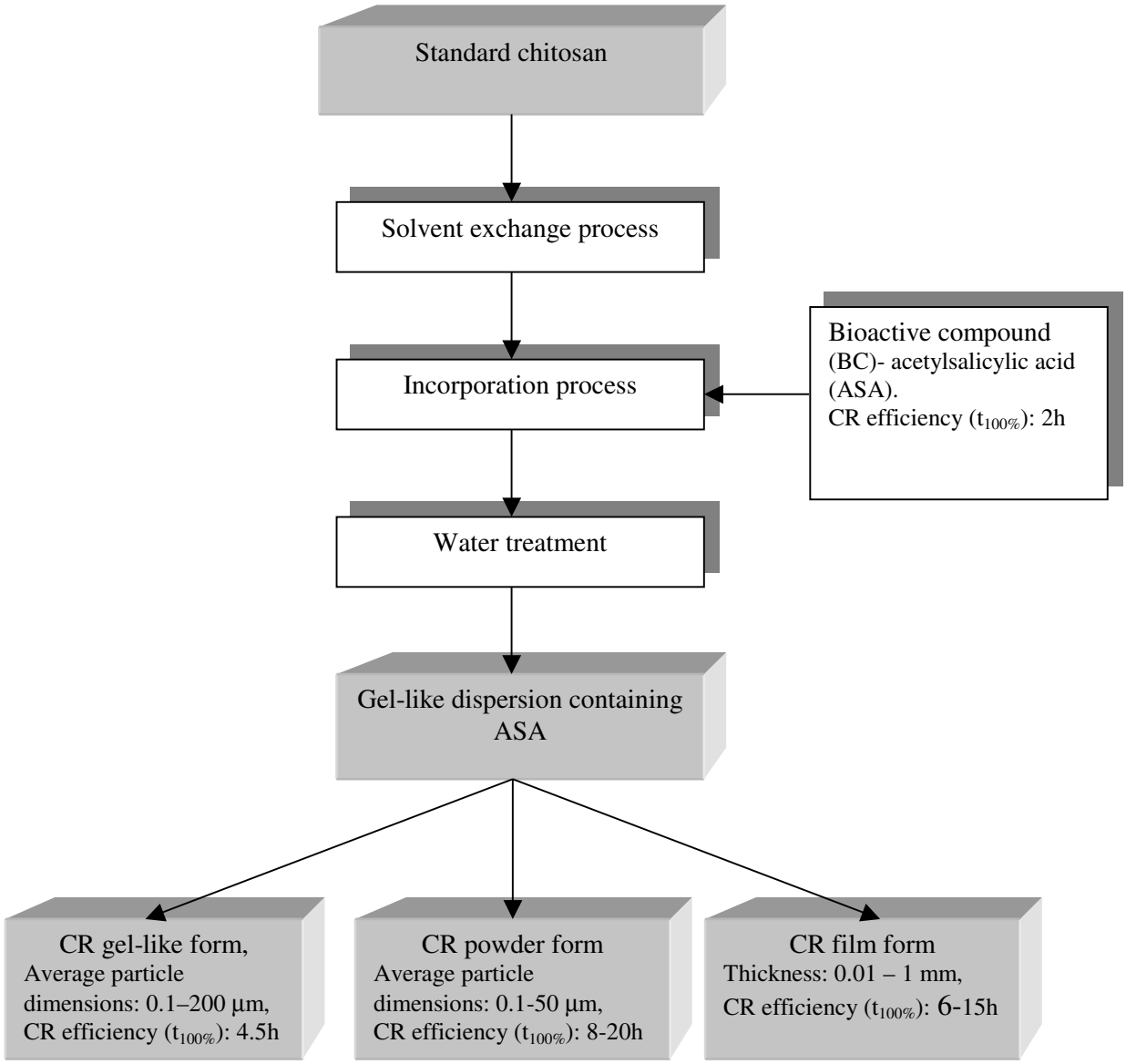

Scheme 7. Preparation of microcrystalline chitosan[112]. (DP- degree of polymerization; WRV – water retention value; Ix – index crystallinity; $E_H$ – energy of hydrogen bonds)

MCCh is unique in several useful properties, partially resulting from the standard chitosan used for its manufacture, such as:

⇒ high water retention behaviour (WRV ranged from 500 to 5,000 %),
⇒ high adhesiveness,
⇒ ability to form powerful hydrogen bonds,
⇒ chelating and sorption properties,
⇒ direct film-forming behaviour (film preparation directly from aqueous dispersion of MCCh),
⇒ high stability of aqueous dispersion,
⇒ controlled biodegradability, higher susceptibility to hydrolytic degradation,
⇒ non-toxicity,
⇒ good miscibility,

⇒ high ability for solvent exchange,
⇒ high chemical reactivity, bioactivity, including bacteriostatic properties, and biocompatibility[110].

The most important and extraordinary property of MCCh seems to be its direct film-forming behaviour just from aqueous dispersion[168]. Films obtained on this basis show excellent adhesion to different types of surface, good elasticity, and water-resistance[169]. Microcrystalline chitosan, especially in the form of gel-like dispersion, can be effectively applied as an auxiliary agent with bioactive action to modify cosmetics: shampoo, liquid soap or bath foam. It was applied as a thickener and viscosity regulator, softener, stabilizator, skin and/or hair-protecting agent, bioactive agent with fungi- and bacteriostatic properties, and controlled release carrier.




Scheme 8. The procedure of formation of controlled release agent containing acetylsalicylic acid (ASA) showed specific release efficiency[112]

The drawback of standard chitosan as a binding agent for nonwovens, as was described relating to fibre coating applications, is the necessity to carry out the conversion of chitosan salts of polymer containing water by treatment with alkali. The use of MCCh solves this dilemma, and with its direct film-forming behaviour enhances the properties of nonwovens[168]. The direct introduction of MCCh to viscose has solved the problem of controlled bioactivity of the modified viscose fibres[111]. Special properties of such fibres make them suitable for fabrics and nonwovens in standard applications and in special uses in medical, pharmaceutical, or cosmetic products.

Standard chitosan is relatively low effective at pH=7.0 for the chelation of metal ions, sorption of dyes and pigments and ion exchange, but the application of MCCh shows an increase in the scope of pH in which MCCh is a sufficient chelating agent. It was found that MCCh is effectively combined with metal ions in media beyond pH=6.5 and remains effective at lower pH similar to a standard chitosan[168]. The enhanced sorption behaviour of MCCh permits the incorporation of bioactive compounds into chitosan structure, as well as the preparation of suitable forms of controlled release (CR), an important function in medical and pharmaceutical applications (Scheme 8.).
The different forms of CR agents showed specific release efficiencies depend on the conditions applied during production[112]. The introduction of Lewis-acid type bioactive compounds proceeds by the solvent exchange process involved in the incorporation of these compounds into the chitosan structure as well as by forming powerful bonds with the polymer.

### 3.5. Chitosan films

Aqueous solutions of chitosan salts very easily form transparent films, which may be regenerated to chitosan films by treatment with alkali solution. The films are distinguished by high water absorbance, biocompatibility and permeability to oxygen, biodegradability, controlled release behaviour, high reactivity, possession of electrochemical properties, and high strength[43]. However, flexible and transparent films are about as water permeable as cellophane films, while their conductivity is similar to that of organic crystals. The possibility to form the films directly on the skin makes it applicable in treating the wounds as well as burns[170]. The change in the behaviour of films by means of the introduction of bioactive components enhances its application as a helpful material for medical agent preparations, which may be used longer and are degradable by human enzymes. The crosslinking of chitosan film is used for improvement in film behaviour as well as for the introduction of modifying agents. However, this type of modification causes an increase in permeability and a decrease in mechanical properties, especially elasticity[171-172]. The water transmission vapour rate of calcium chloride crosslinked films containing glutamate and alginate linearly decreases with the increase in concentration of crosslinking agents[173]. Muzzarelli[104] prepared chitosan films containing collagen or gelatin, increasing their elasticity by treatment with tyrosinase in the presence of phenol. In addition, crosslinking with poly(glutamic acid) to increase polyionic behaviour as well as water sorption parameters was examined[174]. The chitosan-cellulose composite films based on blends of microfibril cellulose and chitosan possess a high tensile strength measured at dry and wet states, complete decomposition in soil or seawater, and relatively fast biodegradability by bacteria *Pseudomonas* sp. H-14 producing cellulase[175]. This type of combined film can be used as a rolled composite film, binder film for dry non-woven fabrics, biodegradable foams, and medical material such as wound dressing.
MCCh is distinguished by its film-forming behaviour directly from its aqueous dispersion, making the preparation process of film more straightforward, faster, and cheaper, which plays a very important role in medicine. The film formed from MCCh gel-like dispersion maintains the behaviour of MCCh[110].
The application of a plasticization agent, which interferes with the hydrogen bonds between the polymer chains facilitates forming of MCCh films. This phenomenon increases the flexibility of the chains and facilitates their relocation. During removal of water, improvement in the degree of chain ordering and the hydrogen linkages between the plasticizer and biopolymer hydroxyl group results from the tight packing of the macromolecules[176]. The addition of low molecular weight compounds, i.e. glycerin, causes augmentation of the elastic behaviour of formed films; however their crystallization at low temperature is one of the disadvantages of glycerin, limiting the application of plasticized chitosan films at low temperature.
The structure of films formed from chitosan significantly differs depending on their origin. However, the existence of more-ordered areas and amorphous regions was confirmed. The films with a low degree of crystallinity formed from high-molecular polymers possess better mechanical endurance.
There is a very wide range of criteria to be applied when considering the selection of film for a particular purpose. In general, they fall into three broad groups:

- ⇒ the first group covers those properties concerned with the mechanical endurance of the film (including tensile strength, impact strength, stiffness, bursting strength, etc.),
- ⇒ the second group contains properties resulting from transmission behaviour (including permeability to gases, vapour, and odors as well as light transmission),

⇒ the last group includes properties concerning the performance of films in converting or packaging equipment[176].

3.6. Paper sheets containing chitosan

Standard paper is held together by hydrogen bonds[177,178,179]. The bonds must span the range between separate segments of fibres that together make up the interfibre bonding area. While several water molecules may or may not be an integral part of the interfibre linkage, it is certain that the existence of larger amounts of water has a disruptive consequence on paper cohesiveness. This can be explained by the fact that an excess of water can disrupt the existing interfibre linkages. Since the hydrogen bonding sites on the fibre surface are then fully occupied by the water molecules, they are in turn hydrogen bonded to such a large excess of free water that the fibres can now be regarded as linked by a macroscopic fluid bridge. The weakness of this bridge is manifested by the so-called wet strength of paper[180].

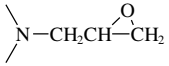
In an attempt to improve this low strength, a number of polymeric materials have been developed. These include urea- as well as melamine-formaldehyde condensates, epoxy-containing polyamides, cationic polyacrylamides, and polyethyleneimines. The most extensively investigated wet strength agent is polyethyleneimines (PEI)[183]. These high molecular weight molecules are globular polyamines containing primary, secondary and tertiary amino groups. However, they do not show film-forming behaviour and have little tensile strength in bulk. Cellulose pulp containing many acidic sites due to the oxidation of this polymer or to the presence of lignin or hemicellulosic residues can be efficient in forming ionic bonds and hydrogen linkages with the basic polyethyleneimine. It is assumed that an excess of water will not rupture these ionic bonds. In addition, covalent bonds between aldehyde and amino groups may be formed by Schiff bases, which may form amide groups from ammonium salts at high temperature[181]. Other derivatives, as aldehydes, formaldehyde, glyoxal, and polyacrolein confer wet strength obviously due to the formation of acetal linkages. The application of small bifunctional compounds, toluene diisocyanate, causes the formation of carbamate linkages on exposure to the alcoholic hydroxyl group of the cellulose fibre surface[182]. Other derivatives used to improve the mechanical properties of paper are shown in Table 6. The potential additive should meet the conditions below:

⇒ be soluble in an aqueous solution for easy application within a conventional papermaking system,
⇒ be substantive to a cellulose corresponding to efficiency of the retention,
⇒ be compatible regiospecificially with cellulose surface so as not to cause the disruption of conventional hydrogen linkages,
⇒ be large enough in molecular weight to give more possibilities to create interfibre bonds,
⇒ be film-forming to offer cohesive resistance to rupture,
⇒ contain a functional group capable of ionic or covalent reaction with celluosic pulp during the formation of paper,be linear to allow accessibility to all functional groups,
⇒ not contain any potential chromophoric groups which later impart colour to the sheet, be non-toxic and biodegradable in order to facilitate compliance with environmental regulations,
⇒ not present problems in the repulping and recycling of paper.

Chitosan meets all of the above-mentioned conditions. The use of chitosan in the papermaking industry was first reported in 1936[183], and several authors have continued to devote much attention to this field[183,184,185].

Chitosan in form of a 1 wt% aqueous solution in acetic acid was used in the past as a surface treatment of various papers such as newsprint, printings, maps, etc.[186] to increase their breaking force, burst resistance, and folding endurance without any effect on brightness[187]. Chitosan is a compatible binder with paper pulp and is claimed to be superior to polyacrylamides as a film former. It is also useable as filler in the preparation of coatings for lithographics paper plates, which accept and hold water and ink[188]. Clays coated with chitosan have been used in paper manufacture to obtain an increase in printing capacity.

Table 6. The chemistry of wet strength treatment for paper[181]

| Wet strength treatment | Mechanism of additive retention | Nature of crosslinking group | Structure of crosslinking group | Type of chemical cross-link | Structure of crosslinking bond |
|---|---|---|---|---|---|
| Heat | None | Hydroxyl | $-CH_2OH$ | Ether | $-CH_2OCH_2-$ |
| | | Carboxyl | -COOH | Ester | -COO- |
| Parchmentizing ($H_2SO_4$) | Impregnation | Hydroxyl | $-CH_2OH$ | Ether | $-CH_2OCH_2-$ |
| | | Carboxyl | -COOH | Ester | -COO- |
| Urea-formaldehyde resin | Cationic center | Methylolamide | $-NHCH_2OH$ | Amidomethyleneether | $-CONHCH_2O-$ |
| | | | | Diamidomethylene | $-CONHCH_2HNCO-$ |
| Melamine-formaldehyde resin | Cationic center | Methylolamine | $-NHCH_2OH$ | Aminomethyleneether | $-NHCH_2O-$ |
| | | | | Diaminomethylene | $-NCH_2HN-$ |
| Glycidylated polyaminoamide | Cationic center | Glycidylamino |  | Ether | -C(OH)-CO- |
| | | | | Amino | -C(OH)-CNH- |
| Polyethyleneimine | Cationic center | *p-, s-, t*-amine | $R_3N$, $R_2NH$, $RNH_2$ | Ionic | $R_3N^+O^-$, $R_2NH_2^+O^-$ $RNH_3^+O^-$ |
| | | | | Amide | -NHCO- |
| | | | | Imine | -N=C- |
| Cationic polyacrylamide | Cationic center | Amine | $-NH_2$ | Amide | -NHCO- |
| | | | | Imine | -N=C- |
| Protein | Cationic center | Amine | $-NH_2$ | Amide | -NHCO- |
| | | Carboxyl | -COOH | Ester | -COO- |
| Carboxyled polymers | Impregnation | Carboxyl | -COOH | Ester | -COO- |
| Formaldehyde | Impregnation | Aldehydo | HCHO | Acetal | $-OCH_2O-$ |
| Glyoxal | Impregnation | Aldehydo | CHOCHO | Diacetal | -OCHCHO- |
| Polyacrolein | Impregnation | Aldehydo | -CHO | Acetal | -CHO- |
| Dialdehyde stretch | Cationic center | Aldehydo | -CHO | Acetal | -CHO- |
| Diisocyanates | Impregnation | Isocyanate | -NCO | Urethane | -NHCOO- |
| Inorganic hydroxides | Cationic center | Inorganic hydroxyl | $-M(OH)_n$ | Ionic, inorganic ester | $-M^+O-$ |
| Neoprene latex | Coagulation | Chloro | =CHCl | Ether | =CHO- |

Softwood bleached kraft pulp slurries have been mixed with chitosan salts and epoxidised polyamides to prepare paper with good dry and wet strength. Chitosan is also used as a flexiblizer of paper sheets applied for the production of the packing materials[189] comprising core layers of paper or paperboard or a polymer[190]. Between the core layer and the outer layer, preferably containing plastic polyethylene, is a barrier layer of polyvinyl alcohol and/or chitosan.

The special quality of moisture holding with the cationic behaviour of this biopolymer increases the yield of anionic paint sprayed during the electrostatic painting of wood-based material. In addition, chitosan painted on plywood reacts with formaldehyde released from the glueline to form Schiff bases[191]. Chitosan is capable of increasing the number of interfibre bonds by virtue of its chemical similarity to cellulose. Chitosan can form low energy hydrogen bonds not only between hydroxyl groups, but also between hydroxyl groups and amine groups or between amine groups. This allows for efficient use of non-ionic fibre surfaces[192]. The idea of microcrystalline chitosan use as a binder for paper is based on its three main attributes, as follows:

⇒ direct film-forming behaviour,
⇒ high adhesion behaviour,
⇒ possibility of modification by compounds, which increase biodegradability, bioactivity, biocompatibility, etc[110.]

The above behaviours, in connection with special properties of microcrystalline chitosan such as bioactivity, controlled biodegradation or biocompatibility, have created a modern binding agent for paper and nonwovens made from different types of fibres or their blends such as polyester, polyamide, polyacrylonitryle cotton, wool, or viscose fibres.

**4. Biodegradation of chitin and chitosan**

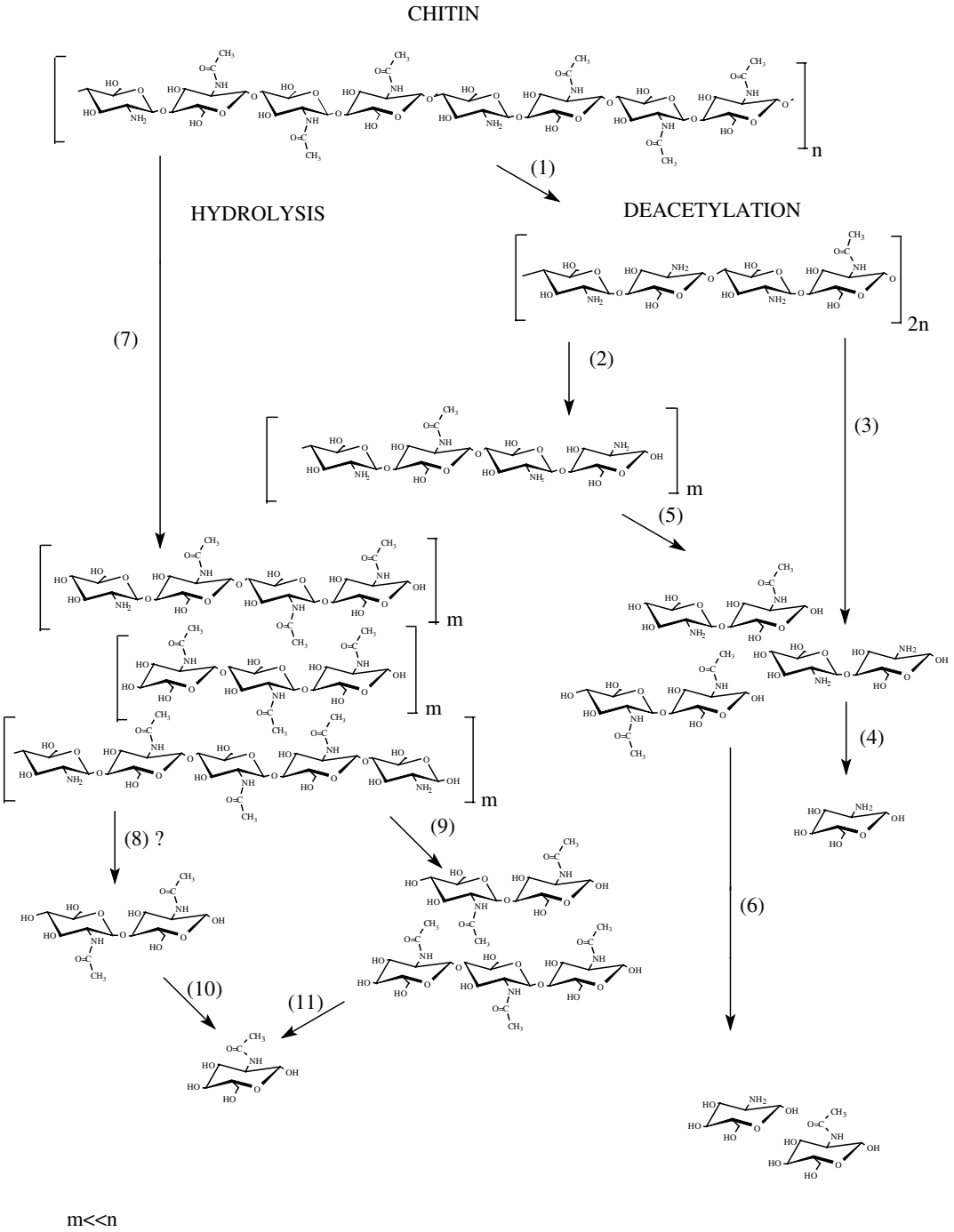
The ability to degrade chitin is thought to involve the action of at least two enzymes: endo-chitinase [E.C. 3.2.1.14] and chitobiase - N-acetyl-β-D-glucosaminidase [E.C. 3.2.1.30] most widely distributed in animals, higher plants, and microorganisms[193]. The synergistic action of chitinases is analogous to that of the cellulase complex. Endo-chitinase (GlcNAcase) randomly hydrolyses biopolymer chains, whereas chitobiosidase (exo-N-N‘-diacetylchitobiohydrolase, [E.C. 3.2.1.29]) existing in fungi *T. harzianum*[194], releases dimeric units from the non-reducing ends of the chains and N-acetyl-β-glucosaminidase cleaves terminal nonreducing N-acetyl glucosamine residue.

However, the process may be more complicated than described above. Endo-chitinase shows liquefying and saccharifying effect[200]. The existence of deacetylase [E.C. 3.5.1.41] in media during hydrolysis of chitin produces faster degradation of polymer. Chitin in the presence of deacetylase is transformed to chitosan cleaved by chitosanases: endo-chitosanase (randomly hydrolyses polyglucosamine chains), exo-chitobiohydrolase (cleaves dimeric units from nonreducing end), exo-glucosaminidase (hydrolyses glucosamine units from nonreducing end) or by chitobiase (Scheme 9).

However, the distribution of chitinases is much wider than chitosanases. Extracellular chitosanases are detected in *Actinomycetes* (especially the species of *Streptomyces*) living mainly in soil[195] and in bacteria (*Bacillus sp., Vibrio sp.,* etc.)[196].

Chitinases are constitutively present in plant seeds, tubers, and flower organs and they are related to the induction of self-defense in response to an exogenous attack by plant pathogens and/or contact with N-acetylchitooligosaccharides[197]. They have been detected in exracellulor, cytosolic, and microsomal fractions from theses organisms. In *Insecta,* the cuticle chitin is digested during the moulting process by a chitinolytic system[198]. In the Antarctic krill before moulting the chitinase and GlcNAcase activities increase shortly to pronounced maximum, indicating the onset of massive resorption of cuticular material[193]. In fungi, they have autolytic, nutritional, and morphogenetic roles, *i.e.*: separation of mother and daughter cells during budding in yeast, local weakening of the wall to permit branching of hyphae or germ tube emergency during spore germination, maintenance of the balance between wall synthesis and lysis at the hyphal tip, and transglycosylation[199].

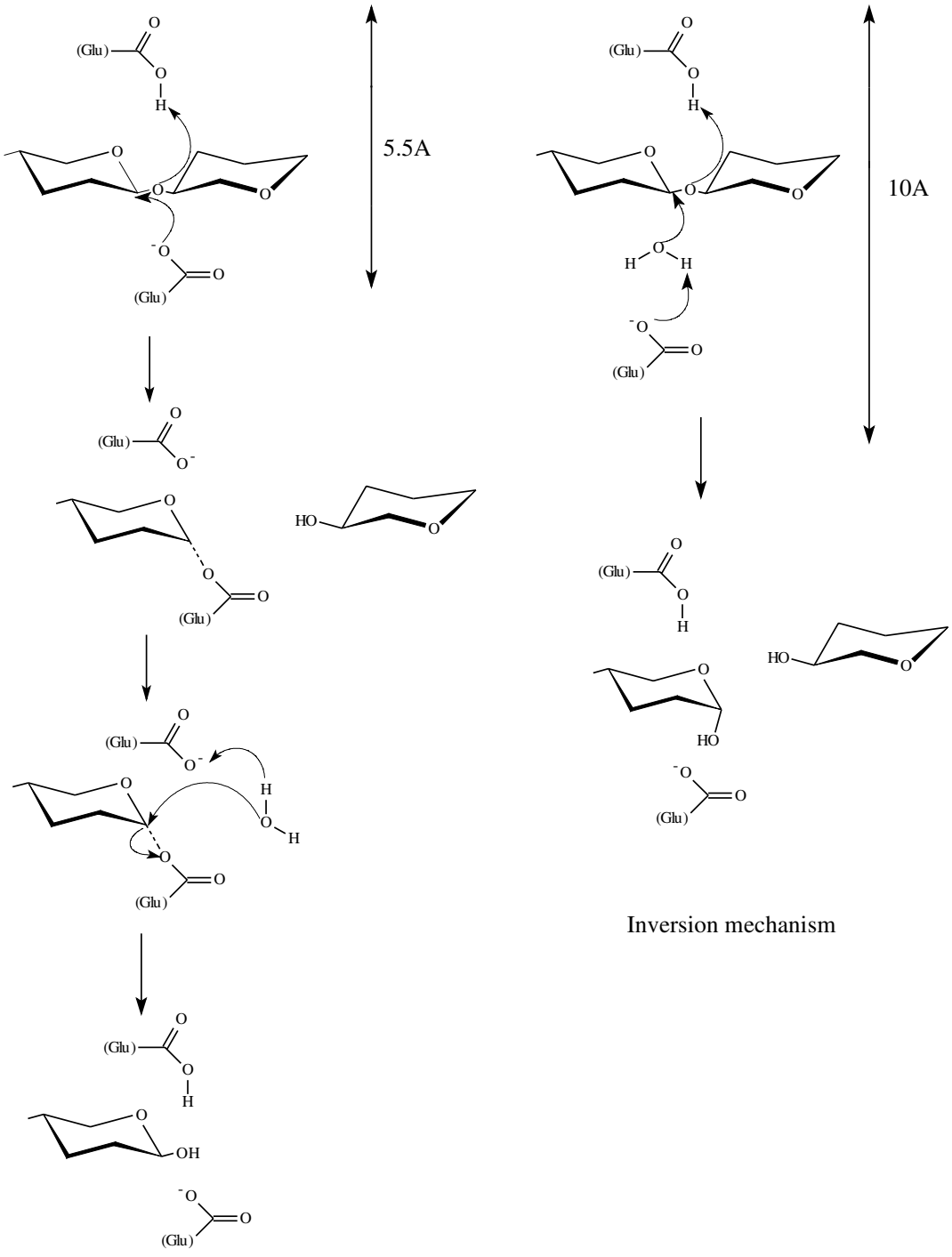
Chitinolytic enzymes also serve as digestive enzymes in fishes, snails, and other marine animals[193]. Lysosomal enzymes are common in human body fluids such as serum, urine, spleen and tears. The presence of chitinases in human serum plays a defense role against chitin-containing phatogens[197].




Scheme 9. Potential enzymatic routes for the degradation of chitin: (1) – chitin deacetylase, (2) – endo-chitosanase, (3),(5) – exo-chitobiohydrolase, (4) – exo-glucosaminidase, - (6) – chitobiase, (7) – endo-chitinase (liquefying), (8) – exo-N-N'-diacetylchitobiohydrolase (?), (9) – endo-chitinase (saccharifying), (10) – exo-N-acetylglucosaminindase, (11) – N-acetylglucosaminidase (N,N'-diacetylchitobiose) [200]

In the fibroblast and some other cell types of higher animals (endothelial cells, lymphocytes, hepatocytes, smooth-muscle cells) no mature forms of lysosomal enzymes are secreted. The macrophages are known to release by mature lysosomal enzymes upon stimulation[193]. Enzymatic hydrolysis of the 1,4-β-gylcosidic bonds takes place by the acid-base catalysis that requires two critical residues: a proton donor and a nucleophile base. This hydrolysis can give a rise to ether and overall retention or an inversion of anomeric configuration.Two variations of the classical acid-base catalysis mechanism common for all glycosyl hydrolases were proposed. The first variation, named "retention mechanism", involves a protonated acidic residue as proton ($H^+$) donor (i.e., carboxyl group of Glu residue) and negatively charged aminoacid, electrostaticlly stabilizing the positive charge of the C1 atom formed during catalysis.

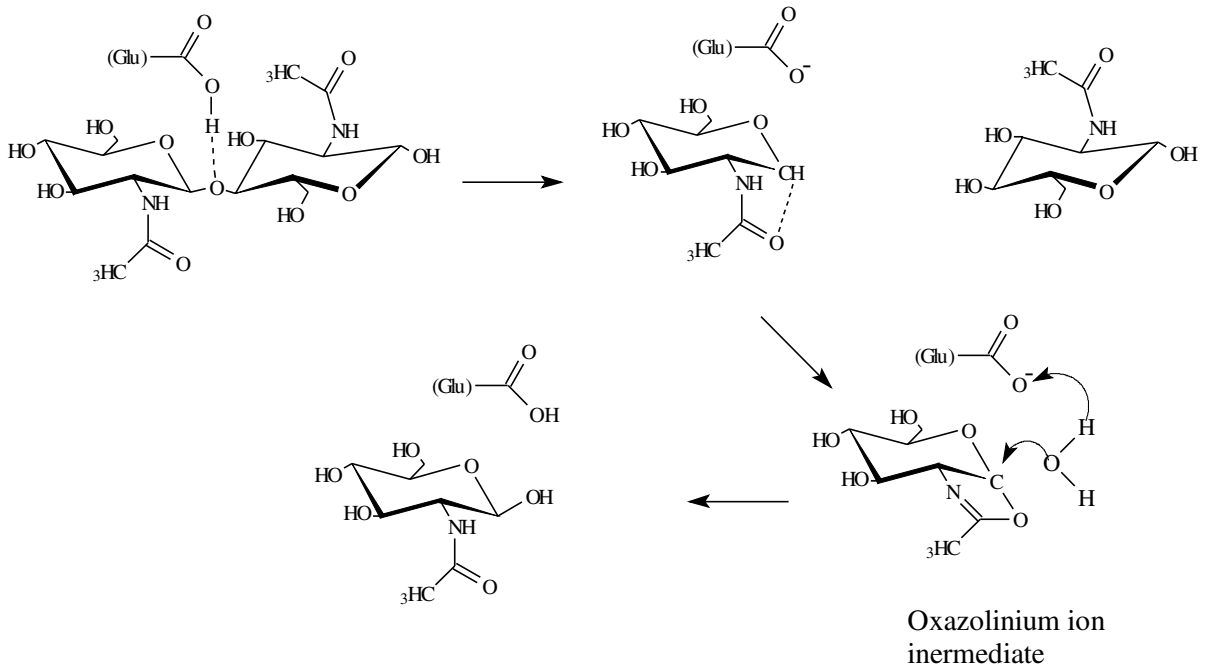
The carboxyl group of aminoacid donates a $H^+$ to glycosidic oxygen, causing it to be a better leaving group due to the polarization of the scissile bond. The product leaves and the remaining sugar acquire a positive charge, called a carbonium ion or oxocarbonium intermediate. The negatively charged residue is thought to stabilize the oxocarbonium ion intermediate, or to act as a nucleophile to form a covalent intermediate (glycosyl-enzyme). Then the intermediate reacts with an activated $H_2O$ (OH-) from the equatorial side, leading to retention of the anomeric configuration of the C1 (Scheme 10).




Scheme 10. Variations of the classical acid-basic catalysis mechanism of the hydrolysis of 1,4 glucosic bond[201]

In the second variation, named “inversion mechanism”, the stabilizing base (negative charged residue) is too far from the C1 atom to stabilize the positively charged carbonium ion intermediate. That

residue facilitates the polarization of the water molecule from the solution attacking directly from the free axial side, leading to an inversion of the anomeric configuration[201].
In the case of hydrolysis of acetamido sugar derivatives (i.e. chitin), this above-described retention mechanism undergoes some modification. The acetamido group of chitin or its oligosaccharides has the particular feature of being able to form an oxazoline intermediate (similar to the chitinases inhibitor allosamidine[202]), from which the reaction proceeds with the retention of the anomeric configuration (Scheme 10).
The initial phase of this reaction is the formation of hydrogen bonding between the catalytic acid (i.e., from Glu) and the oxygen responsible for the glycosidic linkage followed by destruction of this bond. After cleavage of 1,4 glycosidic bonds a N-acetylglucosamine unit is released and the second one stabilized as oxazolinium ion by enzyme. The OH group resulting from water molecule completes hydrolysis. While the catalytic center (catalytic acid) is recovered by proton the second monomer leaves the enzymes (Scheme 11)[201,203].



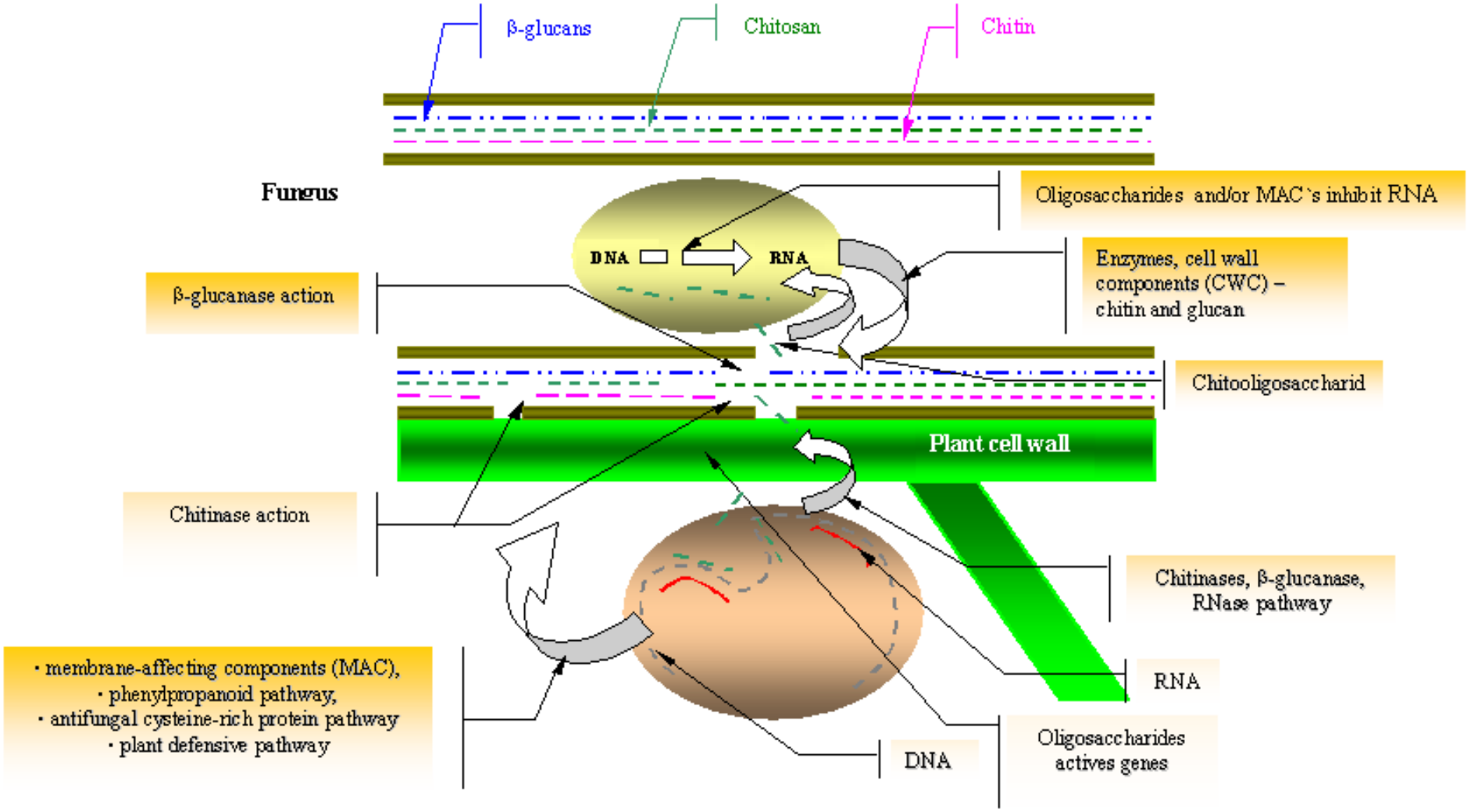

Scheme 11. Reaction mechanism of the hydrolysis of chitobiose by N-acetylglucosaminidase[203]

Other enzymes were reported[204,205] to degrade chitin and/or chitosan. Papain, used in the food industry for the tenderization of meat by controlled hydrolysis of muscle protein, may be applied as a hydrolyzing agent of chitosan. There are many advantages: low cost of commercial enzymes, acceptance in the food industry, etc.
This enzyme prefers the degradation of long chains at room temperature, producing low molecular oligosaccharides, which show a number of interesting biological activites. In addition, the action of several lipases (except human lipase)[204,205], cellulases and hemicellulases[204] on chitosan was reported. This phenomenon resulted from the investigation of hyaluronidase, hexosaminidase, glucoronidase, sulphatase, cathepsins and other proteinases to the synergistic degradation of chitinous materials[193].

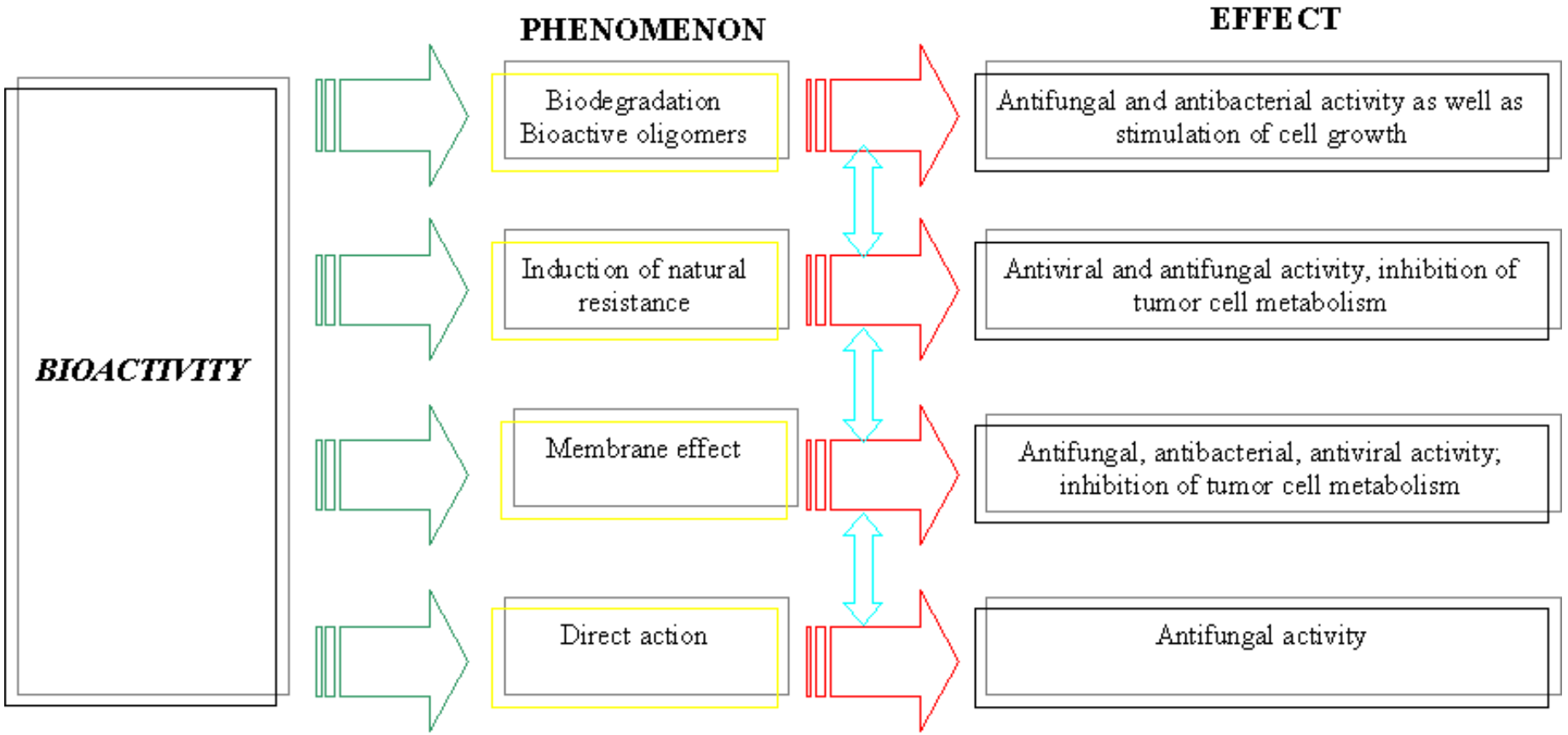
**5. Bioactivity and biocompatibility**

Chitosan, as well as its degradation products, possess antibacterial, antifungal and antiviral properties. However, the mechanism of their action on the pathogen metabolism is still not precisely known. Bioactivity of chitosan and its derivatives is connected with several phenomena, including biodegradation, an induction of natural resistance, membrane effect or direct action against phatogens. Participation of these phenomena in the creation of chitosan bioactivity can be related to the type of chitosan forms, pathogens, or organisms attacked. Chitosan and its derivatives act in dependence on the type of existing hazards, like infection of phytopathogenic bacteria and fungi, viruses or plant growth stimulation, wound healing, and the structure of chitosanous agents[216].

Chitin and chitosan are natural components of the cell walls of many soil fungi that are harmful for plants. During evolution, plants shaped the self-defense mechanism against pathogen fungi. Chitosan is a minor component – about 1 wt% of carbohydrates of the cell wall, however dormant forms of organisms in the soil contain large accumulations of chitosan[170]. This suggests that an increase of chitosan in fungi may initiate the dormant phase, thereby making it harmless to plants. The mechanism of these alterations by chitosan is not well understood, although the preliminary data indicate that it may inhibit the transcription or accumulation of RNA. It is known that the polymer should have a low molecular weight in order to limit the growth of fungus[206]. One possible mechanism is that the positively charged amine groups in chitosan complexes with the cell's DNA[206]. However, another cationic polymer, poly-L-lysine, can also influence cellular structures like membranes, so perhaps the interaction of chitosan and fungal cells is more complex[170]. Chitinolytic enzymes are also synergistic with compounds, which affect cell membranes (membrane-affecting compounds – MAC). MAC`s include plant proteins (osmotin, zeamitin, and other thaumatin-like protein), killer factors (phytoalexin, chemical fungicides), fungal and bacterial antibiotics, and other compounds[207]. Research using radioisotopes in a system containing pea plant and a fungal pathogen (*Fusarium sp.*) has shown that enzymes (endo-β-glucanase and endo-chitinase), existing in lower activity in the pea tissue, break down the fungal cell wall, releasing chito-oligosaccharides, which migrate into fungus and plant cells. Degradation of the fungus cell wall is partially balanced by the deposition of new cell wall components (CWC) by the action of chitin and β-glucan synthases. In the pea, the chito-oligosaccharides become localized in the nucleus, where they appear to enhance the synthesis of about 20 major proteins (endo-chitinases, endo-β-glucanase as well as the phenylpropanoid pathway enzymes creating plant's "killer factors", and other membrane-affecting compounds). This activation may be triggered by the ability of oligosaccharides of chitosan to bind to DNA, thereby altering chromatin structure. Chitosan fragments also enter the fungus cell, where, in some unidentified way, they block the accumulation or synthesis of RNA responsible for the creation of chitin and β-glucan synthases and other compounds[207,208,209,210]. MAC`s alter membranes involved in the production of cell wall compounds and reduce the ability to repair cell wall damage. The increase in the activity of plant cellulase in digesting the cell wall facilitates the penetration of MAC`s, while the increased level of MAC`s reaching their targets supports the action of the lytic enzymes by inhibiting the cell wall turn over and repairing mechanisms[207]. It was reported that transgenic tobacco plants transformed to produce barley chitinases and β-1,3-glucanase were more resistant against *Rhizoctonia solani* than plants expressing those genes individually, providing an argument in favour of synergistic action of those enzymes[211] (Scheme 12.) The initial interaction of polyglucosamine or poly-(N-acetylglucosamine) with plant cells or protoplasts may be affected by their degree of polymerization and/or chemical structure. Chitosan oligomers do not induce lignification in wounded wheat leaves, although chitosan and chitin or its oligomers with DP higher than 4 are effective. Partial N-acetylation or chemical fragmentation of chitosan reduces its ability to elicit callose formation[212]. The same phenomena were observed during viral and bacterial infections in plants as well as animals. The specific response of plants consists of an interaction between chitosan and the plant rather than between chitosan and the virus. Chitosan degree of polymerization and its chemical structure may effect its interaction with plant methabolism[212]. For example, high average molecular weight chitosan did not inhibit viral infections in the tobacco plant but the products of its degradation indicated high antiviral activity[213, 214]. Chitosan may exact influence on induction of specific substances (or messengers), which are systemically transported through the plant where they activate the defense mechanism of the plants against viral infections. It is important to note that other polysaccharide-inhibitors of viral infections – the fungal glucan and cytoplasmic mycolaminaran did not induce systemic resistance. The activity of time-related peroxides (PO), involved in virus diseases, was negatively correlated with the level of systemic resistance induced by chitosan. It may be supposed that the increase of PO activity is an expression of the physiological stress caused by chitosan. PO activity is probably not directly involved in the mechanism(s) of chitosan-induced systemic resistance in bean plants[49]. Low molecular weight MCCh gel-like dispersion, as well as chitosan salts with low and medium molecular weight acted with suitably high effectiveness in the inhibition *in vitro* of selected bacteria[215]. In the case of antiviral activity, MCCh or chitosan salts containing cationic charges with a wide distribution low to medium molecular weights show optimum antifungal activity. However, polyanionic chitosan derivatives such as sulfonated and carboxymethylated chitosan (CMCh) totally lost their bioactivity for bacteria growth[216].




Scheme 12. The model interaction of fungus with pea plants proposed by Hadwiger[170,206]




Scheme 13. Assumed scheme of mechanism for bioactivity of chitosan and its derivatives[216]

Chitosan induces various mechanical defensive reactions in plant cells, such as plugging intercellular spaces with amorphous electron opaque substances and the formation of cell wall appositions, which may be implicated in restricting fungal invasion[217]. Investigations were conducted into the strong inhibitory effect of chitosan salts and MCCh with high average molecular weight on cancer growth by inhibition of lactate formation and a considerable decrease of ATP level in intact Ehrlich ascites tumor (EAT) cells[218]. MCCh with a high degree of deacetylation shows a greater inhibition than chitosan salts with the same $\overline{M}_V$ and lower DD. The removal of neuraminic acid, indispensable for binding of polycations with glycosaminoglycans from the cell membrane or the alteration of environmental pH during the preincubation of EAT cells, eliminates the inhibitory effect of chitosan preparations.

These phenomena indicate that chitosan, acting through the membranes of intact cells, block metabolic cascades connected with the transmission of signals from the cell membrane into its interior and leads to a specific inhibition of the tumor cell metabolism[219]. Low average molecular weight chitosan has lipid-lowering effects by means of its controlled absorption of cholesterol[126].

A bioactivity of chitosan and its modificates seems to be a function of several phenomena related mainly to their structural parameters, including among other:

⇒ average molecular weight,
⇒ polydispersity,
⇒ crystallinity,
⇒ porosity,
⇒ charge character,
⇒ originity,
⇒ type of diseases[220].

At the same time the organism origin plays very often important role in an effectively of chitosan actions. The knowledge of above parameters and phenomena allows creating the chitosan with optimum bioactivity for suitable application. The cationic types of chitosan derivatives, for example, are distinguished by a higher biological action against viruses, compared to the sulphonated chitosan being an anionic type of modificate[220].

Scheme 13 shows the hypothetical mechanism of the bioactivity of chitosan and its derivatives by means of direct action, affecting membrane, action of degradation products, induction of natural resistance, etc.

## 6. The aim of research

The aim of this research is to develop methods for the preparation of biodegradable and/or bioactive films and paper sheets containing chitosan complexed with various proteins.
Another goal is to prepare the composite materials from blends of chitosan with proteins, which can be used as biodegradable packing materials and could also serve as model systems for the construction of enzyme carriers and connective tissue. A considerable amount of work has been published on the interaction of chitosan with collagen[221,222,223,224]. However, it is still little known on its composites with casein and keratin.
The present study is composed of two parts:

⇒ preparation of chitin and chitosan,

⇒ methods and application.

The first part concerning the preparation of chitosans and determination of their properties describes:

⇒ investigation of chitin and chitosan from various sources (crustacean and insect raw materials),

⇒ determination of the physical- and chemical properties of chitin as well as chitosan, especially the degree of deacetylation ( DD ), average molecular weight ( $\overline{M}_v$ ), crystallinity, swelling behaviour and biodegradability,

⇒ development of the most accurate method for determination of the degree of deacetylation,

⇒ comparison of the properties of chitosan prepared from various sources and deacetylated under similar conditions,

⇒ estimation of chitosan biodegradability,

⇒ determination of the composition of the mixture of oligosaccharides released during the degradation of chitosan by the fungus *Aspergillus fumigatus*

⇒ investigation of the inhibition on bacterial and viral phytopathogens by chitosan and/or its oligosaccharides

The second part, developing methods of preparation of chitosan films and paper sheets containing chitosan, is related to:

⇒ preparation of chitosan films from acidic solutions by drying,

⇒ preparation of microcrystalline chitosan (MCCh) gel-like dispersion and MCCh films containing selected proteins,

⇒ estimation of the alteration of average molecular weight and degree of deacetylation during the preparation of microcrystalline chitosan gel-like dispersion,

⇒ determination of the effect of average molecular weight as well as degree of deacetylation on the swelling behaviour of MCCh gel-like dispersion and films,

⇒ determination of swelling behaviour as well as mechanical strength and elastic properties of films,

⇒ estimation of the influence of crosslinking on swelling of MCCh films in acetic solution,

⇒ production of paper sheets containing chitosan-protein compositions,

⇒ assessment of the mechanical properties of paper in dry and wet condition,

⇒ determination of the biodegradability of films and paper sheets containing microcrystalline chitosan prepared in the presence of proteins (biodegradation and mineralization tests).

# EXPERIMENTAL

## Chapter I: Preparation of chitosan from various sources

## 1. Materials

During the research, the chitins from various sources as listed below, were used.
*Chitinous materials from crustaceans:* Technical grade chitin containing approx. 5.0 wt% ash from the Antarctic krill of *Euphausia superba* was used in the form of powder (samples specimen M – DD of 51,5%; K – DD of 52%). This chitin was obtained from the stocks of Fishcontract GmbH, Bremerhaven, Germany.
The shells (samples specimen P) from the North Atlantik shrimp *Pandalus borealis* was obtained as residues of shrimp meat production by Batsfjord, Aarsether AS (Norway). All materials showed a gray colour before demineralization.
*Chitinous material from insects:* Chitin was obtained from the cuticles of larvae of *Calliphora erythrocephala* cultivated in the Ing.-Büro für Aquakultur und Umwelttechnik, Potsdam, Germany.
*Chitin from Hammarus pulex:* As a reference, non-purified chitin obtained from Institute Giprorybflot, St. Petersburg, Russia, was used.
Sodium hydroxide, acetic acid (glacial), 1,4-dioxane, hydrochloride acid (fuming), sodium chloride (extra pure), perchloric acid (70 wt%), potassium hydrogen phthalate were obtained from Fluka, urea was from Merck and N,N-dimethylacetamide from Aldrich.

## 2. Analytical Methods

2.1. Determination of average molecular weight of chitosan and chitin

Viscometry is one of the simplest and most rapid methods of determining average molecular weight. The determination of polysaccharide solution viscosity is usually carried out by comparison of the flow time $t_1$ required for a certain volume of polymer solution to flow through a capillary tube with the corresponding flow time $t_0$ for the pure solvent. If the polymer concentration is given as $g \bullet dL^{-1}$, intrinsic viscosity ($\eta$) will be expressed as $dL \bullet g^{-1}$.
The average molecular weight of chitosan was determined by the viscosimetric method indicated a Limiting Viscosity Number (LVN) for a solution containing:

a) 0.2 M of acetic acid, 0.1 M of sodium chloride and 4 M of urea ( $\overline{M}_V$ in the range from 113,000 to 492,000) using literature value for $K=8.93 \bullet 10^{-4}$ and a=0.71 [225]
b) 0.1 M of acetic acid and 0.2 M sodium chloride ( $\overline{M}_V$ in the range from 90,000 to 1,140,000) using literature value for $K=1.81 \bullet 10^{-3}$ and a=0.93 [225]
c) 0.33 M of acetic acid, 0.3 M of sodium chloride ( $\overline{M}_V$ in the range from 13,000 to 135,000) using literature value for $K=3.41 \bullet 10^{-3}$ and a=1.02 [225]

K and a are constants that are independent of molecular weight over a considerable range of molecular weights. They depend on the polymer, solvent, temperature and, in the case of polyelectrolytes, the nature and concentration of the added low-molecular-weight electrolyte. The constants are normally evaluated from a plot of $\ln[\eta]$ versus $\ln[\overline{M}_v]$ for a series of carefully prepared fractions having very narrow molecular weight distribution values.
The average molecular weight of chitin was determined in a dimethylacetamide solution containing lithium chloride (DMAc-LiCl solvent)[36-37]. The values of $K = 2.2 \times 10^{-4}$ $dl \bullet g^{-1}$, a = 0.88 were used for a LiCl concentration of 50 $g \bullet dm^{-3}$. The increase in LiCl concentration caused the increase of LVN of chitin solution.
The Ubbelohde's viscometer was kept at a constant-temperature by means of a water bath at a temperature of 25.0 ± 0.1°C. The solvent flowing times were preferably greater than 100 s. Chitosan average molecular weight was determined using the Ubbelohde's viscometer Type 53110/I. In the case of chitin, a Type 53113/Ic viscosimeter was used (both from Schott GmbH).
For the preparation of chitosan solution, the weighed determined sample was added, with shaking to a known amount of the solvent system (a, b, or c). When the dissolution was complete, the solution was filtered through a Schott glass sintered filter (No. 1). The correct concentration of dissolved polysaccharide was calculated as the difference between the initial amount of polymer and the insoluble part using below equation:

$$C = \frac{m_1 - (m_2 - m_0)}{V} x100 \quad [1]$$

where:

**C** - concentration of chitosan solution (g/dL),

**V** - volume of chitosan solution (ml),
**$m_0$** - weight of dry filter (g),
**$m_1$** - weight of chitosan sample (g),
**$m_2$** - weight of filter containing insoluble particles after drying (g).

The chitosan solution must be analyzed as soon as possible because of the depolymerization phenomenon (Figure 1).

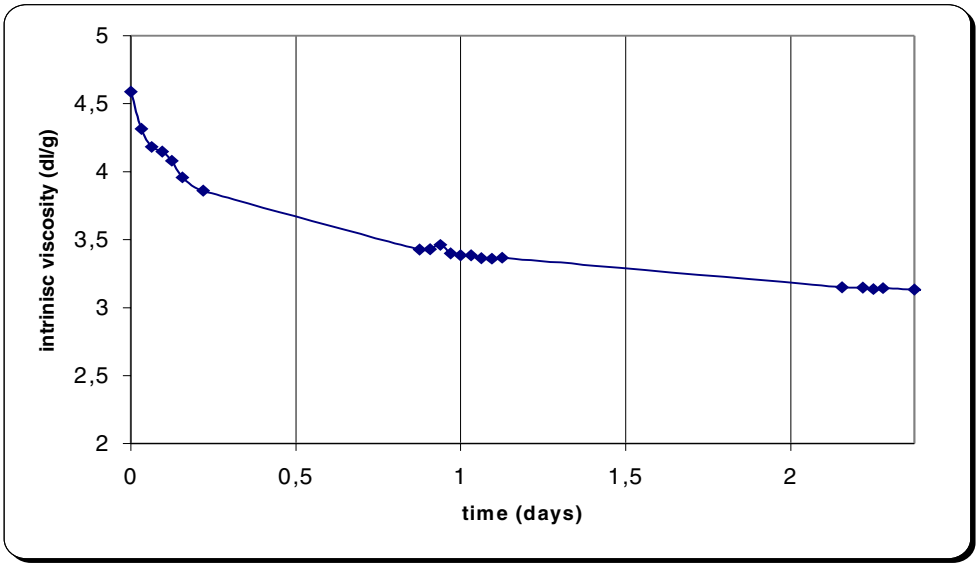



Figure 1. Reduction in the intrinsic viscosity of chitosan in a solvent system containing: acetic acid, sodium chloride and urea in the proportions described above

The system of DMAc/LiCl was prepared by rapidly weighing salt (dried at 130 $^{o}$C before use) and adding the required solvent. The LiCl was dissolved after shaking for 12. The investigated solution of chitin in the DMAc/LiCl solvent was prepared by addition of a weighted polymer to the DMAc/LiCl solvent in a measuring flask (25 cm$^3$). After preliminary swelling, the investigated medium was shacked for a long period (usually for 12-24h). By filtration of the sample through glass sintered Schott filter (No. 1) the swollen, insoluble particles were removed. The concentration of the remaining polymer was determined as described previously (equation 1.).
The intrinsic viscosity was determined according to the equation:

$$\eta_i = \frac{(t_1 - t_0)/t_0}{C} \qquad [2]$$

where:

**C** - concentration of chitosan,
**$t_1$** - flowing time for the chitosan solution,
**$t_0$** - flowing time for the solvent system,
**$\eta_i$** - intrinsic viscosity.

The limiting viscosity number (LVN) was found by the extrapolation of Mark-Houwink's relationship between the intrinsic viscosity and the concentration of chitosan in the investigated solution to concentration of chitosan of zero.
The average molecular weight was obtained according to the equation:

$$\overline{M}_V = \sqrt[a]{\frac{\eta_L}{K}} \qquad [3]$$

where :

**$\overline{M}_V$** - average molecular weight,
**$\eta_L$** - Limiting Viscosity Number,
**K, a** - Mark- Houwink constants

The average molecular weight was calculated as an average of duplicate measurements. The accuracy of it was 10%.

2.2. Determination of the DD of chitosan by potentiometric titration

The DD of chitosan samples was determined by potentiometric titration [23,24] in non-aqueous media (anhydrous acetic acid, 1,4-dioxane). The glass indicator and calomel electrode were connected by a salt-bridge, where a saturated solution of potassium chloride was used. A small quantity of water was introduced into an anhydrous medium of acetic acid by aqueous solution of acetic acid, which was necessary to dissolve the chitosan sample. This amount of water, not more than 4 wt%, does not interfere with the detection of the titration end-point. The DD value was calculated as an average result for the titration of three samples. DD accuracy of the measurement was 3%.

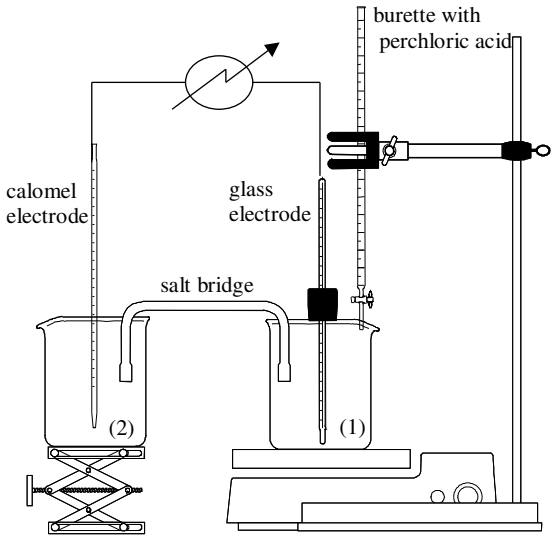



Figure 2. Titration equipment for the potentiometric determination of the chitosan DD: (1) - chitosan sample in the mixture of acetic acid and 1,4-dioxane, (2) - solution of potassium chloride

*Preparation of the samples:* Samples of chitosan (dried at 60°C to a constant mass of 0.05 - 0.075 g, weighed with an accuracy of $\pm 0.0001$ g) were dissolved in 5 $cm^3$ of 1 wt% aqueous acetic acid in 50 $cm^3$ volume measuring flasks. The flasks were filled to 50 $cm^3$ with anhydrous acetic acid and 20 $cm^3$ of this solution were mixed with 20 $cm^3$ of anhydrous acetic acid and 10 $cm^3$ of 1,4 - dioxane was transferred into a measuring vessel.

*Preparation of perchloric acid:* To 5 $cm^3$ of a solution of 2 g of potassium hydrophthalate (previously dried at 105°C, weighed with an accuracy of $\pm 0.0001$ g) in 100 $cm^3$ of anhydrous acetic acid, 20 $cm^3$ of 1,4-dioxane were added, and the volume was filled up to 50 $cm^3$ with anhydrous acetic acid. This solution was titrated with a solution of perchloric acid (17 g of 70% $HClO_4$ filled to 1000 $cm^3$ with 1,4-dioxane). The equivalent weight of perchloric acid was determined according to the equation:

$$M = \frac{0.49}{V} \qquad [4]$$

where :

**V** - volume of $HClO_4$ at neutralization point,

**M** - equivalent weight per $dm^3$.

*Determination of chitosan DD:* Solutions of chitosan were titrated with a solution of $HClO_4$ in anhydrous acetic and the neutralization point was determined potentiometrically. The volume of perchloric acid corresponding to the neutralization point of amino groups was found by correlation of the electromotive power (EMP) with perchloric acid volume. The concentration of amino groups (-$NH_2$) was calculated according to the equation:

$$\left(-NH_2\right) = \frac{M \times v_1 \times V}{m \times V_0 \times 6.211} \qquad [5]$$

where :

**M** - equivalent weight per $dm^3$ of $HClO_4$,

**m** - weight of chitosan sample (g),
$\mathbf{v_1}$ - volume of $HClO_4$ solution at neutralization point ($cm^3$),
**V** - volume of dissolved chitosan solution (100 $cm^3$),
$\mathbf{V_0}$ - determined sample volume (20 $cm^3$),
**6.211** – theoretical concentration of amine group that can be deacetylated.

2.3. FT-IR spectroscopy

IR spectra of investigated samples were recorded on a Mattson FTIR spectrometer. For calculation of DD, the intensity of the amide I bands ($\nu$ = 1650 $cm^{-1}$ and at $\nu$ = 1630 $cm^{-1}$ and $\nu$ = 1660 $cm^{-1}$)[7,20] and were used as the analytical band and that of the hydroxyl band ($\nu$ = 3450 $cm^{-1}$)[20] and C-O band ($\nu$ =1070 or $\nu$ =1030 $cm^{-1}$)[7] as the reference band.
DD values were calculated according to equation [6] either from the height of peaks or the peak area, in this case using Voight's or Lorenzt's function. Background of the spectra was subtracted based on the individual peaks and on the group of peaks, using either the computer software of the instrument or PeakFit for Windows. For calibration, a fully *N*-acetylated chitin sample was taken.

$$y = \frac{a_0}{a_2 \times \sqrt{\Pi}} \times \int_{-\infty}^{\infty} \frac{\exp(-t^2)}{a_3^{\,2} + (\frac{x - a_1}{a_2})^2} dt \qquad [6]$$

where:
$a_0$ – amplitude of the peak,
$a_1$ – maximum of the peak height,
$a_2$ – width of the peak,
$a_3$ – a parameter of the peak form.

The value of the peak area was calculated from the peak obtained by the approximation of Voight's or Lorenzt's function.

*Preparation of KBr disks:* Chitosan samples (2 mg) were dried overnight at 60°C under reduced pressure. Then they were mechanically blended with 100 mg of the KBr and disks were prepared. The thickness of KBr disks was 0.5 mm. These were dried for 24 h at a temperature of 110°C under reduced pressure before measurement [15,226,227,228,229].

*Preparation of chitosan films:* The films were prepared from 1% (w/v) solution of chitosan in a sodium chloride 0.2M/acetic acid 0.1M/water mixture. A strictly defined volume of this solution was applied on a polyethylene substrate to obtain a constant-thickness layer film. To the surface of the specimen, 5% (w/v) NaOH solution in ethanol was added very carefully as a coagulant. During coagulation a jelly-like film was formed. This film was rinsed several times with distilled water and air-dried at a temperature of 70 °C. Dry chitosan films were elastic, transparent, and of uniform thickness[227-228,230].

2.4. Solid state $^{13}C$ – NMR

CP/MAS $^{13}C$ NMR[14,228-229] spectra were recorded on a UNITY 400 (100 MHz) NMR spectrometer at room temperature. Samples of 0.3 $cm^3$ of powdered chitosan were placed into 5 mm ZrO cups and then sealed with Vespel caps. Spinning rate: 5-6 kHz; repetition time: 3 s; radiofrequency field strength used for dipolar decoupling: 50-70 kHz; linebroadening: 10 kHz; number of transients: 5,000; external standard: adamantane were applied. DD was calculated from the intensity ratios of signals for the methyl and anomeric carbons using the special computer software accompanying the spectrometer.

2.5. Determination of WRV (water retention value)

The water retention value (WRV)[167] of chitosan was determined by soaking tested samples in distilled water. After 20 h, the water was removed by the filtration using a filtration fabric and then centrifuged for 10 min. at 4,000 rpm (Hevaeus Sepatech Megafuge 1.0, with a rotor diameter of 225 mm). The sample weight was determined after centrifuging ($m_1$) and after drying to constant weight at 105°C ($m_0$). The water retention value calculated according to the equation:

$$WRV = \frac{m_1 - m_0}{m_0} \times 100\% \qquad [7]$$

where :

**$m_1$** - weight of samples after centrifuging
**$m_0$** - weight of samples after drying.

2.6. Determination of the water content

The procedure for preparing samples was the same as in case of WRV determination. Coefficient of water content (Wc)[231] characterizes the access of water bonding with an internal surface of a polymer. The water content was defined using the following equation:

$$W_C = \frac{m_1 - m_0}{m_1} \times 100\% \quad [8]$$

where:

**$m_1$** - weight of sample after centrifuging,
**$m_0$** - weight of sample after drying.

2.7. Determination of swelling coefficient

The swelling coefficient (Cs)[167] is a measure for the hydrophilicity of the investigated samples. It was determined according to the principles described above for WRV determination. However, the centrifuging of the sample was done at 3,000 rpm for 2 min.

2.8. Determination of the moisture content in chitosan samples

Chitosan samples were weighted before and after drying at 105°C for 4-16h to obtain a constant weight[232]. The moisture content in chitosan samples was determined according to the equation:

$$M_c = (1 - \frac{m_{10}}{m_0}) \times 100\% \quad [9]$$

where:

**$m_{10}$** - weight of samples after drying,
**$m_0$** - weight of samples before drying.

WRV, Cs, Wc and Mc were determined as an average of two repetitions. The accuracy of their was 3%.

2.9. Determination of ash content

Ash content in chitosan was determined gravimetrically. The combustion temperature was 600°C.

2.10. Determination of the crystallinity index

The crystallinity index (CrI) of chitin and chitosan samples was determined by X-ray diffraction using a Rigakin-Denki Diffractometer (Japan), with continuous registration in the angle 2θ range from 5° to 32° with the wavenumber of 0.154 nm using the scintoscopic center and radiation – CuKα. The crystallinity index was calculated from the equation [10], usually used for cellulose[233]:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100\% \quad [10]$$

where:

$I_{002}$ - crystallinity diffraction pattern intensity at 2θ ~20°,
$I_{am}$ - amorphous diffraction pattern intensity at 2θ ~12° 30'.

2.11. Determination of reducing sugar content

Reducing sugar content was determined by the method described by Miller[234] according to the modifications below described:

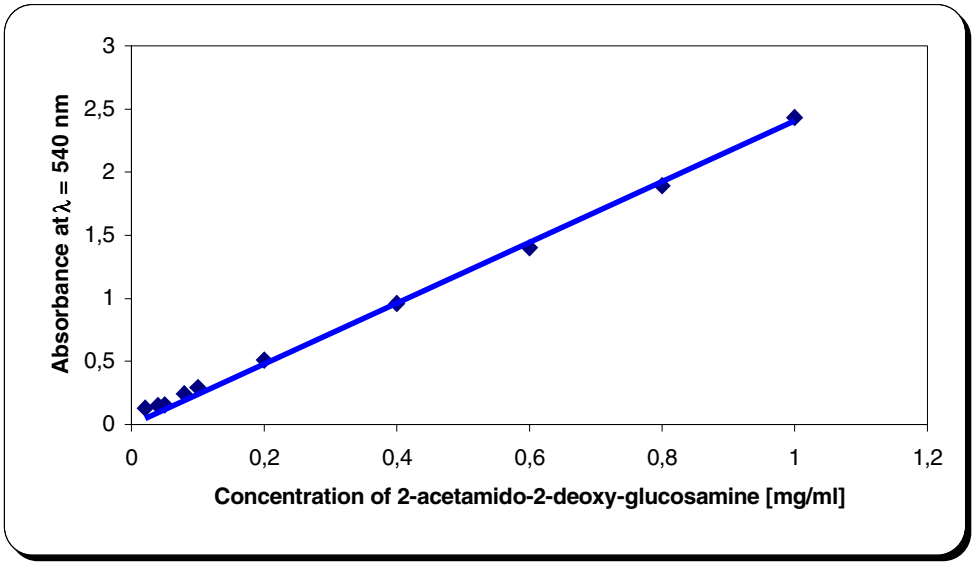



Figure 3. Calibration curve for determination of reducing sugar content in calculation to 2-acetamido-2-deoxy-β-D-glucosamine

An aliquot of the supernatant (1 $cm^3$) obtained after the fermentation of chitosans by fungus *Aspergillus fumigatus* was diluted with $H_2O$ (9 $cm^3$), and 1 $cm^3$ of this solution was mixed with 3 $cm^3$ DNS reagent (5 g 3,5-dinitrosalicylic acid in 100 $cm^3$ $H_2O$ mixed with 8 g NaOH in 75 $cm^3$ $H_2O$, heated, then 201.5 g K-Na-tartrate • 4 $H_2O$ was added, and the solution was filled up to a volume of 500 $cm^3$ with $H_2O$) and incubated in a water bath at 100°C for 5 min. The samples were cooled then with tap water and the absorbency was determined by comparison to the control (1 $cm^3$ water plus 3 $cm^3$ DNS reagent) at λ = 540 nm. GlcNAc (Aldrich) was used as a standard for the preparation of calibration curve.

2.12. HPLC analysis[235]

The HPLC apparatus consisted of a PU-987 pump and a MD-910 diode array detector (JASCO). The mixture before the separation was N-acetylated according to the procedure described below (2.12.1.). The sugars were separated on an Amide 80 column (4.6 x 250 mm, Toso Haas, Stuttgart) with acetonitrile-water 68:32 as the mobile phase, flow rate: 1.0 $cm^3 \bullet min^{-1}$, detection at 205 nm. Samples containing less than 50 μg of reducing sugar in 10 μl of solution were injected. Anomeric mixtures gave two closely eluting peaks of the α- and β-anomer.

2.12.1. N-acetylation of oligosaccharides

Incubation mixtures were filtered through cellulose filters (Schleicher & Schuell GmbH, Germany) and the filtrates were lyophilized. The dry residue was dissolved in 2 $cm^3$ of bidistilled water and 4 $cm^3$ of methanol were added dropwise under agitation, followed by 0.3 $cm^3$ of acetic anhydride. The temperature was adjusted not to exceed 15°C by means of a temperature sensor (IKATRON ETS D3; IKA Labortechnik). Following addition of 2 $cm^3$ of methanol, the mixtures were allowed to evaporate to ca. half of the original volume under air (Figure 4)[205]. The mixture of oligosaccharides was N-acetylated by acetic anhydride-$d_3$ [$(D_3CCO)_2O$] in order to label free amine groups.

2.13. Mass spectrometry

Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed using a Reflexx MALDI-TOF mass spectrometer (Bruker-Franzen, Bremen)[236]. The instrument was calibrated with peptides of known molecular weight. Matrix: 2,5-dihydroxybenzoic acid (dissolved in 25 $g \bullet dm^{-3}$ in 35% acetonitrile, 65% 0.1% TFA. The analytical sample was prepared by mixing 1 $cm^3$ of matrix and 0.2 $cm^3$ of sample containing ca. 2 μg reducing sugar on the target and drying under air.

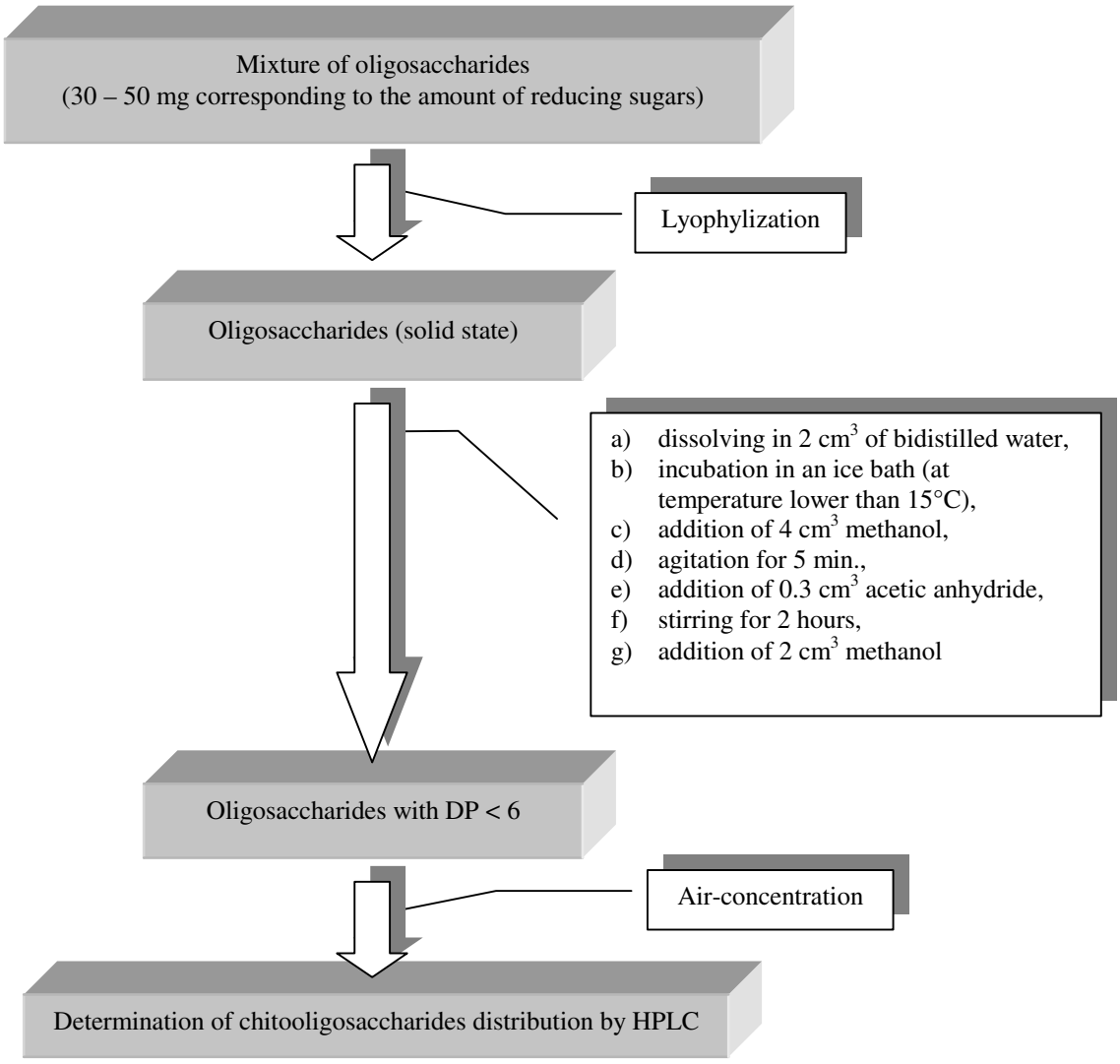



Figure 4. N-acetylation procedure of oligosaccharides

2.14. Gel permeation chromatography (GPC)

The following instrumentation was used: HP 1050 chromatograph (Hewlett-Packard,Waldbronn, Germany) equipped with a RI detector HP 1047A; PL-GFC 4000 A and PL-GFC 300 A columns; and PL CaliberTM GPC/SEC Software Version 5.1 (Polymer Laboratories, Ltd. Shropshire, UK).
As a solvent, acetate buffer at pH = 4.3 was applied. Calibration was carried out using the narrow standards: polyethylene glycol and polyethylene oxide.
70 µl of 0.1 wt% of investigated solution was injected to the columns set at the ambient temperature. K and a of sample was $K=7.4 \times 10^{-6}$, a=0.76 and for standards $K=6.5 \times 10^{-6}$, a=0.625 according to Rinaudo[237].

**3. Preparative methods**

3.1. Processing of chitin and chitosan from *Pandalus borealis*

3.1.1. Separation of chitinous materials

*Deproteinization procedure:* 400 $cm^3$ of aqueous 2 M sodium hydroxide solution was added to 20 g of shells of shrimp (calculated as dry weight) of *Pandalus* and the mixture was stirred for 2h at 100°C (at 400 rpm). The deproteinized shells were washed with distilled water until neutrality. The shells were then air-dried and ground to a coarse powder in a limping mill.

*Demineralization procedure:* 350 $cm^3$ of 0.5 M hydrochloric acid solution was added to 15 g deproteinized shells and this mixture was stirred for 3 h at a room temperature. After that the crude chitin was washed using distilled water to obtain pH=7.0 and then air-dried.

*Decolouration procedure:* Pigments were extracted with a 10-fold amount of ethyl alcohol (relatively to dry weight) for 2h at a temperature of 50°C with shaking at 100 rpm. The chitin was then washed with distilled water, next with 50 $cm^3$ ethanol and air-dried. Decolouration was also effected during the deacetylation of chitin using strong alkali.
The overall procedure of removing the discussed compounds is presented in Figure 5.

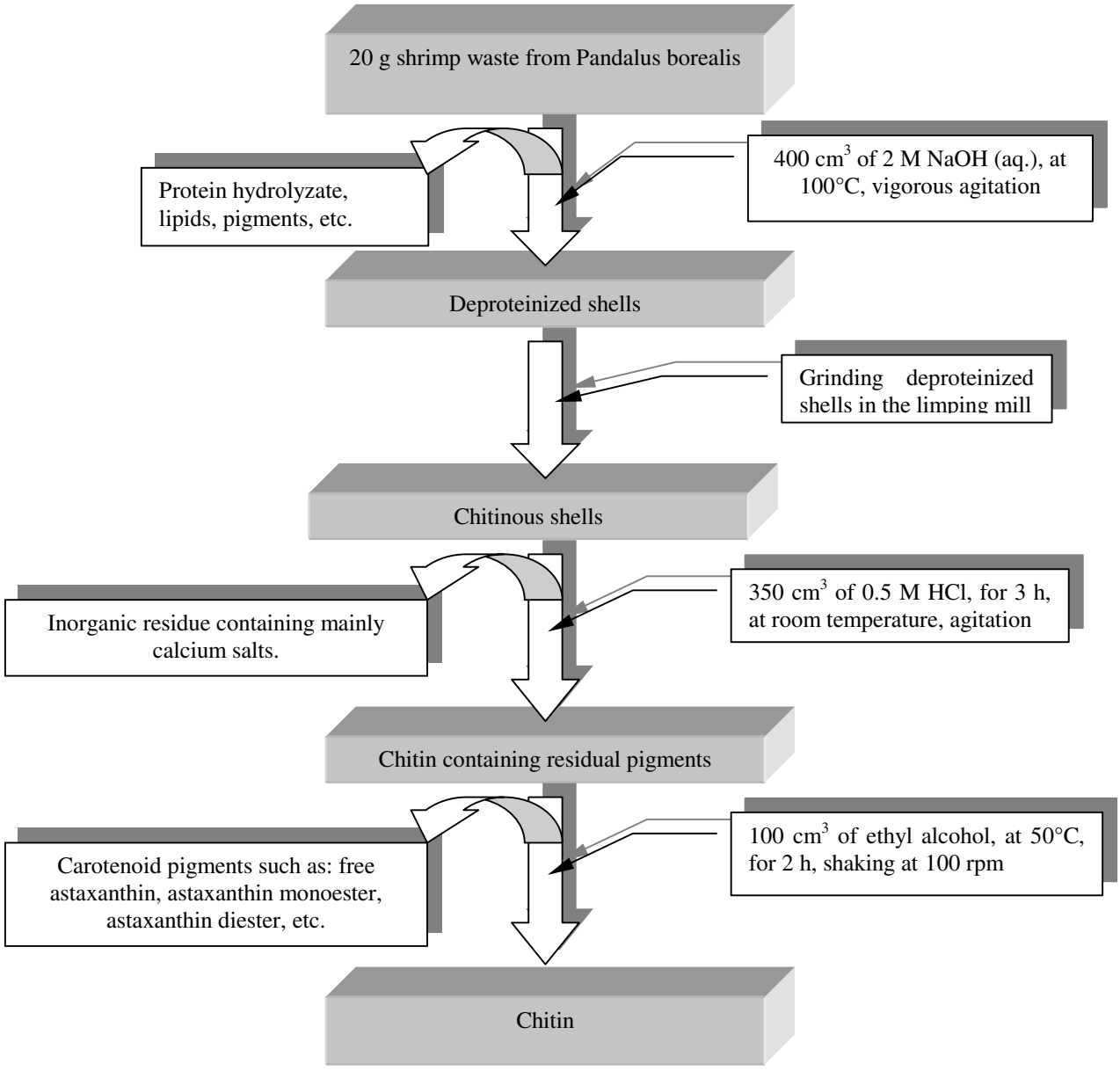



Figure 5. The process of chitin preparation of *Pandalus borealis* shrimp shells

3.1.2. Deacetylation of chitin

*Primary procedure:* 250 $cm^3$ of 50 wt% aqueous sodium hydroxide solution was added to 7.8 g of chitin in a glass flask. The mixture was stirred for period ranging from 2 to 7 h at temperature of 100°C (PA) and 120°C (PB). After that, the chitosan was washed using distilled water to pH 7.0 and air-dried.
Figure 6 shows the equipment used for the deacetylation process of chitin.

*Additional deacetylation:* Because the deacetylated product was still not soluble in 1 wt% acetic acid it was necessary to carry out additional deacetylation.

3.9 g partially soluble samples after primary deacetylation carried out at 100°C or 120°C for 5h or 7h were additionally treated with 125 $cm^3$ 50 wt% sodium hydroxide solution at 100°C (PA) or 120°C (PB) for 1h (PA7, PB7), 3h (PA7, PB7) or 4h (PA5, PB5). After that, the samples were washed until neutrality and air-dried.

3.2. Preparation of chitosan from *Euphausia superba*

Because the ash content of chitin prepared from *Euphausia superba* shell was over 4.0 wt% it was necessary to remove the residual calcium carbonate by an additional demineralization step.

3.2.1. Demineralization of chitin

350 $cm^3$ of 0.5M aqueous solution of hydrochloric acid was added to 8.0 g of powdered chitin and this dispersion was stirred for 3h at room temperature. The purified chitin obtained was then washed with distilled water until pH=7.0 and then air-dried.

3.2.2. Deacetylation of chitin

Chitosan was obtained from both purified and non-purified krill chitin according to the conditions described in section 3.1.2. The deacetylation process was carried out using a solution of sodium hydroxide with a concentration of either 40 wt% or 50 wt% at temperature of 100°C (A), 120°C (B) or 130°C (C).

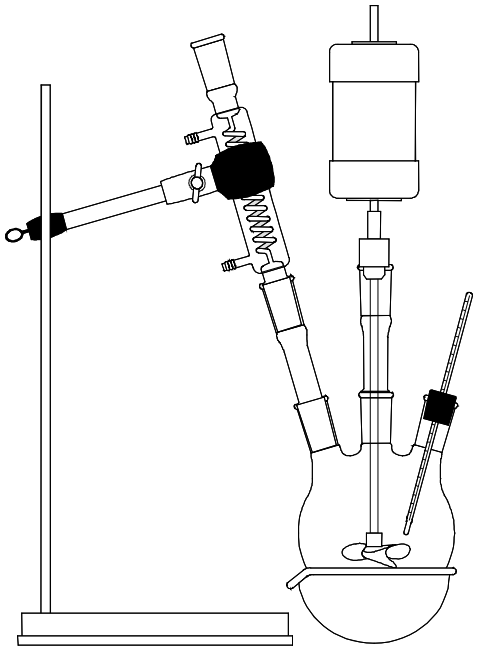


Figure 6. Equipment for the chitin deacetylation process

3.2.3. Gradual deacetylation of chitin (multiple process)

Chitosan samples with a high average molecular weight and high level of DD may be produced by a 3-step deacetylation process (multiple treatment).
250 $cm^3$ aqueous solution of 50 wt% sodium hydroxide was added to 7.8 g of chitin and then dispersion was stirred for 1 h at a temperature of either 100°C or 120°C. After that, the product was separated, washed to pH=7.0 using distilled water, and air-dried. The above procedure was repeated two times.

3.3. Processing of chitin from *Calliphora erythrocephala*

3.3.1. Preparation of chitin cuticles

<u>*Cuticles I and III:*</u> Frozen larvae (-20°C) of *Calliphora erythrocephala* were washed with distilled water and homogenized using homogenizer type 240A (Krups, Ireland) for 15 s at 80 rpm, and the slurry was poured on a Büchner and washed repeatedly. Homogenization and washing were repeated twice.

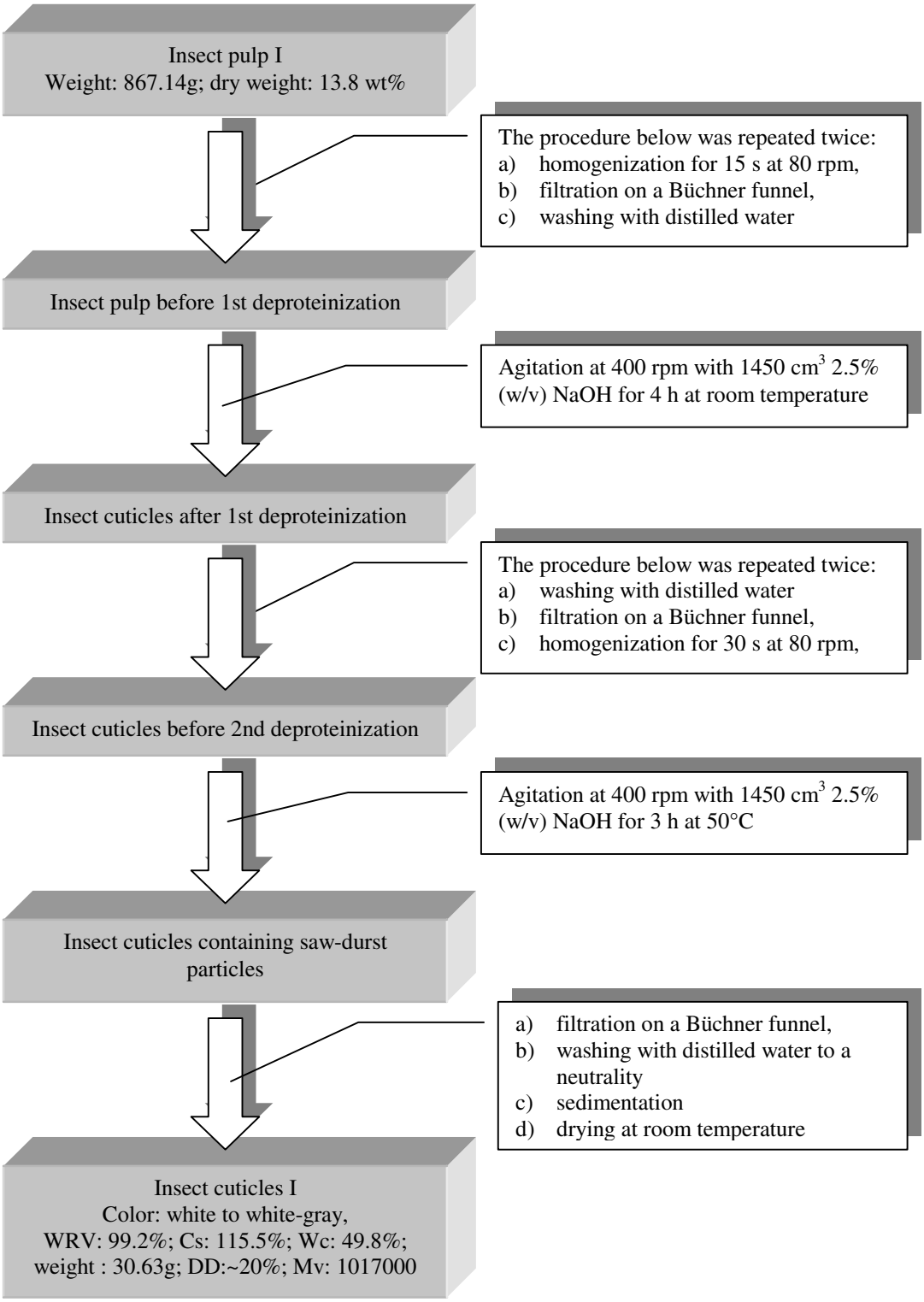



Figure 7. Scheme for the preparation of larval chitin I

The primary deproteinization process was carried out in 1450 $cm^3$ of a 2.5% (w/v) 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 $^oC$ 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 partially removed by sedimentation. In a

typical example, 867.1 g (wet weight) frozen larvae yielded 119.7 g (dry weight; 13.8%) crude cuticles and 30.6 g (dry weight; 3.5%) chitin of white to light gray colour. Figure 7 shows the procedure of chitin preparation from larvae of *Calliphora erythrocephala.*

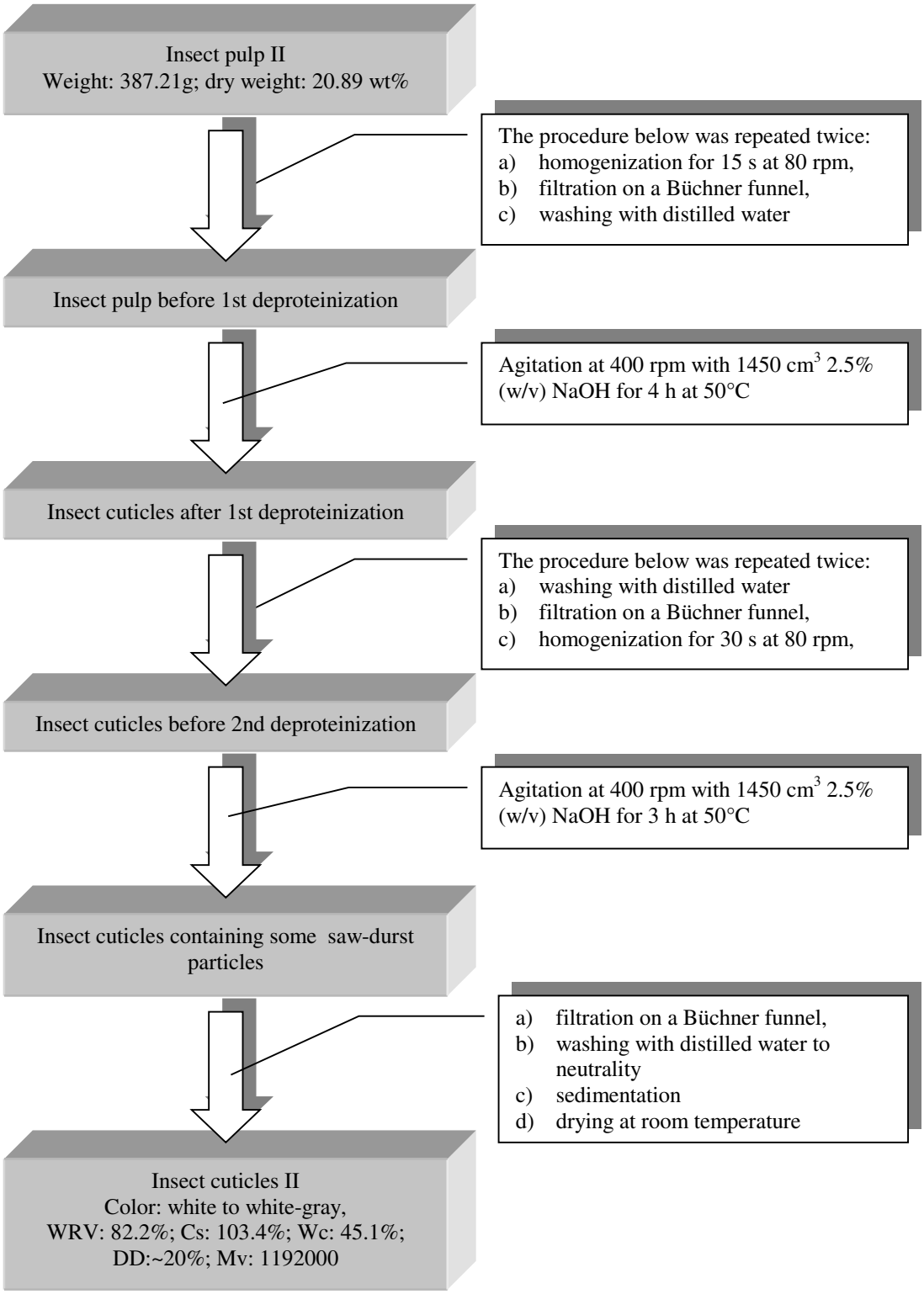



Figure 8. Scheme for the preparation of larval chitin II

*Cuticles II:* The procedure for the purification was the same as described above, except that the temperature for the deproteinization was 50 °C in both steps (Figure 8).

3.3.2. Preparation of chitosan

The chitin cuticles (8.1 g) were treated with 40 wt% or 50 wt% of an aqueous solution of sodium hydroxide (250 $cm^3$) for 1, 2, 3 or 4 h at a temperature of either 100 °C or 120 °C, with vigorous agitation. The product was then washed with distilled water until neutrality and air-dried.

3.3.3. Gradual deacetylation of chitin cuticles (multiple process)

Cuticles containing partially-deacetylated chitosan (obtained after 3h deacetylation with 50 wt% aqueous solution of sodium hydroxide at 100 °C) were treated in a second step with 50 wt% sodium hydroxide solution at 100°C for 1h (Figure 9.).

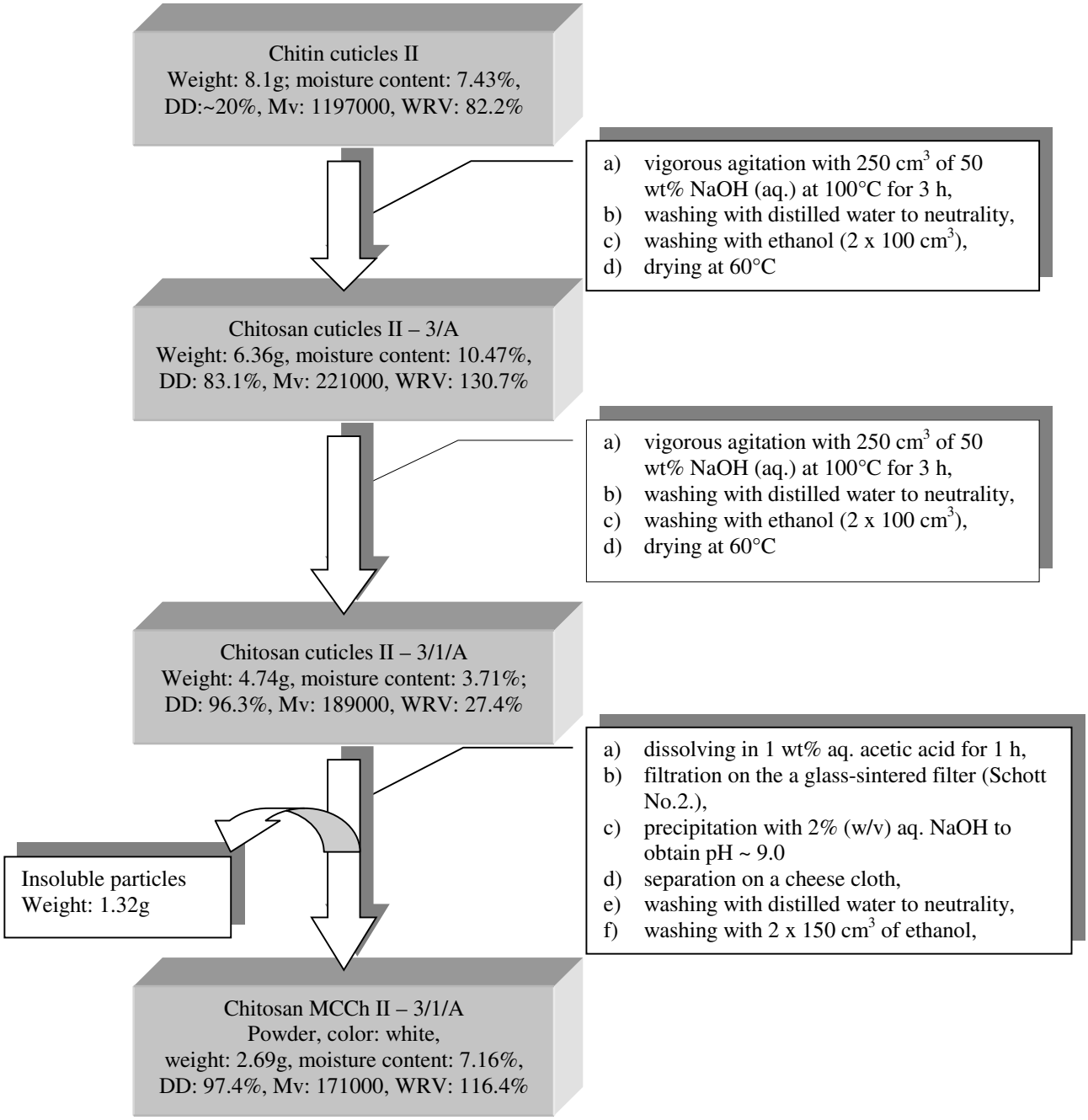



Figure 9. Preparation of microcrystalline chitosan proceeded by a multiple process of deacetylation

3.3.4. Preparation of microcrystalline form of chitosan (MCCh)[112]

Deacetylated chitosan cuticles contained a lot of saw-durst, therefore it was necessary to carry out purification step as fallow:

Chitosan cuticles 3/1/100-I (ca. 5g) were dissolved in 1% (v/v) 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 the addition of a 2% (w/v) aqueous solution of sodium hydroxide to yield pH = 9.0. The MCCh gel-like dispersion was adjusted immediately to pH=8.0 with 1% (v/v) hydrochloric acid (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 obtain a powder (Figure 9.).

The effect of the acidity of the solvent on $\bar{M}_V$ of MCCh was studied, using 1% (v/v) aqueous solution of acetic acid instead of hydrochloric acid.

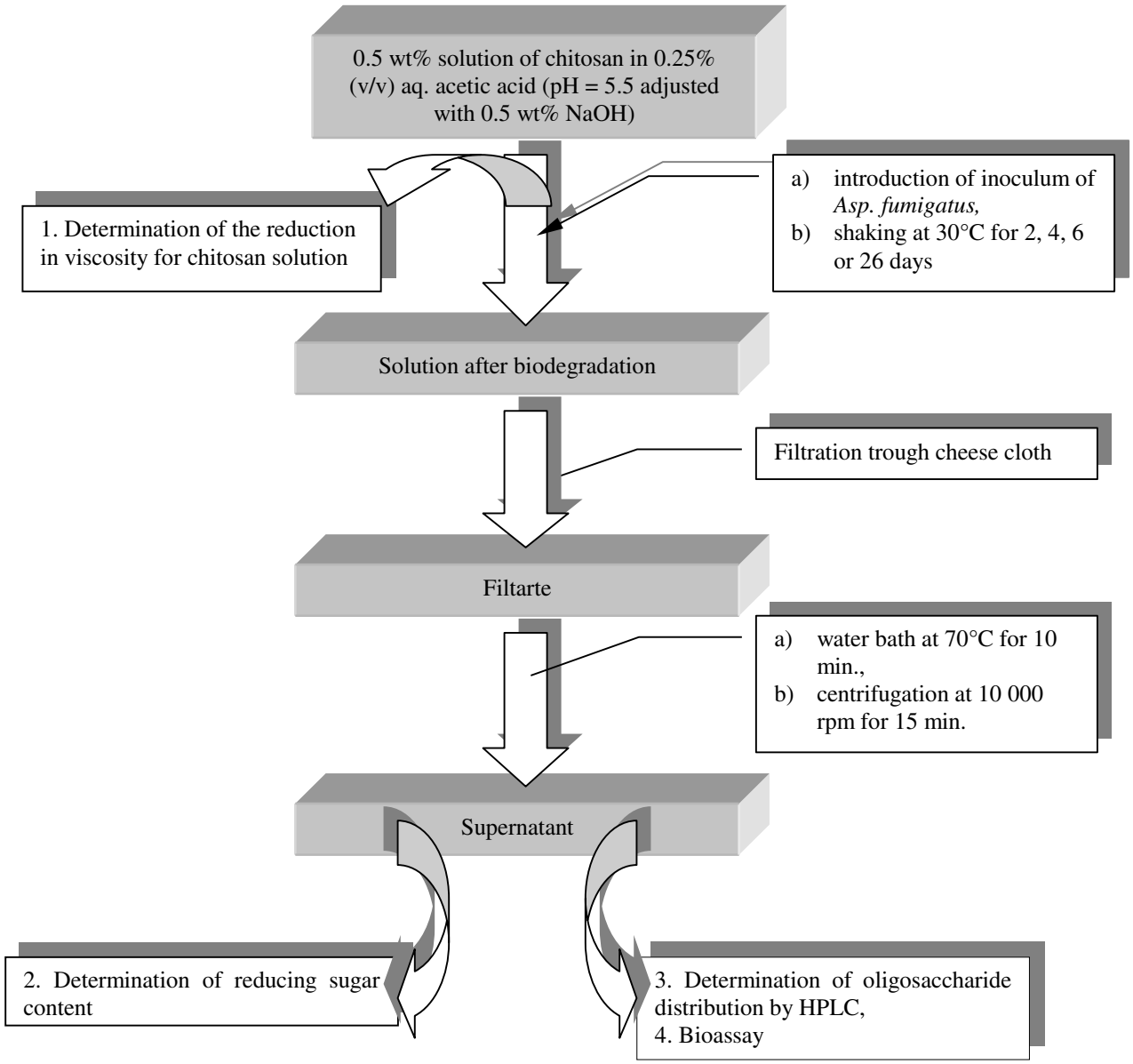



Figure 10. Degradation of chitosan by *Aspergillus fumigatus* and analysis of the reaction products

3.3.5. Comparison of chitosan properties prepared from various sources

The chitinous materials obtained from insect cuticles (*Calliphora erythrocephala*), shrimp shells (*Pandalus borealis*) or krill (*Euphausia superba*) were milled for 5 min. at 20,000 $min.^{-1}$ using the rotary analytic mill A10 (IKA Labortechnik, Germany). The powdered chitin was then added to 50 wt% sodium hydroxide solution at a ratio of 33,3 $cm^3$ NaOH per g of dry chitin. The suspension was agitated at a temperature of 100°C for 3h, and then the chitosan was separated using a glass-sintered filter (Schott No.2.), washed until neutrality, and dried at a temperature of 70°C. The properties of

chitosan (WRV, Cs, Wc, DD and $\overline{M}_v$) were compared with the values obtained during the original preparation of the mentioned chitosans.

3.4. Biodegradation of chitosan

3.4.1. Degradation of chitosan by Aspergillus fumigatus

The strain of *Aspergillus fumigatus* was cultivated in the Instytut Ochrony Roslin (Plant Protection Institute), Poznan, Poland[228]. Solutions of chitosan samples were prepared using a concentration of 5 mg•cm$^{-3}$ in 0.25% (v/v) aqueous acetic acid solution.
The solutions were filtered after 24 h of stirring and the pH was adjusted to 5.7 with 0.5 wt% aqueous sodium hydroxide solution. 3 mg of dry fungus was added to 50 cm$^3$ of chitosan solution, followed by incubation in a shaking water bath (Julaba SW 20, Germany) at 30°C at 160 rpm for 2, 4, 6 or 27 days. The incubation mixtures were filtered through cheesecloth and the filtrates were heated at a temperature of 70°C for 10 min. Finally, the mixtures were centrifuged at 10,000 rpm for 15 min and the supernatants were removed for further analysis and for the determination of the bioactivity (Figure 10).The viscosity of solutions was measured using an Ubbelohde's viscometer, capillary No. of 53210/I, instrument constant K = 0.009831 mm$^2$•s$^{-2}$ at 30°C.
UV-absorption at $\lambda$ = 205 nm was measured by a UV/VIS double beam UVICON 941 Plus spectrophotometer (Kontron).

3.4.2. Bioassay[213]

Antiviral activity was tested on two plant-virus models: bean - Alfalfa mosaic virus and tobacco *Nicotiana tabaccum sp.* Xanthi nc - tobacco mosaic virus. The surface of leaves was rubbed with carborundum as an abrasive. Either the test samples were sprayed with a mixture of equal amounts of the virus suspension and sample solution or, alternatively, the samples and viruses were applied separately onto the plant leaves. Antiviral activity was calculated as the percentage of reduction in the number of local lesions caused by virus infection on the treated leaves in comparison to the control sample (no chitosan or degradation products added).
Antibacterial activity was determined by the minimum inhibitory concentration (MIC) method. A drop of a solution of the test samples was applied to the surface of agarose plates containing cultures of *Escherichia coli, Clavibacter michiganesis subsp. Michiganesis*, *Pseudomonas syringae pv. Phaseolicola* or *Pseudomonas syringae pv. Tomato*.

## 4. Results and Discussion

4.1. Assessment of the properties of chitin obtained from various sources

Various types of chitin sample were characterized with respect to: degree of deacetylation (DD), average molecular weight ( $\overline{M}_v$ ), ash content as well as WRV, Wc, and Cs.
The demineralization process of chitin prepared from *Euphausia superba* reduced $\overline{M}_v$ of around 8-9% in both charges of K and M chitins, while no change in DD was observed. However, the ash content was decreased considerably (approx. 70%) with a decline of WRV after additional demineralization (Table 7, Figure 11).
The particle size of chitin samples was most critical: coarse particles or shells resulted in the lowest deacetylation yield (low DD and high $\overline{M}_v$). WRV as well as Cs values of powdered chitin from Antarctic krill were significantly higher than that chitin from shrimp shells (Table 8). The DD was not drastically decreased during demineralization (chitin M – DD 38,7%; chitin Mm – DD 42,7%) as the result of the removal of degraded, low molecular oligosaccharides containing a high amount of N-acetyl aminoglucose units[21].
The lowest DD was found for chitin prepared from shells of *Pandalus borealis,* but $\overline{M}_v$ in this case was the highest. WRV and Cs values indicated a low level due to the large size of shrimp shell. Particle size distribution affects the swelling behaviour of chitin[32,58]. Chitin with smaller particles requires a shorter time for swelling and results in higher deacetylation yield[76,84]. The above results confirmed this observation.
High ash content may disturb the deacetylation process because of the increase in swelling time (Table 8).

Table 7. Properties of chitin from Antarctic krill

| Type of chitin | Ash content (%) | Moisture content (%) | $\bar{M}_v$ (Da) | DD by FTIR (%) | WRV (%) | Cs (%) | Wc (%) |
|---|---|---|---|---|---|---|---|
| "K" | 4.9 | 2.01 | 1,011,000 | Detected as determined | 132.4 | 180.8 | 58.2 |
| "Km" | 1.4 | 3.16 | 932,000 | Detected as determined | 130.4 | 156.4 | 56.2 |
| "M" | 4.7 | 2.78 | 994,000 | 38.7 | 126.4 | 124.1 | 55.8 |
| "Mm" | 1.2 | 3.33 | 904,000 | 42.7 | 101.7 | 136.7 | 50.4 |

m – demineralized chitin from Antarctic krill, WRV – water retention value, Cs – swelling coefficient, Wc – water content coefficient, K, M, - chitin specimen (see section 1),

Table 8. Properties of chitin from *Pandalus borealis*

| Type of chitin | Ash content (%) | Moisture content (%) | $\bar{M}_v$ (Da) | DD by FTIR (%) | WRV (%) | Wc (%) | Cs (%) |
|---|---|---|---|---|---|---|---|
| "P" | 2.8 | 3.41 | 1,365,000 | 7.4 | 59.4 | 37.3 | 63.5 |

WRV – water retention value, Cs – swelling coefficient, Wc – water content coefficient,

When the deproteinization process of cuticles prepared from *Calliphora erythrocephala,* carried out at a higher temperature (50°C) the chitin sample possessed generally higher DD of prepared chitin cuticles (Table 9). The $\bar{M}_v$ of chitin obtained at higher temperature were higher (ca. 10-15%). It is probably connected with the faster degradation of the shorter, less ordered regions at higher temperature, which are removed by washing. The longer, crystalline region are degraded much slower, therefore the value of $\bar{M}_v$ was higher (Table 9, Figure 11).

Table 9. Properties of chitin cuticles from the insect larvaes of *Calliphora erythrocephala*

| Type of chitin / harvest | Moisture content (%) | $\bar{M}_v$ (Da) | DD by FTIR (%) | WRV (%) | Wc (%) | Cs (%) |
|---|---|---|---|---|---|---|
| chitin cuticle - I | 7.19 | 1,017,000 | 24.7 | 99.2 | 49.8 | 115.5 |
| chitin cuticle - II* | 7.43 | 1,192,000 | 30.1 | 82.2 | 45.1 | 103.4 |
| chitin cuticle - III | 7.30 | 1,067,000 | 25.1 | 79.6 | 44.3 | 108.2 |

WRV – water retention value, Cs – swelling coefficient, Wc – water content coefficient,
* - both deproteinization steps was carried out at 50°C.

However, this larvae chitin had better susceptibility for deacetylation, because produced chitosans showed a lower $\bar{M}_v$ and higher DD than polymers deacetylated at the same condition but from other harvest of larvae (see Chapter I, Section 4.2.3.).
WRV of deproteinized cuticles showed similar levels as in the case of shrimp shells. The Cs coefficient was considerably higher and approached the value obtained for powdered chitin from Antarctic krill.

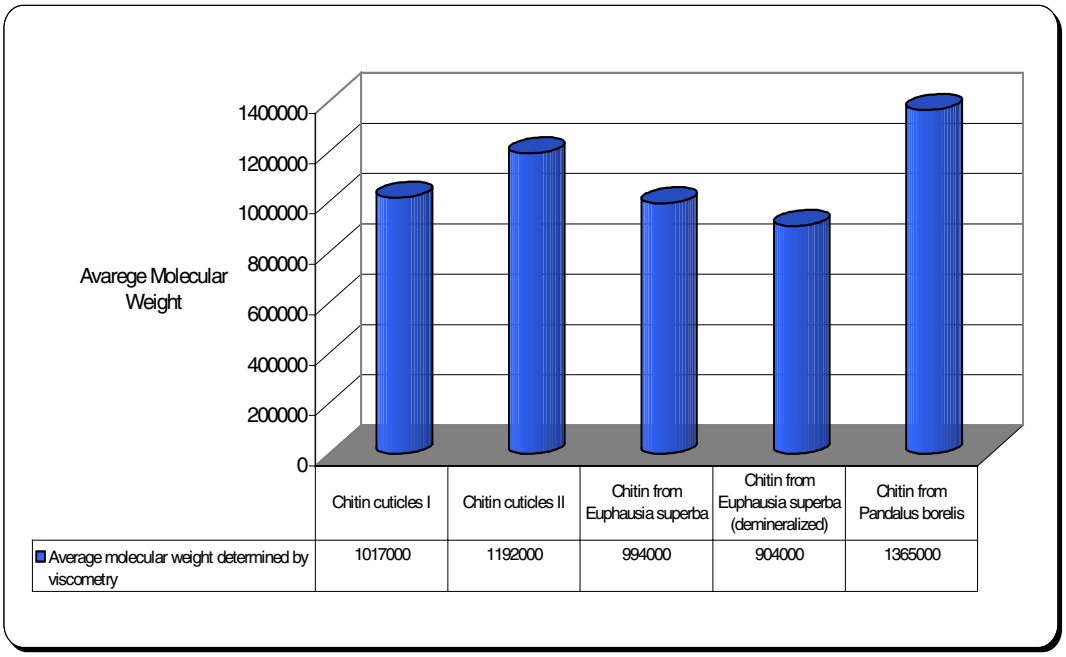



Figure 11. $\overline{M}_v$ of chitin obtained from various sources

4.1.1. FTIR spectroscopy of chitin prepared from various sources

The IR spectra of chitins obtained from various sources demonstrated on Figure 12 are compared to the IR spectrum of raw chitinous material from *Hammarus pulex*.

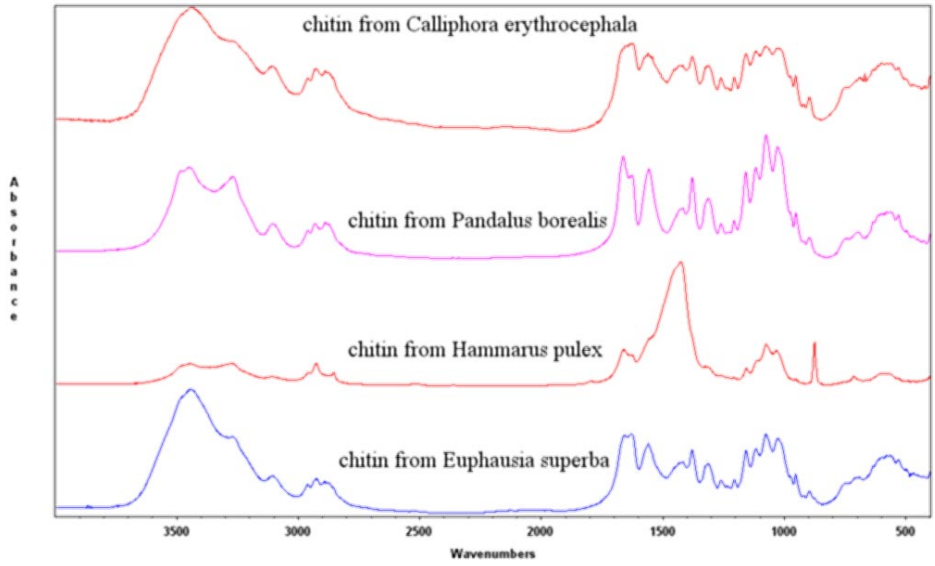



Figure 12. FTIR spectra of various chitins

A low DD of chitin from *Pandalus borealis* was confirmed by the presence of two bands in the IR spectrum resulting from the hydrogen-bonded NH group of the amide group at 3120 $cm^{-1}$ (symmetric stretch) and at 3310 $cm^{-1}$ (asymmetric stretch). There were not resolved for chitins with higher degree of deacetylation (from *Euphausia superba*, *Calliphora erythrocephala* or *Hammarus pulex*). The well resolved intensities at 1100 $cm^{-1}$ are usually observed for highly N-acetylated chitins. The singly and doubly hydrogen-bonded amide I band also existed in all spectra. The presence of peaks at 1730 $cm^{-1}$ resulting from the C=O stretch of ester of contaminates for this type of chitin was observed. The high amount of mineral residues (carbonates) was manifested by the absorption at the wavenumber of 1400 $cm^{-1}$ as well as at 855 $cm^{-1}$ (IR spectrum of chitin from *Hammarus pulex*). The strong band at 1380 $cm^{-1}$ can be from the C=C stretch of fatty acids.

No strong reduction was observed in the weak band at frequency of 1400 $cm^{-1}$ and 860 $cm^{-1}$ (responsible for carbonate[238]) before and after demineralization of krill chitin (Figure 13).

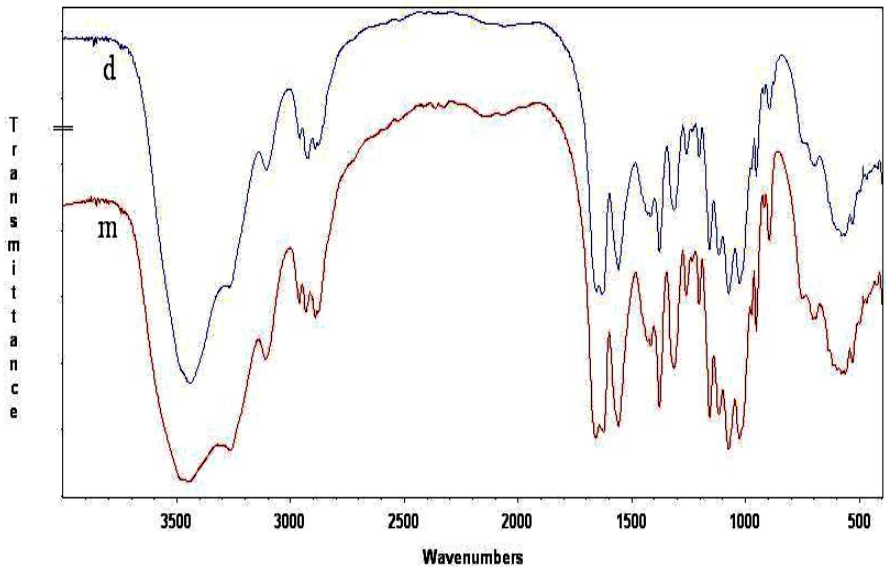



Figure 13. Changes in IR spectrum of chitin from *Euphausia superba* caused by demineralization; (m) - chitin before demineralization, (d) - chitin after demineralization

Comparison of the IR spectrum of *Hammarus pulex* with the spectra obtained for chitin from *Euphausia superba* and *Pandalus borealis* confirmed the complete absence of additional, not derived from chitin, bands. The spectrum of chitin from *Calliphora erythrocephala* shows additional intensities (at 1550 $cm^{-1}$, 1770 $cm^{-1}$) corresponding to the hard-to-remove sclerotized protein and/or catechol, which may be removed after deacetylation by the dissolution of chitosan in acid solution and subsequent precipitation.

4.1.2. Solid state $^{13}$C-NMR

Figure 14 shows the NMR spectra of chitins from *Pandalus borealis*, *Euphausia superba, Calliphora erythrocephala* and non-purified *Hammarus pulex* chitin additionally.

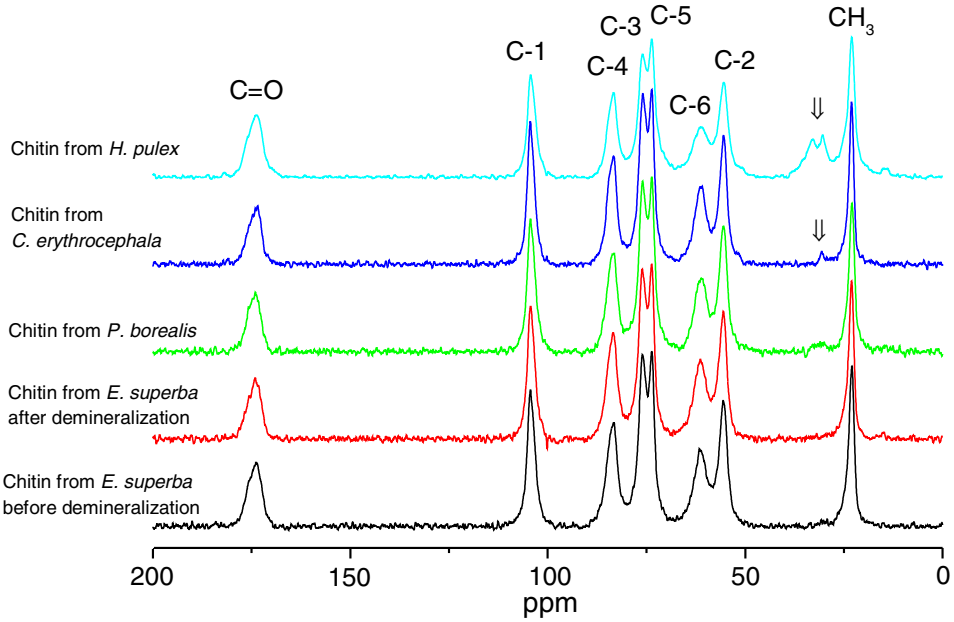



Figure 14. $^{13}$C-NMR spectra of chitins from various sources; (⇓) – indicates the presence of contaminants (mostly protein, catecholamines and possibly lipids).

Table 10. $^{13}C$ chemical shifts of chitin from various sources (in ppm)

| | C=O | C1 | C4 | C5 | C3 | C6 | C2 | $CH_3$ |
|---|---|---|---|---|---|---|---|---|
| | | | | *α-chitin* | | | | |
| Crab shell[1)] | 173.7 | 104.5 | 83.2 | 76.0 | 73.6 | 61.0 | 55.4 | 23.1 |
| Shrimp shell | 173.9 | 104.4 | 83.3 | 76,0 | 73,7 | 61,0 | 55.5 | 23.0 |
| Krill chitin | 173.8 | 104.4 | 83.3 | 76.0 | 73.7 | 61.5 | 55.6 | 23.0 |
| Krill chitin (demineralized) | 174,1 | 104.4 | 83.5 | 76.1 | 73.7 | 61.3 | 55.5 | 23.0 |
| *Hammarus pulex* | 173.9<br>179.8* | 104.3 | 83.3 | 76.0 | 73.7 | 61.1 | 55.4 | 23.0 |
| Insect chitin | 173.5<br>179.1* | 104.1 | 83.8 | 76.0 | 73.9 | 61.0<br>61.2 | 55.1 | 22.6 |
| | | | | *β-chitin* | | | | |
| *Tevinia* tube[1)] | 175.5<br>176.4 | 105.3 | 84.4 | 75.4 | 73.1 | 59.8 | 55.2<br>56.0 | 22.7 |
| Squid pen[1)] | 174.8 | 104.8 | 83.7 | 75.4 | 74.5 | 59.3<br>61.0 | 56.0 | 23.3<br>24.1 |

[1)] – Tanner et al.1990, spectrometer Bruker CXP-300, external reference adamantane[239],
* - $^{13}C$ NMR signals which cannot be assigned to chitin

The process of demineralization of krill chitin seems to be effective as revealed by the presence only one carbonyl signal at ca. 174 ppm (i.e. absence if carbonate). Chitin from insect is contaminated with low amounts of protein and/or catechol, as indicated by the low intensity peaks at ca. 30 ppm. For the shifts of *Hammarus pulex* chitin extra intensities at ca. 30 ppm arising from contaminates such as proteins, minerals or/and fatty acids were detected.
The solid state $^{13}C$-NMR spectra of demineralized chitins from *P. borealis* and *E. superba* indicated the absence of intensities at ca. 30 ppm leading to the conclusion that the process of chitin isolation from this sources form rather pure chitin.
The chemical shifts of α-chitins and β-chitins presented in Table 10 confirm that all the purified chitinous materials were α-chitins. The comparison of the relative intensities of insect chitin with the intensities of the chitin from *Hammarus pulex* suggests that insect chitin did not contain inorganic contaminates (absence of the additional signals near 174 ppm), but possibly some protein.

4.2. Assessment of the properties of chitosan derived from various sources

4.2.1. Chitosans manufactured from deep-water shrimp (*Pandalus borealis*)

Chitosan samples derived from *Pandalus borealis* shrimp shells possessed low DD and high $\bar{M}_v$ ( $\bar{M}_v$ of chitosan PA10 540,000), although the longest time of deacetylation was applied (Table 11, Figure 15.).
Deacetylations carried out longer than 7 h produced samples, which were not fully soluble in 1% (v/v) aqueous acetic acid. Therefore, these products were additionally deacetylated. The low value of DD after the first step carried out in 50% solution of aqueous sodium hydroxide for the longest time, was probably the result of the lowest level of swelling parameters, high crystallinity of chitin, high content of ash or low initial DD[36,45,240,241] (Table 8, 11 and 18). The DD was significantly increased leading also to the decrease in $\bar{M}_v$. The DD of "PB" chitosan charge increased by ca. 10% - 20% in comparison with chitosan deacetylated at 100°C. At the same time, the $\bar{M}_v$ was reduced by ca. 50%.
The absence of the solubility of polymer deacetylated during one stage of the deacetylation process in DMAc/LiCl, usually used for chitin dissolution, with partial solubility in 1 wt% acetic acid was observed. This fact supports the conclusion that the DD of the above-mentioned samples were too high to obtain dissolution in DMAc/LiCl[38-39]. Moreover, partial solubility in an aqueous acetic acid may confirm DD range from ca. 40% to 50%[41].
The lower $\bar{M}_v$ for chitosan produced at 120°C confirmed the influence of higher temperature on a more extensive degradation of polymer chains. A lower temperature affected less drastically the molecular structure of the polymer (Figure 15.). During deacetylation of chitin, the amorphous regions are more susceptible to alkaline treatment and are more degraded than the ordered segments.

Table 11. Properties of chitosan produced from *Pandalus borealis*

| Symbol of sample | DD by titration (%) | $\bar{M}_v$ (Da) | Deacetylation time(h)/ temp. (°C) | WRV (%) | Cs (%) | Wc (%) | Moisture content (%) |
|---|---|---|---|---|---|---|---|
| Concentration of sodium hydroxide of 50 wt% | | | | | | | |
| PA2 | --[a)] | --[a)] | 2/100 | 40.1 | 56.1 | 28.6 | 9.61 |
| PA3 | --[a)] | --[a)] | 3/100 | 50.2 | 67.6 | 33.4 | 9.79 |
| PA4 | --[a)] | --[a)] | 4/100 | 82.0 | 108.8 | 45.1 | 9.88 |
| PA5 | --[a)] | --[a)] | 5/100 | 72.2 | 88.2 | 38.1 | 9.66 |
| PA7 | --[a)] | --[a)] | 7/100 | 65.9 | 72.0 | 39.7 | 9.79 |
| PB2 | --[a)] | --[a)] | 2/120 | 55.3 | 68.0 | 35.6 | 10.34 |
| PB3 | --[a)] | --[a)] | 3/120 | 53.6 | 74.5 | 34.9 | 10.56 |
| PB4 | --[a)] | --[a)] | 4/120 | 64.2 | 78.3 | 37.2 | 9.46 |
| PB5 | --[a)] | --[a)] | 5/120 | 66.0 | 73.6 | 37.7 | 12.7 |
| PB7 | --[a)] | --[a)] | 7/120 | 52.5 | 63.9 | 34.4 | 11.58 |
| PB8 | 89.9 | 248,000 | 7+1/120 | 65.2 | 72.1 | 37.6 | 11.05 |
| PB9 | 92.5 | 261,000 | 5+4/120 | 62.8 | 71.8 | 36.8 | 10.98 |
| PB10 | 98.4 | 227,000 | 7+3/120 | 56.8 | 65.4 | 35.0 | 9.89 |
| PA9 | 77.4[a)] | -[a)] | 5+4/100 | 65.2 | 73.1 | 37.6 | 10.32 |
| PA10 | 83.2 | 543,000 | 7+3/100 | 63.2 | 71.9 | 37.0 | 9.46 |

[a)] this sample was not soluble in the solvent system usually used for chitin dissolution or partially soluble in 1% (v/v) acetic acid (aq.),; A, B: code for notation of reaction temperature - A: 100°C, B: 120°C. Digits indicate reaction time, which may be two sequential reactions, as indicated

The increase of the temperature causes the increase in the degradation of less- as well as more-ordered regions, yielded the significant decrease in $\bar{M}_v$ [88].

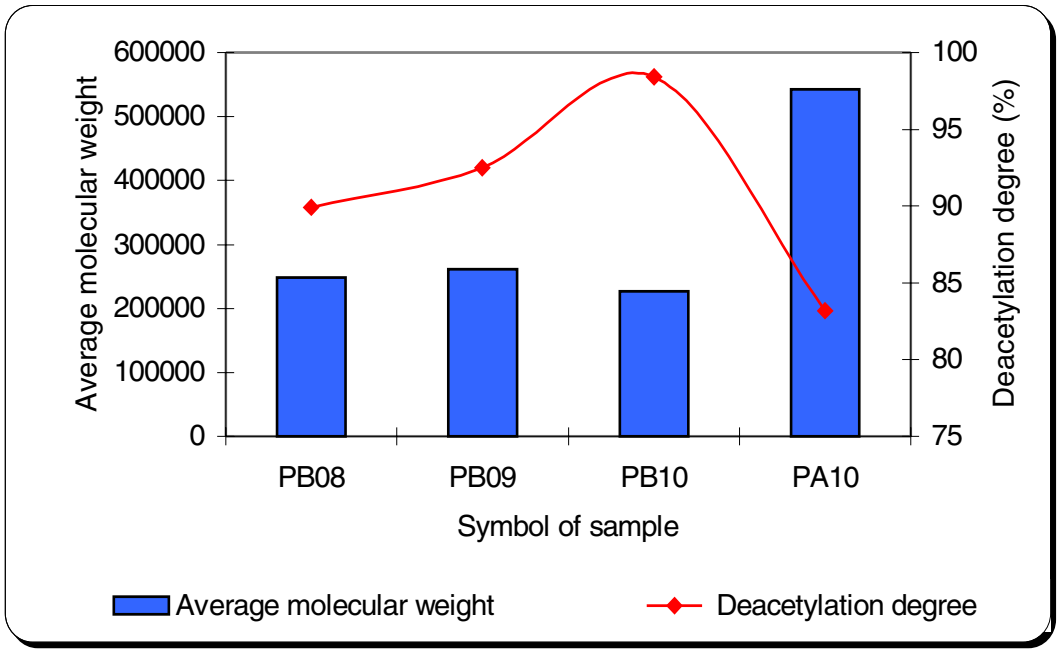



Figure 15. Changes in $\bar{M}_v$ and DD during deacetylation of chitin from the *Pandalus borealis*

The changes in WRV and Cs during deacetylation of chitin from *Pandalus borealis* indicated the destructive influence of temperature on the swelling behaviour connected with the changes in an internal structure of polymer (Figure 16.). During the first stage of sodium hydroxide treatment, for a period ranging from 1h to 2h, WRV and Cs decreased, especially when deacetylation was carried out

at a lower temperature. During prolongation of the process, an improvement of swelling parameters was observed.
This fact can be explained by the smaller influence of the deacetylating agent on polymer degradation in the more ordered region when the deacetylation was carried out at lower temperature. The changes in the WRV and Cs for chitin deacetylated at 120 °C were not drastic, but a decrease of these parameters after 4 h of deacetylation correlated with the degradation of the crystalline regions.

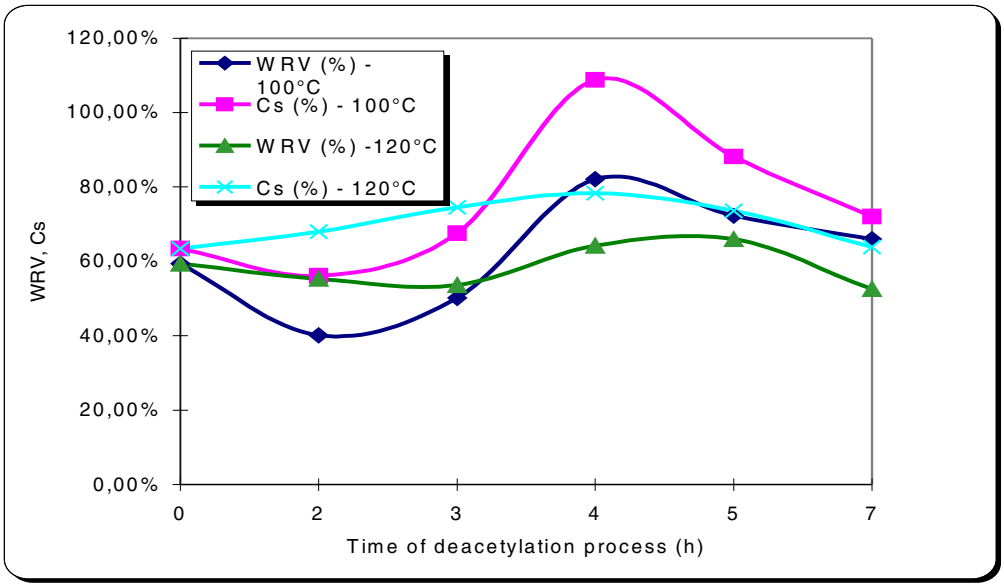



Figure 16. Changes in WRV and swelling coefficient during deacetylation of chitin from *Pandalus borealis* at 100 °C or 120 °C

4.2.2. Chitosan prepared from Antarctic krill (*Euphausia superba*)

In the case of chitosan from krill chitin (charge "M" and "K"), it was found that a high content of minerals was correlated with a lower DD, higher $\overline{M}_v$, and an increase in swelling properties.

Table 12. Properties of chitosans produced from Antarctic krill – samples series "K"

| Symbol of sample | DD by titration (%) | $\overline{M}_v$ (Da) | Deacetylation time(h) /temp. (°C) | WRV (%) | Cs (%) | Wc (%) | Moisture content (%) |
|---|---|---|---|---|---|---|---|
| | | | Concentration of sodium hydroxide of 50 wt% | | | | |
| K1A[a)] | 67.9 | 323,000 | 1/100 | 245.9 | 294.3 | 93.0 | 11.67 |
| K3A | 79.1 | 256,000 | 3/100 | 238.7 | 287.1 | 90.8 | 12.48 |
| K1mA[a)] | 68.6 | 305,000 | 1/100 | 204.7 | 253.1 | 80.4 | 10.80 |
| K3mA | 81.7 | 198,000 | 3/100 | 132.9 | 181.3 | 58.4 | 14.03 |
| K1B[a)] | 69.8 | 224,000 | 1/120 | 120.4 | 168.8 | 54.5 | 11.05 |
| K3B | 78.2 | 131,000 | 3/120 | 104.1 | 152.5 | 49.5 | 11.58 |
| K1mB | 68.5 | 221,000 | 1/120 | 119.2 | 167.6 | 54.2 | 12.06 |
| K3mB | 84.6 | 118,000 | 3/120 | 92.1 | 140.5 | 45.8 | 11.90 |
| K1mC | 78.9 | 122,000 | 1/130 | 141.6 | 190.0 | 61.0 | 11.07 |
| K3mC | 88.4 | 116,000 | 3/130 | 90.9 | 139.3 | 45.5 | 12.34 |

[a)] this sample was only partially soluble;
A, B, C: code for notation of reaction temperature: A - 100 °C, B - 120 °C, C - 130 °C.
Digits indicate reaction time, which may be two or three sequential reactions, as indicated, "m" denotes that chitin was demineralized

The product of deacetylation of chitin "K" and "Km" showed lower DD and higher $\overline{M}_v$ than chitosan from chitin "M" deacetylated under similar conditions.

This observation suggests that chitins prepared from various charges, however having similar DD and $\overline{M}_v$ (Tables 12-13) but differing in the crystallinity and swelling behaviour, produced chitosans differing in DD and/or $\overline{M}_v$ (Figure 17).

Table 13. Properties of chitosans produced from Antarctic krill – samples M

| Symbol of sample | DD by titration (%) | $\overline{M}_v$ (Da) | Deacetylation time(h) /temp. (°C) | WRV (%) | Cs (%) | Wc (%) | Moisture content (%) |
|---|---|---|---|---|---|---|---|
| | | | Concentration of sodium hydroxide of 50 wt% | | | | |
| M1A [a)] | 68.2 | 336,000 | 1/100 | 204.5 | 252.9 | 80.3 | 11.73 |
| M3A | 73.5 | 187,000 | 3/100 | 171.1 | 219.5 | 70.1 | 11.41 |
| M1mA [a)] | 67.4 | 313,000 | 1/100 | 179.0 | 227.4 | 72.5 | 11.47 |
| M3mA | 83.3 | 148,000 | 3/100 | 126.8 | 175.2 | 56.5 | 11.08 |
| M6mA | 87.8 | 137,000 | 6/100 | 109.6 | 158.0 | 51.2 | 10.24 |
| M1B | 67.8 | 273,000 | 1/120 | 162.7 | 211.1 | 67.5 | 11.62 |
| M3B | 84.2 | 155,000 | 3/120 | 98.5 | 146.9 | 47.8 | 12.38 |
| M1mB | 69.0 | 206,000 | 1/120 | 141.7 | 190.1 | 61.1 | 12.26 |
| M3mB | 90.5 | 143,000 | 3/120 | 99.8 | 148.2 | 48.2 | 11.95 |
| M6mB | 93.7 | 116,000 | 6/120 | 104.3 | 152.7 | 49.6 | 10.62 |
| M1mC | 85.0 | 111,000 | 1/130 | 135.9 | 184.3 | 59.3 | 11.18 |
| M3mC | 89.0 | 94,000 | 3/130 | 98.9 | 147.3 | 47.9 | 10.58 |
| M6mC | 92.6 | 64,000 | 6/130 | 88.7 | 137.1 | 44.8 | 10.73 |
| GM2B | 73.8 | 197,000 | 1+1/120 | 94.8 | 135.0 | 48.7 | 10.12 |
| GM3B | 76.8 | 135,000 | 1+1+1/120 | 99.7 | 148.1 | 48.9 | 10.41 |
| GM2A [a)] | 67.1 | 298,000 | 1+1/100 | 152.9 | 201.3 | 60.5 | 10.13 |
| GM3A [a)] | 66.3 | 264,000 | 1+1+1/100 | 153.6 | 202.0 | 60.6 | 11.25 |
| | | | Concentration of sodium hydroxide of 40 wt% | | | | |
| M1mA - 40 [a)] | 63.9 | 410,000 | 1/100 | 197.9 | 246.3 | 78.3 | 10.41 |
| M3mA - 40 | 74.2 | 295,000 | 3/100 | 139.3 | 187.7 | 60.3 | 11.27 |
| M6mA - 40 | 78.0 | 200,000 | 6/100 | 126.0 | 174.4 | 56.2 | 10.38 |
| M1mB - 40 | 70.4 | 241,000 | 1/120 | 148.4 | 196.8 | 63.1 | 9.41 |
| M3mB - 40 | 86.8 | 157,000 | 3/120 | 108.8 | 157.2 | 51.0 | 10.12 |
| M6mB - 40 | 89.4 | 125,000 | 6/120 | 100.9 | 149.3 | 48.5 | 9.84 |

[a)] this sample was only partially soluble;
A, B, C: code for notation of reaction temperature: : A - 100 °C, B - 120 °C, C - 130 °C.
Digits indicate reaction time, which may be two or three sequential reactions, as indicated, "m" denotes that chitin was demineralized

After the demineralization, samples "Mm" showed a higher DD by ca. 2-5% and lower $\overline{M}_v$, for 1h – ca. 10%, for 3h – ca. 20%, as well as lower swelling parameters. These results confirm that the presence of the residual minerals in the chitin raw materials has affected a decrease in DD, most likely due to the limitation of the diffusion of sodium hydroxide (Figures 17-18)[89].
Chitosans deacetylated for 1h at 100 °C or 120°C have been identified by the same DD and $\overline{M}_v$, WRV and Cs. This fact suggests that during the first stage of the deacetylation, degradation of the amorphous regions of chitin occurred. However, the DD determined by titration method, is less accurate in the case of samples containing insoluble particles. A higher DD has been produced for the chitosan sample deacetylated at 130°C (sample with notation *C*).

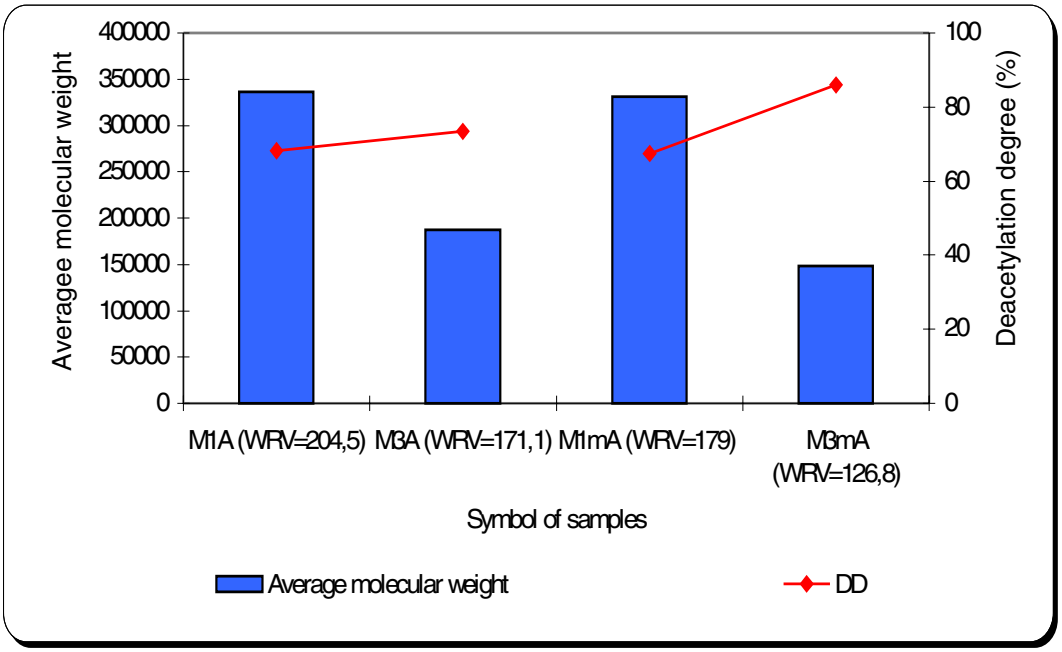



Figure 17. Influence of deacetylation carried out at 100°C on $\overline{M}_v$ and DD of chitosan "M" with or without demineralization

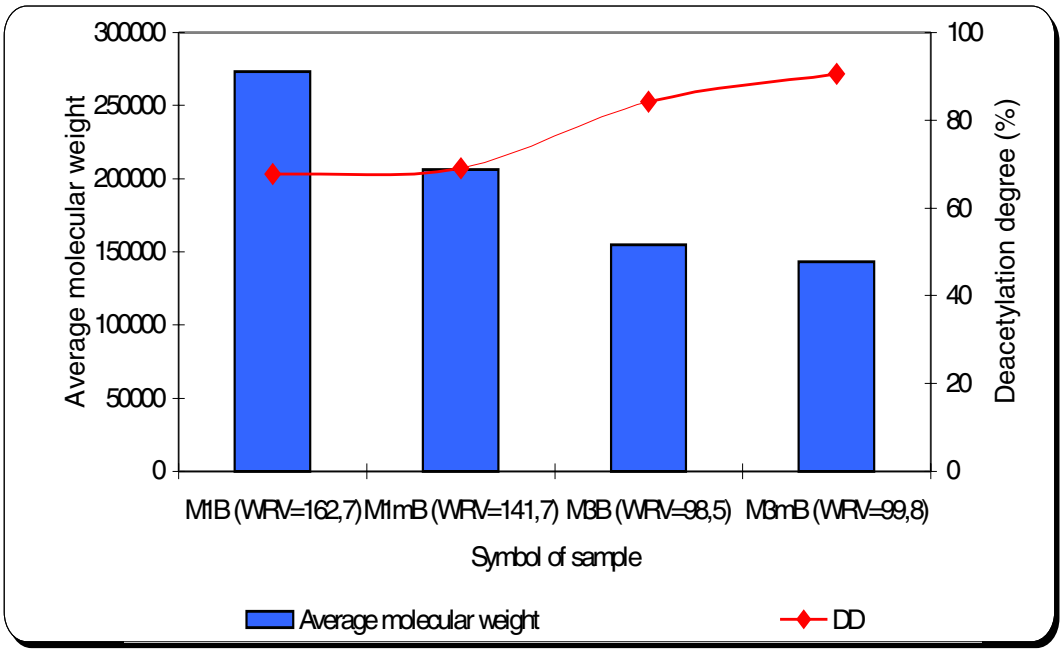



Figure 18. Influence of deacetylation carried out at 120°C on $\overline{M}_v$ and DD of chitosan "M" (before and after demineralization)

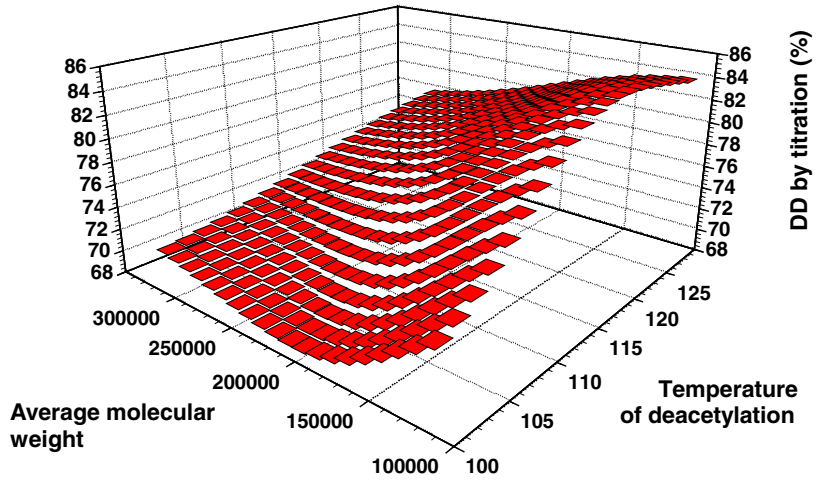



Figure 19. Deacetylation of chitin "M" for 1 h with 50 wt% aqueous sodium hydroxide at various temperatures

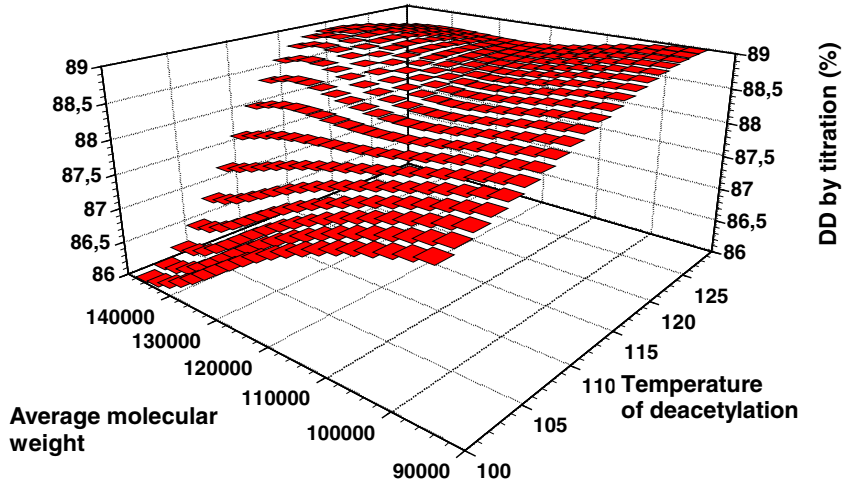



Figure 20. Deacetylation of chitin "M" for 3 h with 50 wt% aqueous sodium hydroxide at various temperatures

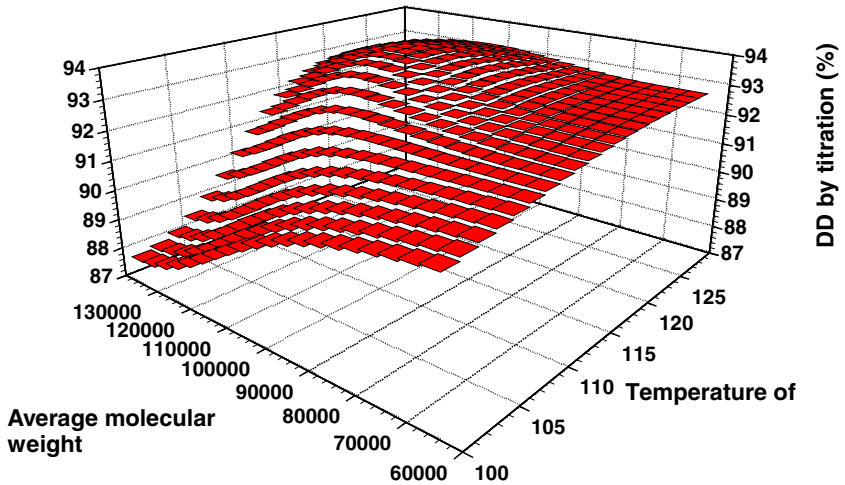



Figure 21. Deacetylation of chitin "M" for 6 h with 50 wt% aqueous sodium hydroxide at various temperatures

At the same time, the $\overline{M}_v$ was reduced by about 60% in comparison with those samples deacetylated at 100°C. When the deacetylation was carried out at 120°C and 130 °C a more extensive degradation of the polymer was observed (Figures 19-21).

Chitosan samples "M" and "K", after 3 h of deacetylation, showed a ca. 50% lower $\overline{M}_v$ and WRV, as well as a higher DD. The change in DD was decreased compared to the treatment carried out for 1h.

Moreover, the highest reduction in $\bar{M}_v$ has been found for the sample deacetylated at 130°C (Figures 22-24.).

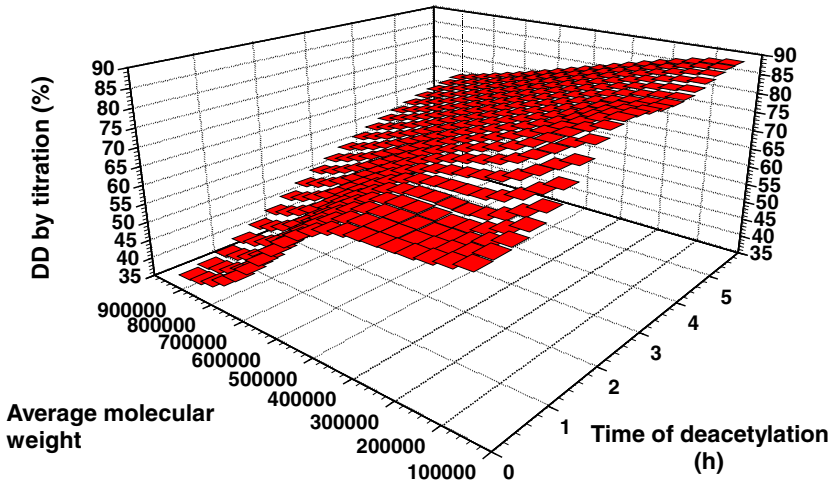



Figure 22. Deacetylation of chitin "M" at 100 °C with 50 wt% aqueous sodium hydroxide

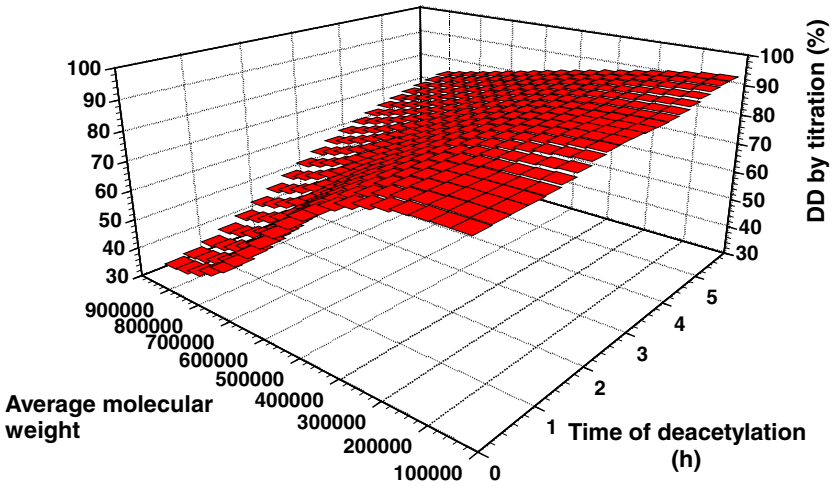



Figure 23. Deacetylation of chitin "M" at 120 °C with 50 wt% aqueous sodium hydroxide

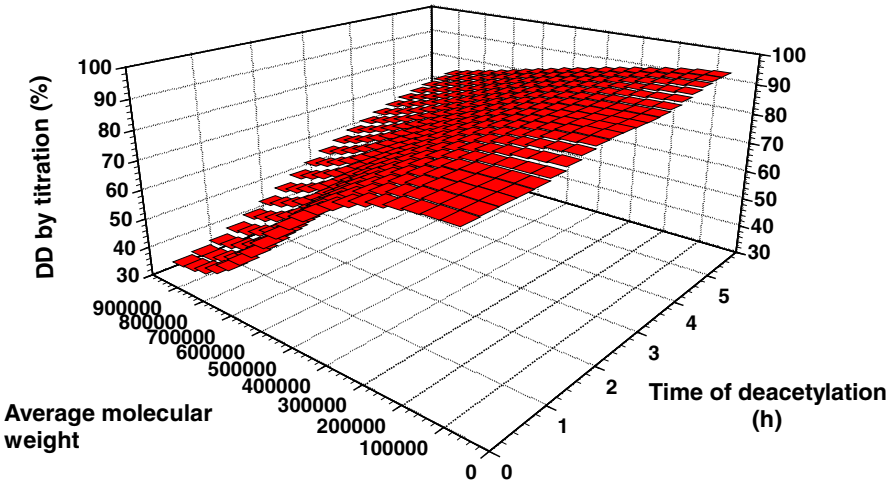



Figure 24. Deacetylation of chitin "M" at 130 °C with 50 wt% aqueous sodium hydroxide

The sample Gm3A, deacetylated by multiple treatment at 100°C for 1h showed consecutively a lower DD, and higher $\bar{M}_v$ as well as WRV than chitosan produced under similar conditions by the standard procedure (3h at 100°C).
The DD obtained was similar to DD for the sample deacetylated once for 1h at 100°C (M1mA). Nevertheless, sample was partially soluble in aqueous acetic acid and DD was determined only for its dissolved part. This suggests that the above DD value is considerably lower.
The gradually (three times) deacetylated sample at 120°C (G3mB) showed a lower DD and a similar $\bar{M}_v$ and WRV as the chitosan samples prepared after 3h-deacetylation process at 120°C (M3mB).

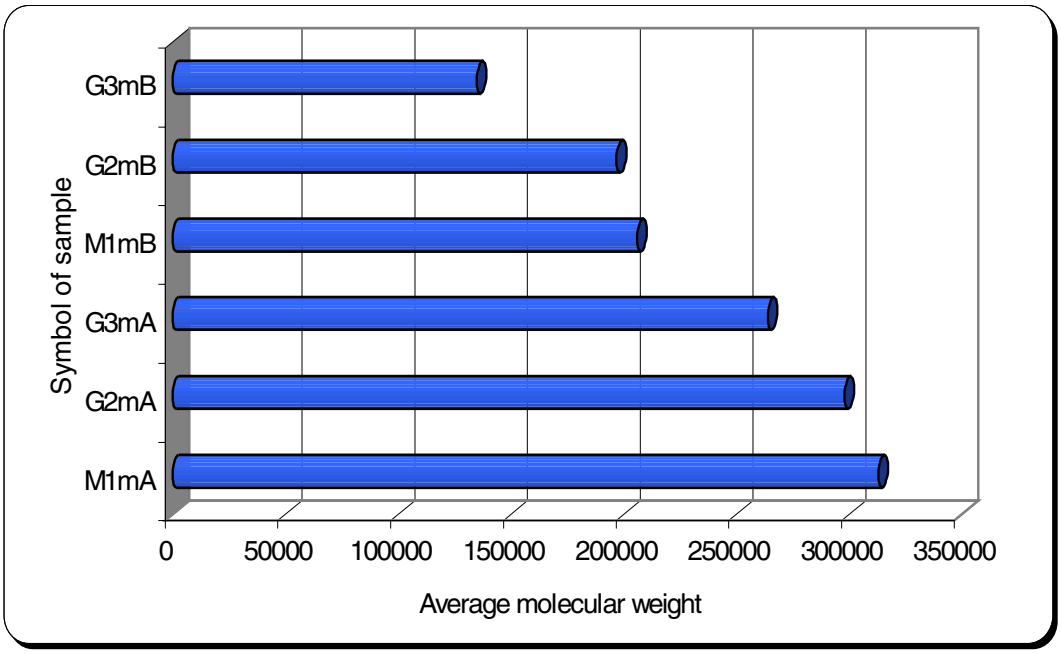



Figure 25. The changes in $\bar{M}_v$ during gradual deacetylation of chitin "M"

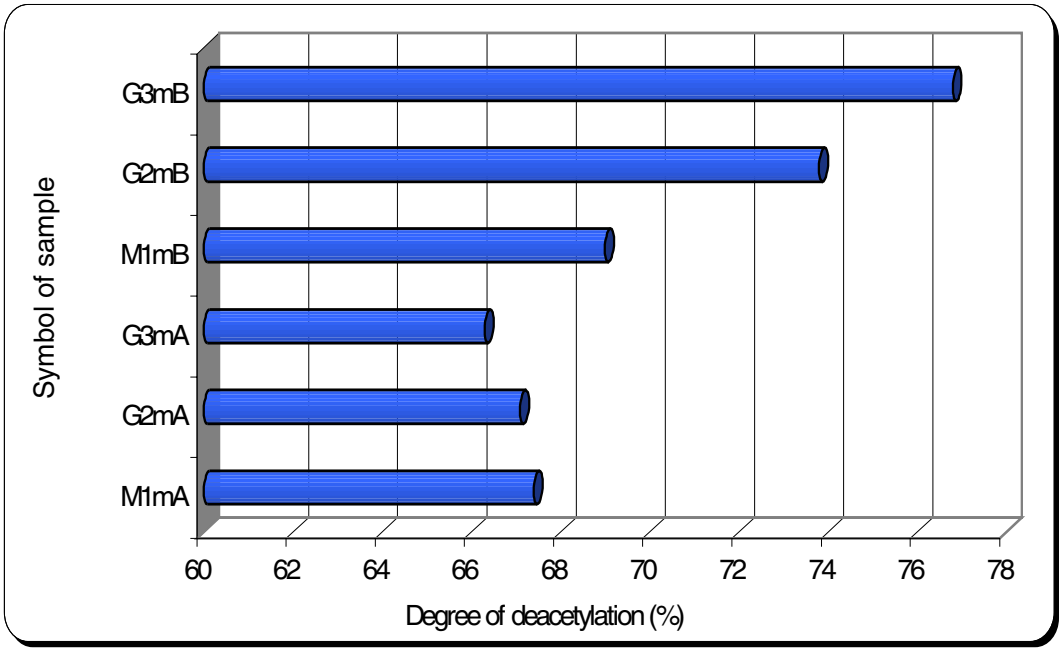



Figure 26. The changes in DD during gradual deacetylation of chitin charge "M"

These results lead to the conclusion that the temperature of the deacetylation markedly affects the degradation of the polymer.
A lower temperature of the process seems to affect the degradation of the amorphous regions, and a higher temperature of the deacetylation process produces a more rapid softening of the

polymer structure (Figures 25-26.). The highest difference between the DD of chitin and the product was found during 1h of the deacetylation stage.
Prolongation of the deacetylation procedure affected a falling tendency in the DD increase. The reduction in $\overline{M}_v$ indicated a steady decrease during up to 3h of the deacetylation process.

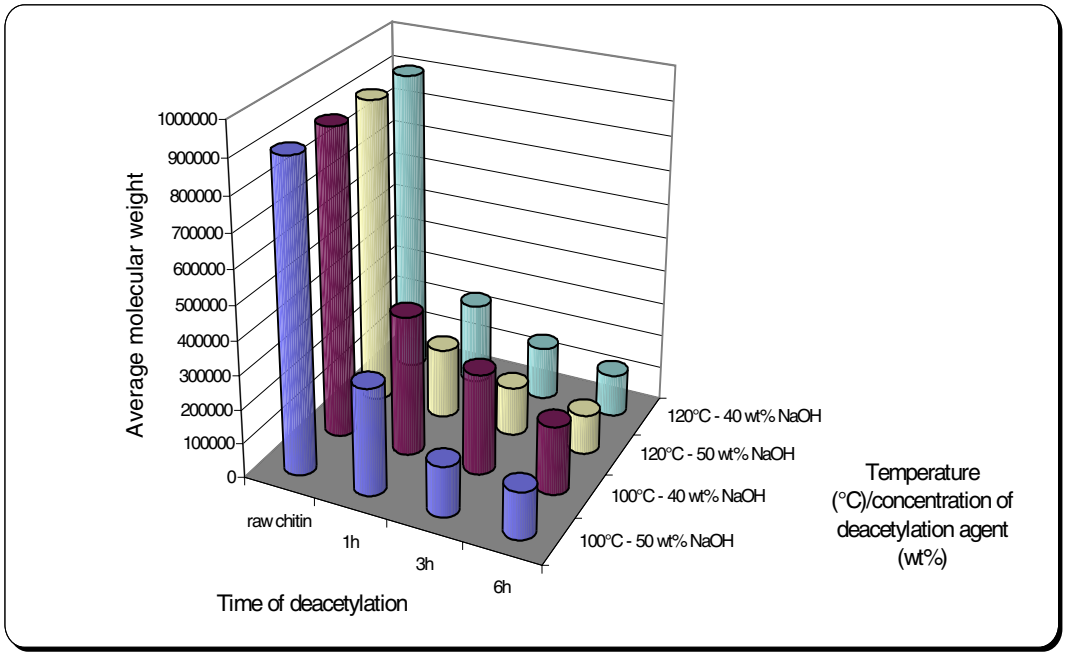



Figure 27. Influence of NaOH concentration and temperature of deacetylation on $\overline{M}_v$ of chitosan (for chitin“M”)

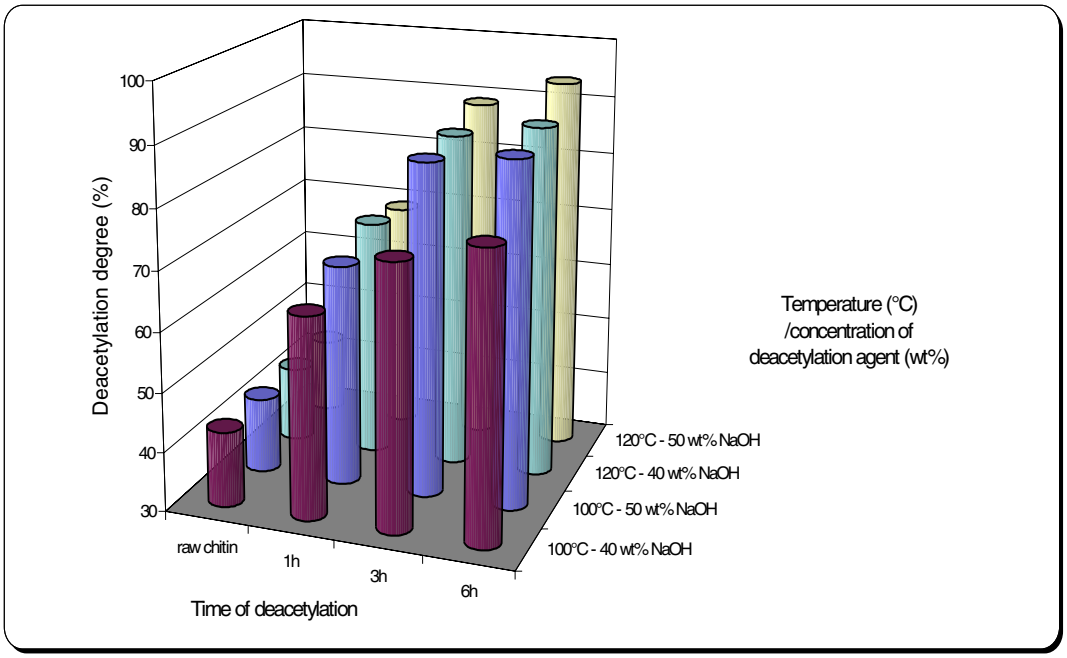



Figure 28. Influence of NaOH concentration and temperature on DD of chitosan “M”

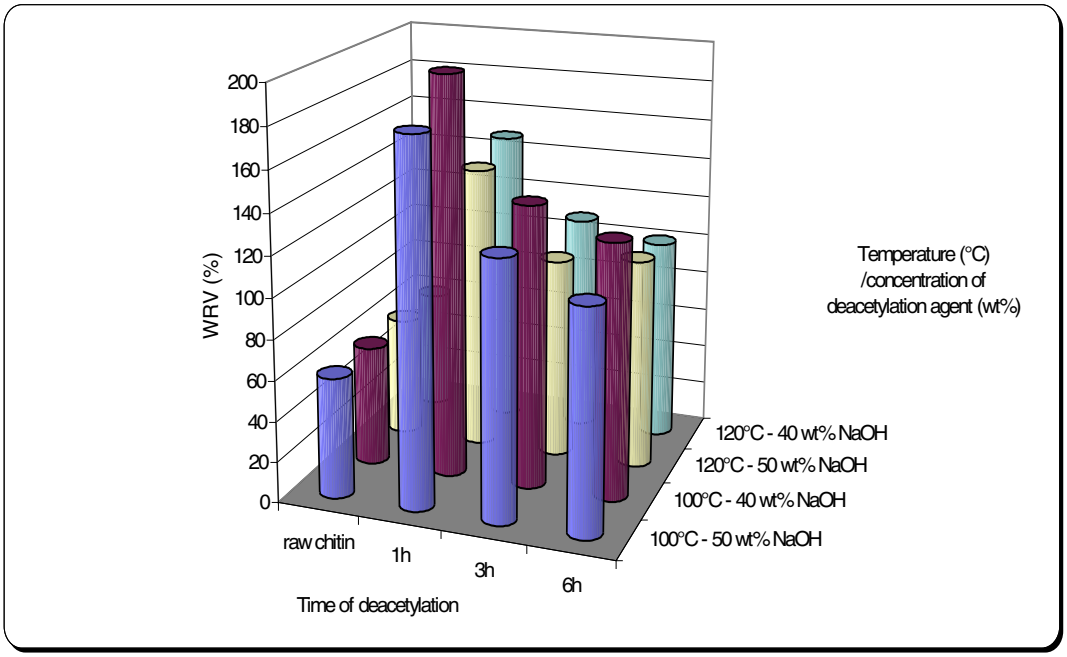



Figure 29. Influence of NaOH concentration and temperature on WRV of chitosan "M"

Prolongation of the treatment caused a drastic reduction in $\overline{M}_v$, especially for the samples deacetylated at temperatures of 120°C and 130°C (Figures 20-21). Reduction in the concentration of sodium hydroxide solution results in an increase in $\overline{M}_v$ as well as DD for chitosan deacetylated at 100°C. Increase of the process temperature caused a more extensive degradation of the polymer chain, similar as noted for deacetylation carried out with a higher concentration of sodium hydroxide (Figures 27-28). At the same time, WRV and Cs were comparable with the results where 50 wt% NaOH was used at 100°C and 120°C (Figure 29). An increase in the swelling parameters (WRV, Wc and Cs) during 1h of deacetylation process was observed. The greatest increase in these parameters was found for samples deacetylated at a lower temperature (100°C) as well as with lower concentration of sodium hydroxide (40 wt%). During prolonged deacetylation, WRV and Cs were significantly decreased. The initial increase of WRV, Wc and Cs may be explained by the rapid as well as extensive degradation of amorphous regions, which are more sensitive to sodium hydroxide solution.
A basic structure of polymer with hydrogen bonded chains remains but the absence of non-ordered structure permits a better and deeper penetration of water into the polymer structure.
Prolongation of the deacetylation or increase in the temperature or NaOH concentration are factors which cause a more extensive degradation of crystalline regions with a decrease in $\overline{M}_v$ and reduction of swelling (Figure 29).

4.2.3. Chitosan from insects larva of *Calliphora erythrocephala*

Chitin samples obtained from the larvae of *Calliphora erythrocephala* are generally deacetylated under milder conditions as compared to chitins from Crustacea as shown by the comparably lower degree of depolymerization during reactions under similar conditions (Table 14).
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 reduction of $\overline{M}_v$ by ca. 81 %; *E. superba* chitin required at least 3 h at 120 °C and the average molecular weight decreased by ca. 86%, whereas *Calliphora erythrocephala* chitin required 3 h at 100 °C and the decrease in average molecular weight was only ca. 67%.
Thus, insect chitin seems to be a superior starting material when a high average molecular weight and high degree of deacetylation are required.
The absence of inorganic materials as well as the lower crystallinity of chitin from the insects is useful for the conduct of a non-degradable process in the preparation of high-deacetylated chitosan as a product (Figures 30-31.). The relative decrease of DD with the prolonged reaction time was also observed. Multiple treatments with sodium hydroxide accelerated of high deacetylation and reduction in $\overline{M}_v$ as well as swelling behaviour (Figure 32.).

The application of multiple deacetylation resulted in rapid decrease in $\bar{M}_v$ and WRV. The increase in DD by 5.3% was only minor.

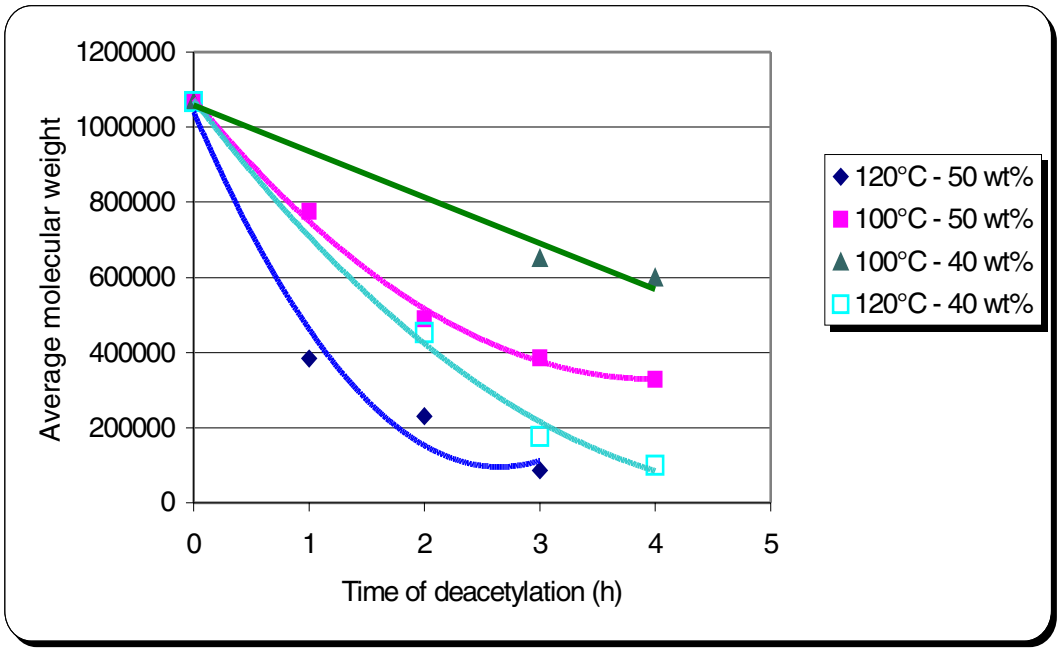



Figure 30. Effects of NaOH concentration and reaction temperature on the reduction in $\bar{M}_v$ of insect chitin

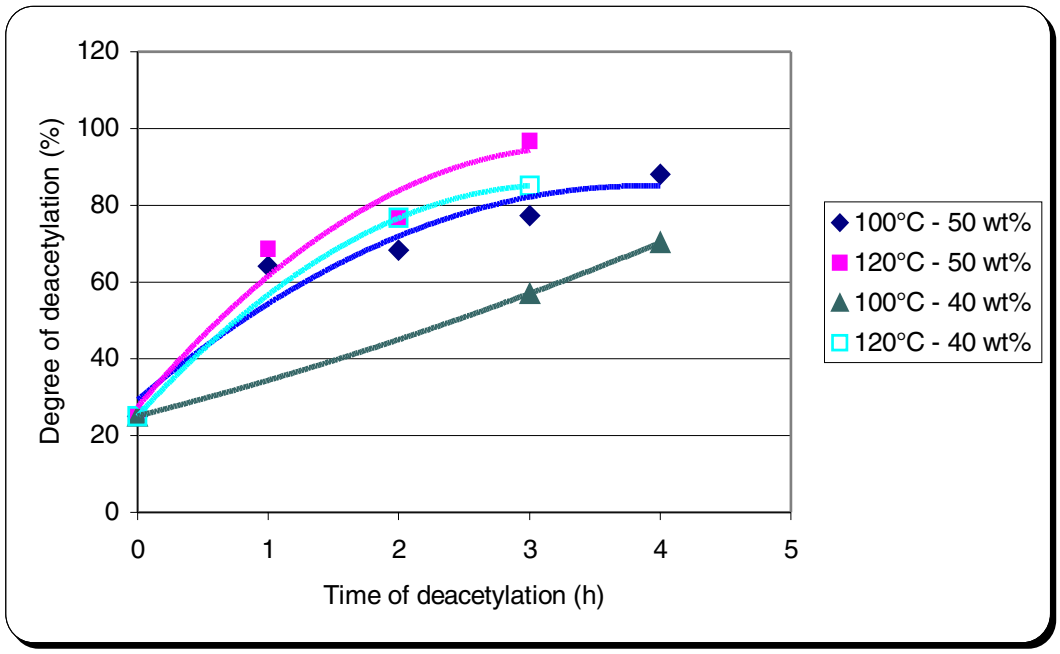



Figure 31. Effects of NaOH concentration and reaction temperature on the DD during deacetylation of insect chitin

Table 14. Properties of chitosans from insects larves of *Calliphora erythrocephala*

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

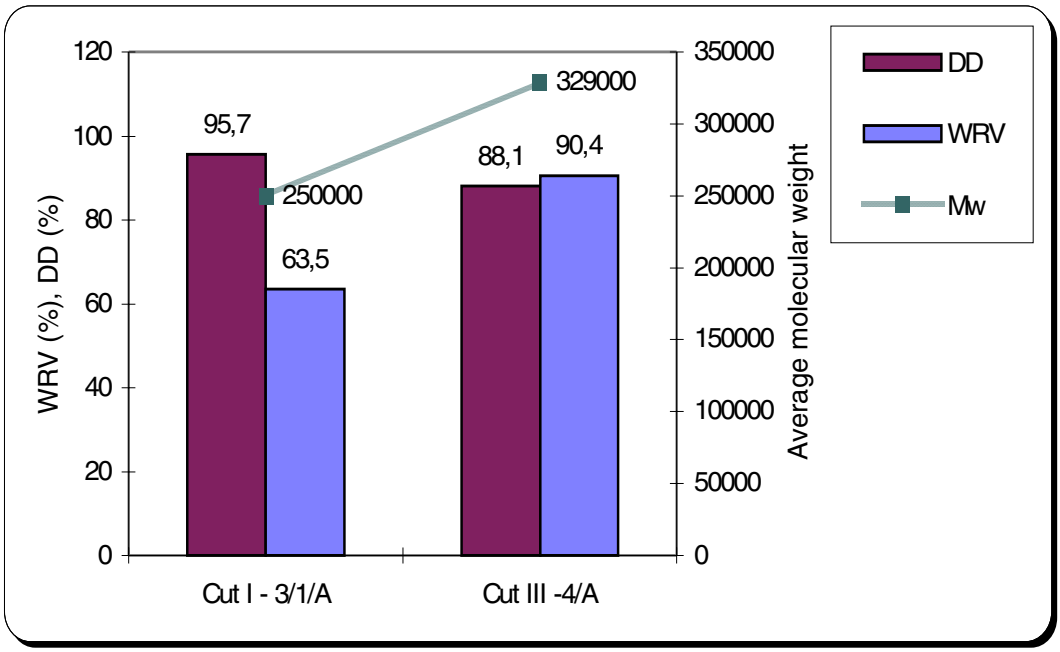



Figure 32. $\bar{M}_v$ DD and WRV in two chitosan samples; Cut I-3/1/A was deacetylated in two consecutive steps, Cut III-4A was deacetylated according to the standard procedure

The swelling parameters reflecting the internal surface of polymer (WRV, Wc and Cs) were reduced with an accompanying decrease in $\bar{M}_v$ after reactions of at least 1h (Figure 33). However, an increase of there parameters was observed at shorter reaction time.

Degradation of ordered structures during the first stage of the deacetylation may explain this observation. The increase in swelling of chitosan is due to the destructions of crystalline regions during degradation resulting in a most extensive penetration of water into the polymer structure. The transformation to microcrystalline chitosan (MCCh) has little effect on $\bar{M}_v$, but rather high WRV and Wc were observed.

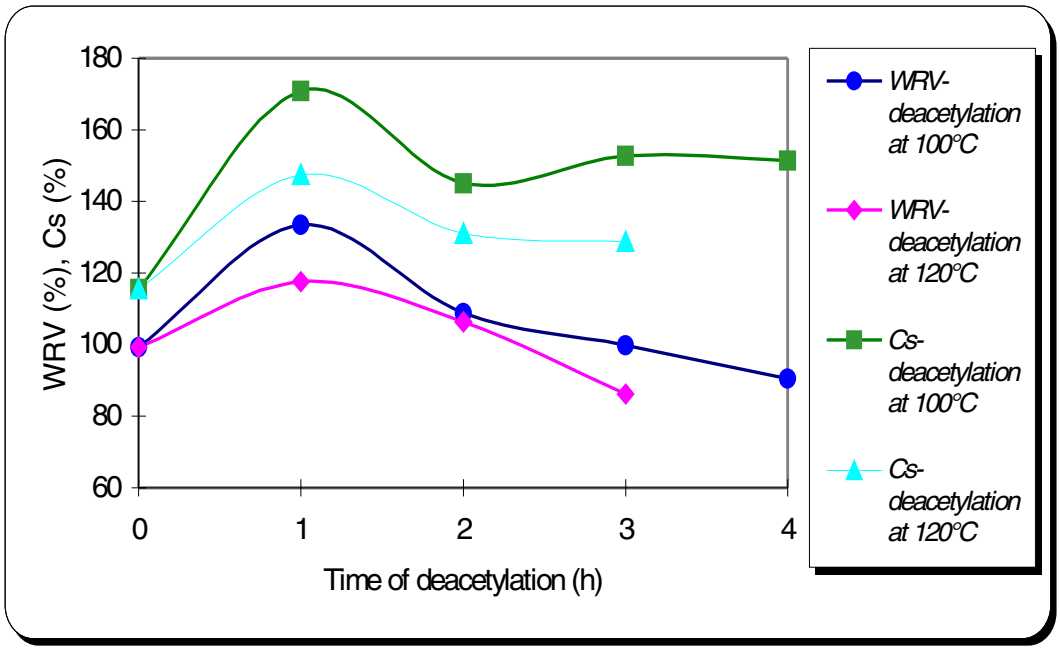



Figure 33. Change in WRV and Cs coefficients during deacetylation of insect chitin at various temperatures

The small reduction in $\overline{M}_v$ during MCCh preparation can be explained by partial degradation of the polymer by hydrochloric acid (sample MCCh I-3/1/A). Moreover, low molecular fractions were removed during precipitation of the MCCh gel-like dispersion.
When acetic acid was used as a solvent, the $\overline{M}_v$ remained unchanged (sample MCCh II-3/1/A).

4.2.4. Comparison of the properties of chitosan from various sources

A decrease in particle size of chitin raw material improved its swelling behaviour during its deacetylation, affecting the increase of shrimp chitosan solubility in acetic acid. The above observation corresponded to the increase in DD and reduction in $\overline{M}_v$ in the resultant chitosan. No notable alteration of the above-mentioned parameters was found for powdered chitosan derived from the insect larvae and krill chitin in contrast to non-grinded forms (Table 15). Nevertheless, a weak reduction in $\overline{M}_v$ (ca. 10%) for insect chitosan was observed. In the case of products obtained from shrimp shells, the swelling behaviour of powdered chitosan increased (high $\overline{M}_v$ at relatively low DD) in contrast to flakes, which parameters was not determined, because of partial solubility.
The decrease in $\overline{M}_v$ affected the reduction in swelling coefficients for the chitosan produced from the insect material. The change between WRV, Wc, and Cs of chitosan from krill was not significantly changed (Table 16).

Table 15. Properties of chitosan prepared from grinded chitin in contrast to chitin from insect cuticles and flakes

| Origin of chitin | $\overline{M}_v$ (Da) powdered chitosan | $\overline{M}_v$ (Da) not grinded chitosan | DD (%) powdered chitosan | DD (%) not grinded chitosan |
|---|---|---|---|---|
| krill | 145,000 | 148,000 [a)] | 82.1 | 83.3 [a)] |
| insect | 347,000 | 386,000 [b)] | 76.3 | 77.3 [b)] |
| shrimp | 703,000* | Insoluble [c)] | 68.5* | Insoluble [c)] |

* - properties of the soluble part, [a)] – krill chitin, [b)] – insect chitin cuticules, [c)] – shrimp chitin flakes.

Table 16. Swelling behaviour of chitosans

| Origin of chitin | WRV (%) powdered chitosan | WRV (%) not grinded chitosan | Cs (%) powdered chitosan | Cs (%)not grinded chitosan | Wc (%) powdered chitosan | Wc (%)not grinded chitosan |
|---|---|---|---|---|---|---|
| krill | 135.0 | 126.8 [a)] | 185.6 | 175.2 [a)] | 57.2 | 56.5 [a)] |
| insect | 88.1 | 99.8 [b)] | 132.8 | 152.2 [b)] | 46.8 | 50.0 [b)] |
| shrimp | 76.8 | 50.2 [c)] | 98.4 | 67.6 [c)] | 43.3 | 33.4 [c)] |

[a)] – krill chitin, [b)] – insect chitin cuticles,[c)] – shrimp chitin flakes.

The parameters of chitosan shown in Tables 15-16, produced under similar conditions, lead to the conclusion that the form of chitin (powder, flakes or cuticles) has significant influence on the properties of chitosan. The small-size particles of ground *Pandalus borealis*'s chitin were deacetylated faster than flakes, and produced a soluble product with higher swelling properties. The small decrease in $\overline{M}_v$ and the increase in swelling behaviour with no correlation to DD were found for insect chitosan.
Chitin from insect larvae of *Calliphora erythrocephala* seems to be a good substrate for production of chitosan of high $\overline{M}_v$ and relatively high DD. The highest $\overline{M}_v$ product was obtained from shrimp chitin; however, it was only partially soluble. It can be concluded that quality, for the preparation of good chitosan from shrimp chitin, it is necessary to prolong the deacetylation. However, extended deacetylation time will result in products having lower $\overline{M}_v$.

4.3. Changes in morphology during deacetylation

It was noted[88] that deacetylation process carried out under heterogeneous conditions favorably degraded the less-ordered area of chitosan with DD not higher than ca. 70%, keeping the crystallinity structure characteristic of chitin. For the range of chitosan DD from 70% to 90%, the crystalline structure is not detected. However, relatively low number of N-acetylglucosamine units (GlcNAc) in chitosan with DD higher than 90% caused the higher chitosan crystallinity.

The treatment by sodium hydroxide at first affected the destruction of amorphous regions and then proceeded from the surface to the center of the crystallinity region, thereby resulting in an increase in block-type copolymers of GlcNAc and GlcN segments. This fact suggests that the crystallinity of chitosan deacetylated long time arises from those chain segments originally present in the crystalline region of the chain and which are only deacetylated in the later stages of treatment.
So far, the following six crystalline polymorphs have been examined for chitosan: tendon-chitosan, 1-2, L-2, form I, from 2 and annealed [242-243]. The last one is anhydrous form prepared by heating a hydrated crystal of chitosan in water at a high temperature, such as 240°C [244]. The single molecular chain in these polymorphs has always been observed to extend the 2-fold helical structure similar in chitin and common cellulose[245-246].

4.3.1. Solid state $^{13}$C-NMR study

The $^{13}$C chemical shifts of crustacean chitin from *P. borealis* and *E. superba* are similar to the chemical shifts of α-chitin from crabs presented in Table 17.
The spliting of the intensity of C-4 signal into a pair of sharp and broad components after deacetylation has been observed. At the same time, a signal of C-4 was shifted from 83.3 ppm (observed in chitin; see Section 4.1.2., Table 10) to ca. 81.8 ppm and 86.7 ppm with an increase in their rel. intensity.
This observation is especially noticeable for highly deacetylated chitosan (DD between 93.2% and 99.2%). The presence of doublet for C4 carbons was first interpreted in terms of a non-equivalence of alternate glycosidic linkages along the molecular chain. VanderHart[247] et al. proposed that the multiplicity reflects the presence of more than two anhydroglucose residues per unit cell in the crystal. This observation was also explained by conformational non-equivalence of two independent chains caused by the presence of loosely bound water between the chitosan chains[7].

Table 17. $^{13}$C chemical shifts of solid chitin and chitosan samples prepared from various sources (in ppm).

| | C=O | C1 | C4 | C5 | C3 | C6 | C2 | $CH_3$ |
|---|---|---|---|---|---|---|---|---|
| | | | *Chitosan* | | | | | |
| Crab shell[1)] | -------- | 105.0 | 85.6<br>81.1 | 75.5 | -------- | 60.3 | 56.4 | -------- |
| Shrimp shell (*P. borealis*)<br>(PB10; DD=99.2%) | -------- | 105.2 | 86.1<br>81.2 | 75.6 | -------- | 60.1<br>61.0 | 57.2 | -------- |
| Krill shell (*E. superba*)<br>(M3mB; DD=93.2%) | -------- | 105.8 | 86.7<br>81.9 | 75.9 | -------- | 61.9<br>60.4 | 57.2 | -------- |
| Chitosan cuticles I-3/1/A | -------- | 105.1 | 85.5<br>81.8 | 75.4 | -------- | 61.1<br>60.2 | 57.1 | -------- |
| Insects MCCh I-3/1/A | -------- | 105.7 | 86.1<br>81.8 | 75.8 | -------- | 61.2<br>60.4 | 57.2 | -------- |

1) – Seito et al., 1987, spectrometer Bruker CXP-300, external reference: liquid benzene[248]

It is assumed that the highly deacetylated polymer is composed of two types of polymorphs. This observation is similar to that detected for cellulose[246]. The typical region for the less-ordered cellulose contaminates is detected as a several signals between 80 ppm and 86 ppm (corresponding to xylan, hemicellulose, accessible or inaccessible fibril surfaces etc.). Signals in the range from 86 ppm to 90 ppm are detected in the crystalline cellulose polymorphs (Iα, Iβ, para-crystalline, etc.)[246].
The decrease in intensity in methyl ($CH_3$) and carbonyl (C=O) signals of chitosan when DD decreased, till the complete absence of mentioned signal in fully deacetylated chitosan and shift of C-2 signal resulted in the deformation of polymer chains (elimination of acetyl group) was noted (Figure 34). No additional signals for the chitosans prepared from various crustacean chitins were detected. This fact suggests the removal of non-chitinous contaminates.
The $^{13}$C-CP/MAS spectra of chitosans are listed in Table 17. There are no significant differences in the appearance of the spectra of chitosans and chitins obtained from Crustacea or from insects.

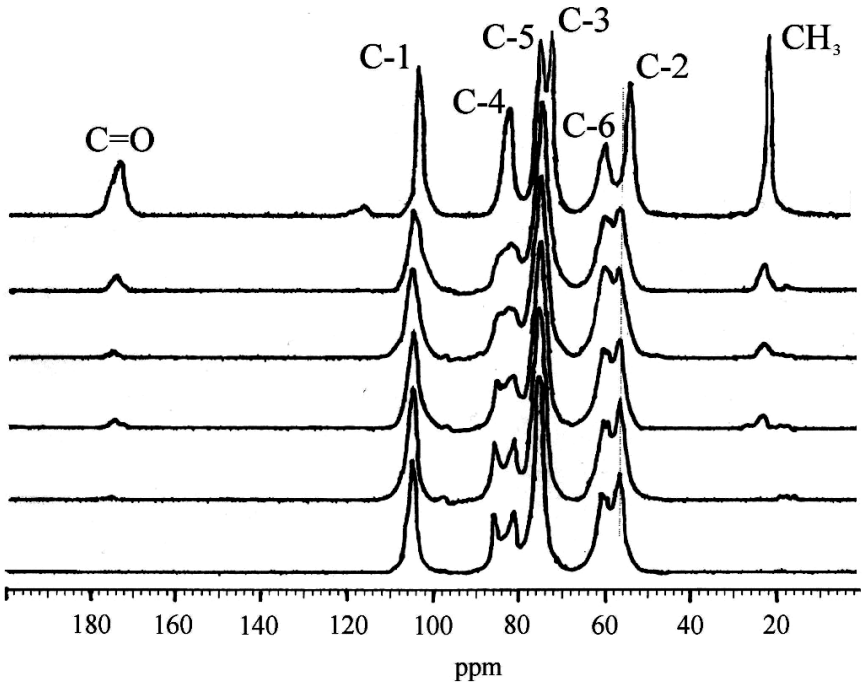



Figure 34. $^{13}C$ CP-MAS NMR spectra of chitin from *P. borealis* (DD=4.6%), chitosan G3mA (DD=58.5%), M3A (DD=69.4%), G3mB (DD=76.0%), M3mB (DD=93.2), PB10 (DD=99.2%)

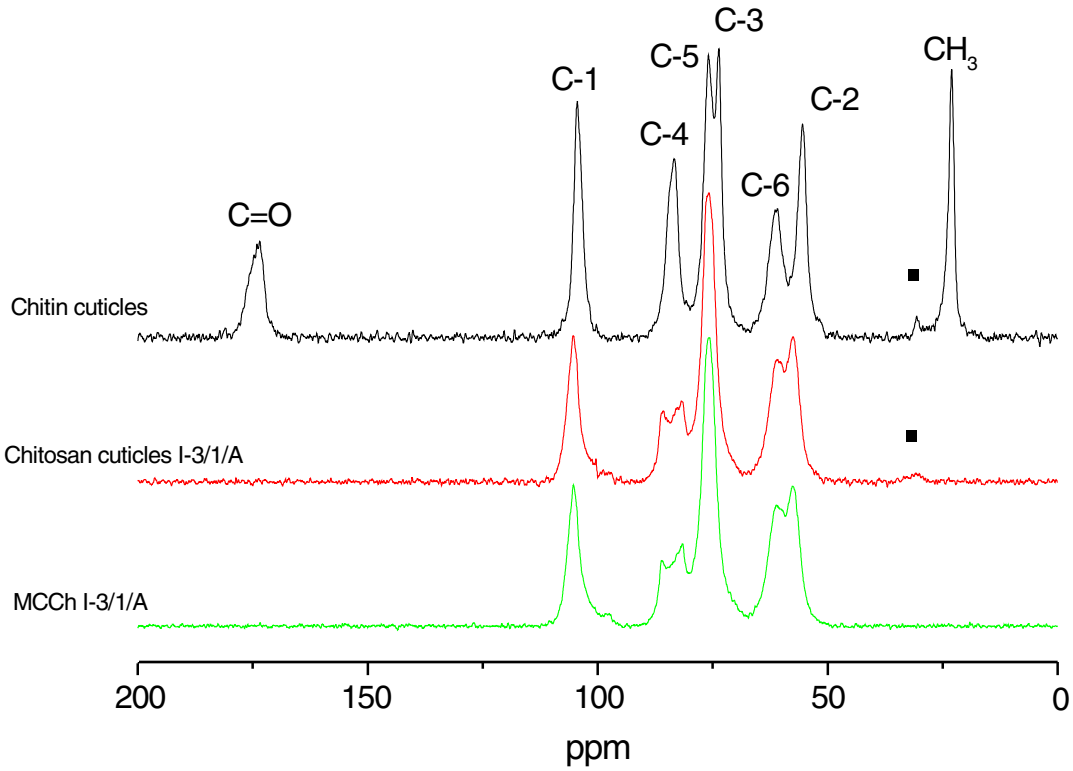



Figure 35. $^{13}C$ CP-MAS NMR chemical shifts of chitosan from *Calliphora erythrocephala* chitosan cuticles and purified insect MCCh. ■ - signals derived from contaminants (residual proteins and catechol)

However, the insect chitosan still contains a small amount of contaminants (residual proteins and/or catechol), which is absent in its MCCh form. Thus, preparation of MCCh also means a higher degree of purity of chitosan.
The spectrum of chitosan from insect cuticles showed the complete absence of the relaxation characteristic for methyl-carbon signal (at 23 ppm) as well as carbonyl-carbon (at 174 ppm). This fact suggests that above-mentioned chitosan was fully deacetylated (DD determined by titration as 99%). The duplication of C-4 signal with shifting to a lower frequency as well as the relocation of C-2 signal after deacetylation was also observed in the case of high-deacetylated insect chitosan (Figure 35).

4.3.2. FTIR spectroscopy

IR spectra of chitin and chitosan show characteracteristic absorptions as follows:

- Superimposition of hydroxyl and N-H stretch at 3450 $cm^{-1}$ ,
- Absorption at 2950 $cm^{-1}$ – 2880 $cm^{-1}$ corresponding to aliphatic C-H stretch,
- Amide I band at 1660 $cm^{-1}$ resulting from C=O stretch in the amide group of N-acetylglucosamine units,
- C-O-C stretches (asymmetric) at 1150 $cm^{-1}$ and (symmetric) at 1100 $cm^{-1}$ – 1020 $cm^{-1}$.

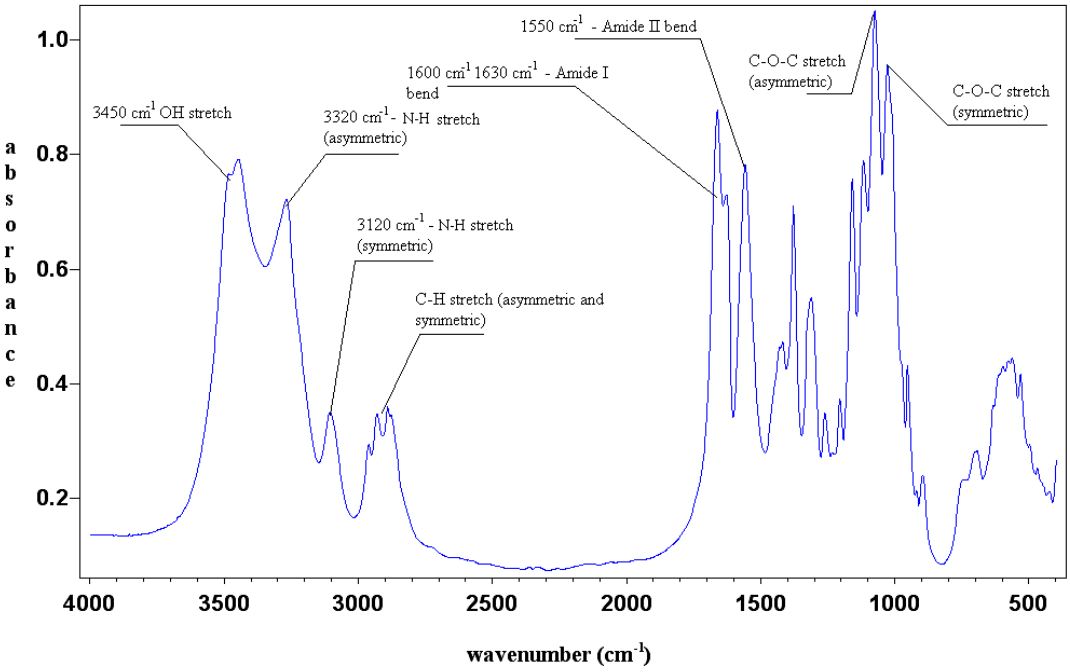



Figure 36. FTIR spectrum of chitin P (from *Pandalus borealis*)

Some additional bands in the IR spectrum of chitin from *Pandalus borealis* in the wavenumber range from 1600 $cm^{-1}$ to 1200 $cm^{-1}$ was observed (Figure 36). The pair of N-H stretches, superimposed with O-H stretch, at 3320 $cm^{-1}$ (asymmetric) and at 3120 $cm^{-1}$ (symmetric) was found for IR spectrum of high N-acetylated chitin (DD lower than 30%). In addition, two absorption bands of amide I were resolved at 1660 $cm^{-1}$ and 1630 $cm^{-1}$. This fact suggests the existence of two types of hydrogen-bonded carboxyl groups in amide I[249]. The singly hydrogen-bonded group absorbed at 1660 $cm^{-1}$ and the doubly- bonded at 1630 $cm^{-1}$. The intensity of 1550 $cm^{-1}$ corresponding to the N-H stretches in the amide group (amide III), in the IR spectrum of chitin was observed. The strong intensities at 1100 $cm^{-1}$ characteristic for highly N-acetylated chitin were sharply resolved.
After deacetylation of crustacean chitins under heterogeneous conditions, a decrease in the intensities of the amide I band with shifting of amide II to lower frequencies was observed. The generation of the $NH_2$ group was reflected by the appearance of a new band at 1590 $cm^{-1}$. The duplication of N-H stretches at 3300 $cm^{-1}$ and 3120 $cm^{-1}$ was not resolved for highly deacetylated chitosans (Figure 37).
Similar results were observed upon deacetylation of chitin cuticles prepared from *Calliphora erythrocephala* (Figure 38).

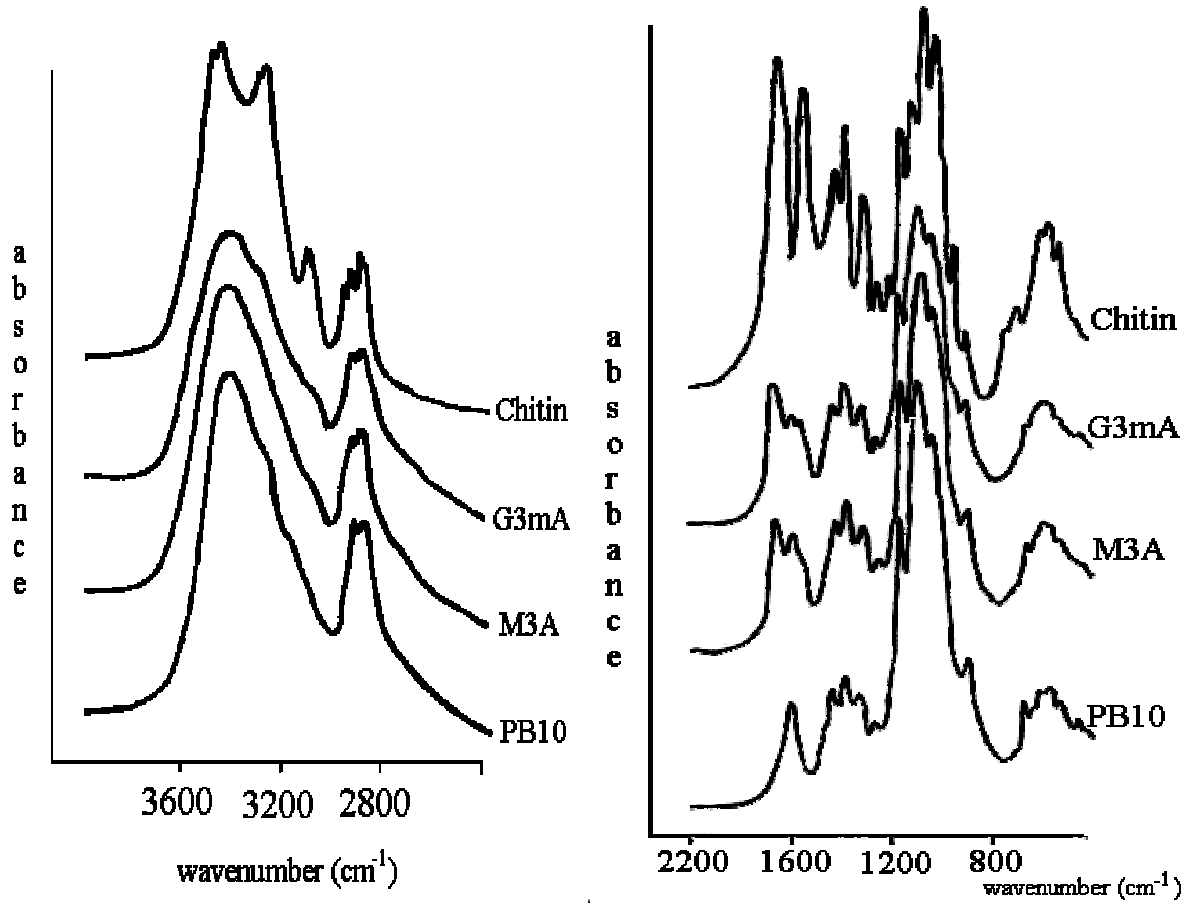



Figure 37. IR spectra (2000 $cm^{-1}$ to 400 $cm^{-1}$ and from 4000 $cm^{-1}$ to 2400 $cm^{-1}$) of chitin from *Pandalus borealis* (DD=7.4%) as well as chitosan PB10 (DD=98.3%), G3mA (DD=58.2%), M3A (DD=66.7%)

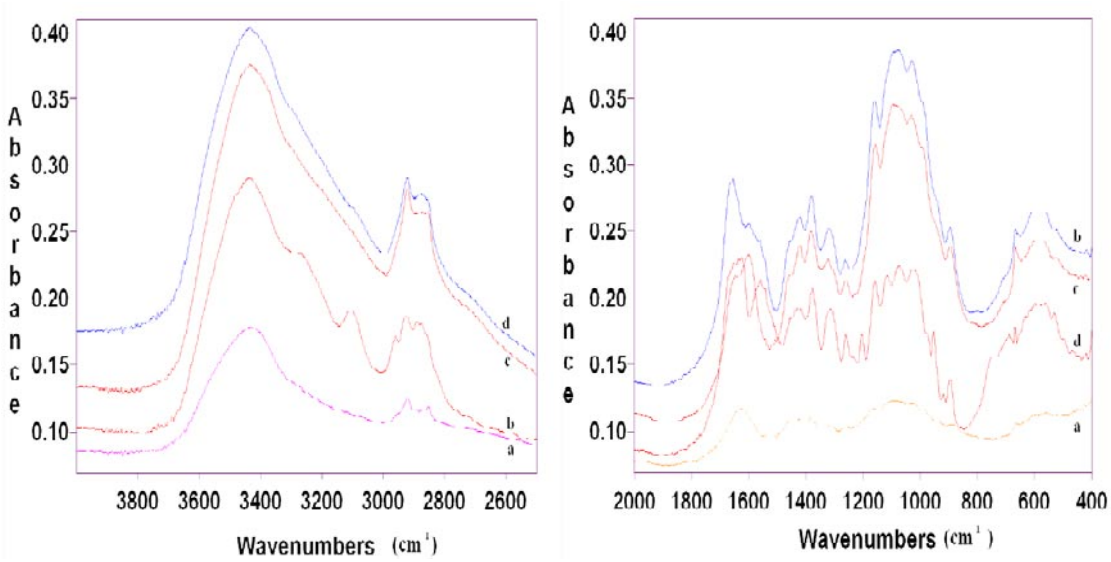



Figure 38. IR spectra (2000 $cm^{-1}$ to 400 $cm^{-1}$ and from 4000 $cm^{-1}$ to 2400 $cm^{-1}$) of insect chitin from *Calliphora erythrocephala* (b - DD=24.7%) as well as chitosan cuticles I – 3/A (d - DD=78.8%), chitosan cuticles I – 3/1/A (c -DD=95.7%), MCCh I – 3/1/A (DD=97.6%)

The pair of N-H stretches were not resolved confirming their absence for chitin or chitosan with DD higher than 20%. The appearance of strong absorption of amide I for chitosan cuticle indicated the presence of protein, which was not found in the IR spectrum of microcrystalline chitosan (MCCh I-3/1/A. The presence of protein may cause some deviations in the determination of DD by the IR

method because of the additional intensities for amine and/or amide groups. The IR spectrum of MCCh presented the same pattern as the IR spectrum of fully deacetylated chitosan (chitosan specimen PB10).

4.3.3. X-ray diffraction

It is well known that the crystalline structure of chitin influences its properties, including its susceptibility to hydrolysis and deacetylation yield. The occurrence of crystallinity improvement for the prolonged deacetylation of biopolymer was also confirmed in the existing literature data[88,250].
Table 18 shows the indexes of crystallinity (CrI) and the lattice angle 2θ of crystallinity as well as the amorphous regions of chitins produced from various sources and deacetylated chitosan.

Table 18. Index of crystallinity and the lattice angle (2θ) of crystallinity and amorphous peaks of chitins and heterogeneous deacetylated chitosan

| Sample | Crystallinity diffraction pattern intensity | Amorphous diffraction pattern intensity | CrI (%) |
|---|---|---|---|
| Chitin M | 19.3° | 9.7° | 73.3 |
| Chitin Mm | 19.4° | 9.8° | 67.1 |
| Chitin P | 19.2° | 9.5° | 63.5 |
| Chitin cuticles I | 19.8° | 9.9° | 39.4 |
| Chitosan M3A | 20.0° | 9.7° | 36.6 |
| Chitosan M6mC | 20.5° | 11.0° | 44.1 |

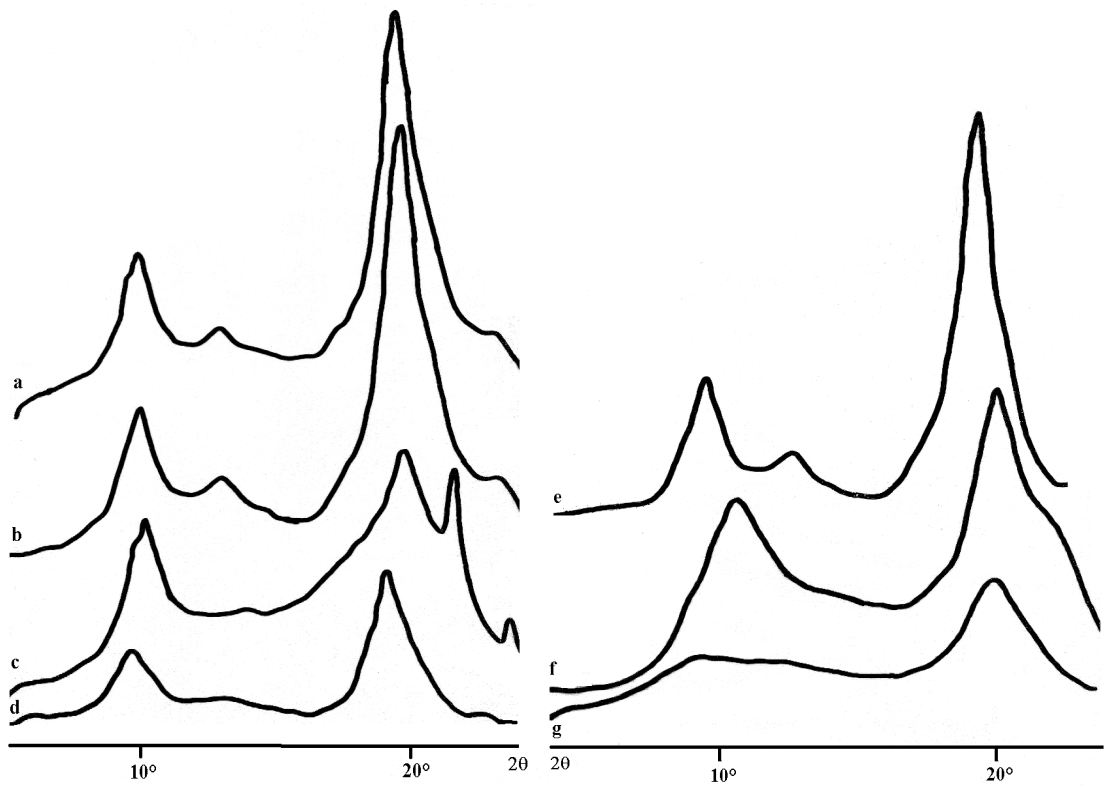



Figure 39. X-diagrams of chitins from various sources and heterogeneous deacetylated chitosan: a) - chitin from Antarctic krill, b) and e) - chitin from Antarctic krill after demineralization, c) – chitin cuticles from *Calliphora erythrocephala*, d) – chitin from *Pandalus borealis*, f) – chitosan M6mC (DD – 92.6%), g) chitosan M3A (DD – 73.5%)

The chitin from *C. erythrocephala* shows the lowest CrI, which is consistent with their facility of deacetylation under relatively mild conditions. The X-ray diffraction patterns also reveal some protein contaminations, as has been observed by NMR and FT-IT spectroscopy (lattice angle 2θ at 22.0° and 24.2°) (Table 18, Figure 39).

The demineralization of krill chitin caused a low decrease in crystallinity (CrI of chitin Mm after 3 h demineralization is lower ca. 6%).
In the case of chitin from *Pandalus borealis,* the low DD and high CrI prolonged process of deacetylation for obtaining the soluble chitosan. Chitosan samples with DD lower than 75% have a more-ordered structure, however CrI decreased gradually with further increase in DD.
Long deacetylation time, giving chitosan with DD higher than 92%, resulted again in higher CrI, though the lattice angle at $2\theta = 9.8°$ and 19.4° (chitin Mm) had shifted to $2\theta = 11.0°$ and 20.5° (chitosan M6mC). This fact suggests that the crystallinity diffraction pattern of these chitosans increased for the chain segments that already exist in the more ordered region of chitin, which are therefore susceptible to deacetylation only at longer reaction times[88,241]

4.4. Analysis of degree of deacetylation in chitosan from various sources

The degree of deacetylation (DD) considerably affects the solubility of chitosan and this determines the potential applications of this biopolymer. Therefore, a rapid and accurate method for determining DD of chitosan is important both for studies into this interesting polysaccharide and for the technologies involved in the use of chitosan in medicine, agriculture, dressing materials, cosmetic, papermaking, etc. Titration of amine groups [12,23,24,26, 25,251] and IR spectroscopy[15,16,17,18,19,20] are the most frequent procedures reported in the literature.
The titration technique seems to be accurate method. It is rapid and accurate, but as the degree of deacetylation lowers and thereby the solubility of chitosan decreases, it becomes more less applicable. This restriction does not apply to the IR spectroscopic technique, which can be used even with insoluble materials (KBr disk technique). However, for low-deacetylated chitosans the IR method becomes decreasingly accurate, being affected by several factors such as sample preparation, selection of analytical and reference bands, mathematical methods applied to process the data[76,252].
A comparison of the spectra recorded using KBr disks or films showed that the latter method does not yield reproducible data. Therefore, all spectra were recorded from KBr disks. In this study, IR spectra were analyzed by three methods as described in the literature. Muzzarelli et al.[20] and Domard[25] used the quotient $A_{1650}/A_{3450}$, while Sannan et al.[15] recommended $A_{1550}/A_{2900}$, i.e. the Amide II bands as the analytical and the CH band as the reference. Another method has been suggested by Shigemasa et al.[7] who used the C-O at 1070 and 1030 $cm^{-1}$ as the reference and the Amide I at 1630 and 1660 $cm^{-1}$ as the analytical bands.
Furthermore, a calibration coefficient has to be applied (calculated using the absorbance ratio of $A_{1650}/A_{3450}$ for fully N-acetylated chitin), the value of which was given by Muzzarelli et al. [20]as 0.75, and by Domard[25] as 1.15. In this case, comparison of IR spectra with NMR and potentiometric titration revealed a calibration coefficient of 0.86. Table 19 shows DD values calculated from the ration $A_{1650}/A_{3450}$ without applying calibration coefficients.

Table 19. DD values obtained for calculations based on the ratio $A_{1650}/A_{3450}$

| Sample | Baseline separation based on group of peaks | Baseline separation based on individual peak |
|---|---|---|
| G3mA | 51.6 | 57.8 |
| M3A | 61.4 | 66.2 |
| G3mB | 73.3 | 76.1 |
| M3mB | 94.8 | 95.7 |
| PB10 | 98.0 | 99.8 |

All DD results were calculated as an average of three repetitions, with accuracy of 3%,

It was found that calculations based on the area of the peaks yield large variations and thus are not reliable. However, there is a good agreement in the values based on individual peaks or groups of peaks. The data shown in Table 20 are evaluated by application of the various calibration coefficients discussed above, and compared with the DD values obtained by NMR and potentiometric titration, respectively.

The best correlation was found using a calibration coefficient of 0.86 (individual peak) and 0.75 (group of peaks) when comparing the IR data with the potentiometric method, if DD is in a range between 85% and 100%. (Figure 40).

This is explained by the increasing solubility of chitosan with increasing DD values. It is advised to check the solubility of a given chitosan sample by means of dissolving it in 1% of aqueous acetic acid before carrying out potentiometric measurements.

Table 20. DD values (%) of chitosans obtained for the ratio $A_{1665}/A_{3450}$ with various calibration coefficients.

| Samples | CP/MAS $^{13}C$ – NMR | Titration | IR spectroscopy: baseline separation based on | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | On individual peak | | | Group of peaks | | |
| Calibration coefficient | | | 0.751 (1) | 0.86 (2) | 1.15 (3) | 0.751 (1) | 0.86 (2) | 1.15 (3) |
| G3mA | 58.5 | 66.3 | 68.0 | 63.2 | 51.1 | 63.6 | 58.2 | 44.3 |
| M3A | 69.4 | 73.5 | 74.6 | 70.8 | 61.2 | 71.0 | 66.7 | 55.6 |
| G3mB | 76.0 | 81.8 | 82.0 | 79.3 | 72.5 | 79.9 | 76.9 | 69.3 |
| M3mB | 93.2 | 90.5 | 96.8 | 96.3 | 95.1 | 96.1 | 95.5 | 94.0 |
| PB8 | 95.6 | 89.9 | 96.9 | 96.5 | 95.3 | 96.4 | 95.9 | 94.5 |
| PB10 | 99.2 | 98.4 | 99.9 | 99.8 | 99.8 | 98.5 | 98.3 | 97.7 |
| M1mC | 79.0 | 85.6 | 86.6 | 84.5 | 79.5 | 84.8 | 82.5 | 76.8 |
| M6mC | 85.1 | 92.6 | 90.6 | 89.2 | 85.6 | 90.0 | 88.5 | 84.7 |
| MCCh I – 3/1/A | 95.6 | 97.6 | 99.9 | 99.7 | 99.8 | 98.4 | 98.2 | 97.6 |

All DD results were calculated as an average of three repetitions, with accuracy of 3%,
(1) - according to R.A.A. Muzzarelli [20]; (2) - coefficient equal calculated for the average value of $A_{1665}/A_{3450}$ for fully N-acetylated chitin; (3) - according to A. Domard[25]

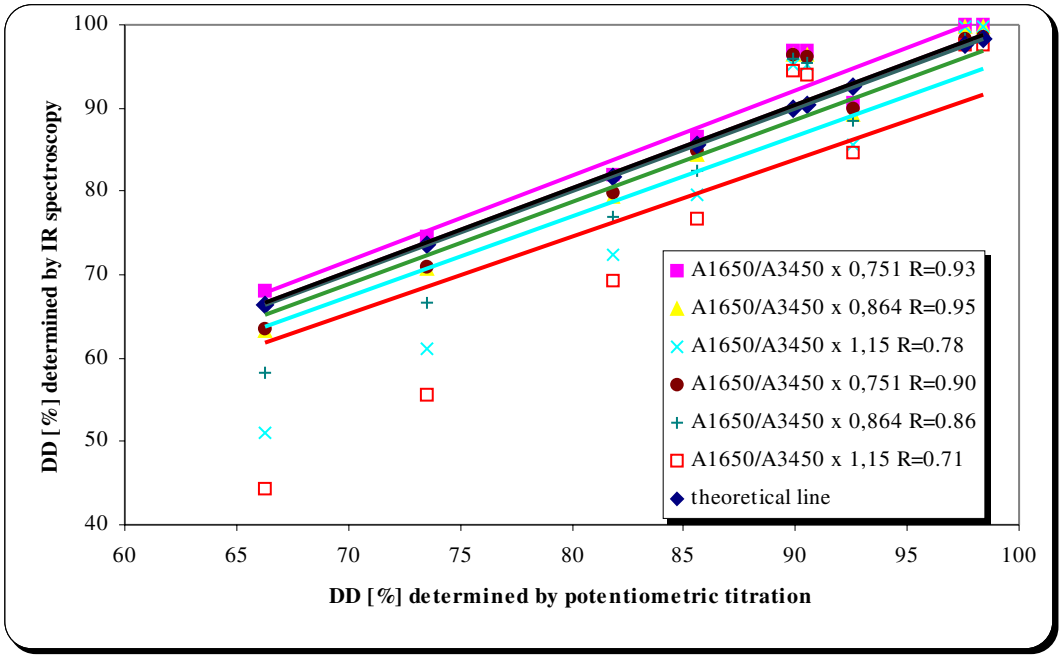



Figure 40. Relationship between DD values determined by potentiometric titration and IR spectroscopy (■. ▲. x - baseline separation according to individual peak; ●. +. ◘ -and group of peaks; R - coefficient of correlation; ◆ - theoretical line)

A good correlation of DD values was also found in comparison of IR with NMR using the calibration coefficient 0.864, as shown in Figure 41.

Ideally, the correlation line should cross the origin of the coordinates. This criterion most closely met when the calibration coefficient 0.864 is applied in the case of potentiometric titration as well as NMR spectroscopy.

Minke et al.[226] reported that chitin contains two types of amide groups and both form C=O----H-N, but one is intermolecularly hydrogen bonded to C(6) hydroxyl groups.

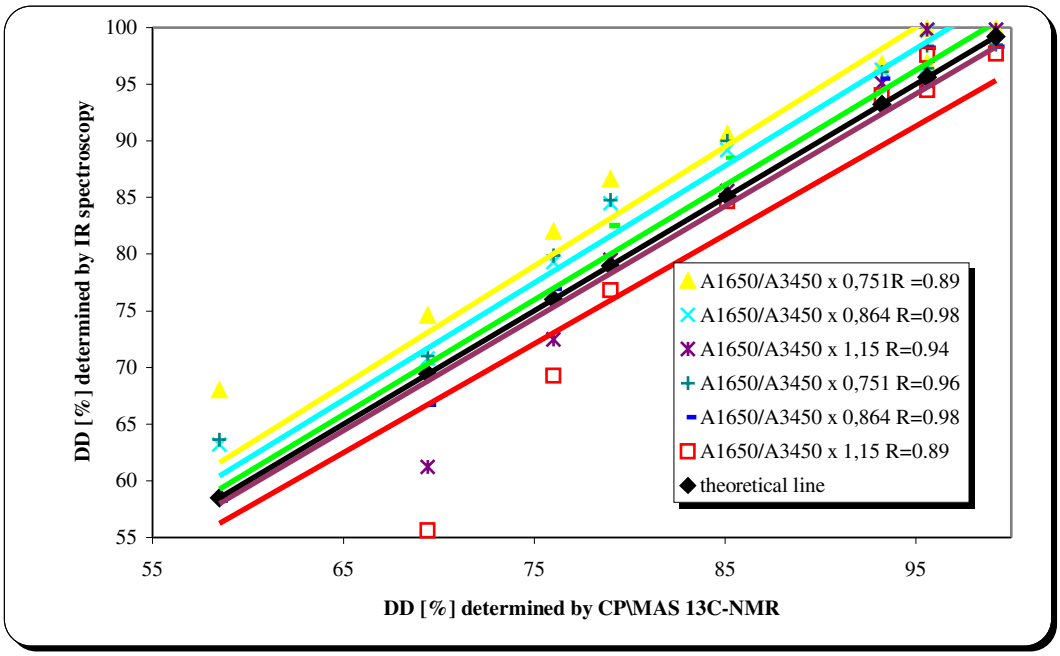



Figure 41. Relationship between DD values determined by CP/MAS $^{13}$C-NMR and IR techniques (▲. x .x- baseline separation according to individual peak ; +. -. ◘ group of peaks ; **R** - coefficient of correlation; ◆ - theoretical line)

Table 21. Degree of deacetylation calculated according to the Shigemasa's method[7]

| Samples | DD (%) determined by: | | | |
|---|---|---|---|---|
| | Potentiometric titration | CP/MAS $^{13}$C - NMR | IR spectroscopy | |
| Calculation according to: | | | $(A_{1630}+A_{1660})/A_{1070}$ | $A_{1660}/A_{1070}$ |
| G3mA | 66.3 | 58.5 | 56.8 | 44.0 |
| M3A | 73.5 | 69.4 | 68.0 | 61.5 |
| G3mB | 81.8 | 76.0 | 75.1 | 67.0 |
| M3mB | 90.5 | 93.2 | 93.2 | 90.8 |
| PB8 | 89.9 | 95.6 | 95.7 | 93.6 |
| PB10 | 98.4 | 99.2 | 100.0 | 100.0 |
| M1mC | 85.6 | 79.0 | 78.5 | 74.5 |
| M6mC | 92.6 | 85.1 | 84.8 | 77.0 |
| MCCh I –3/1/A | 95.6 | 97.6 | 100.0 | 100.0 |

Therefore, Shigemasa et al.[7] proposed to use the sum of the Amide I bands ($A_{1650}$ and $A_{1630}$ $cm^{-1}$) as the analytical band and $A_{1070}$ as the reference band. However, in study this approach gave rather poor correlation with the data obtained by potentiometric titration (Table 21, Figure 42) whereas the correlation with NMR was very good (Figure 43).

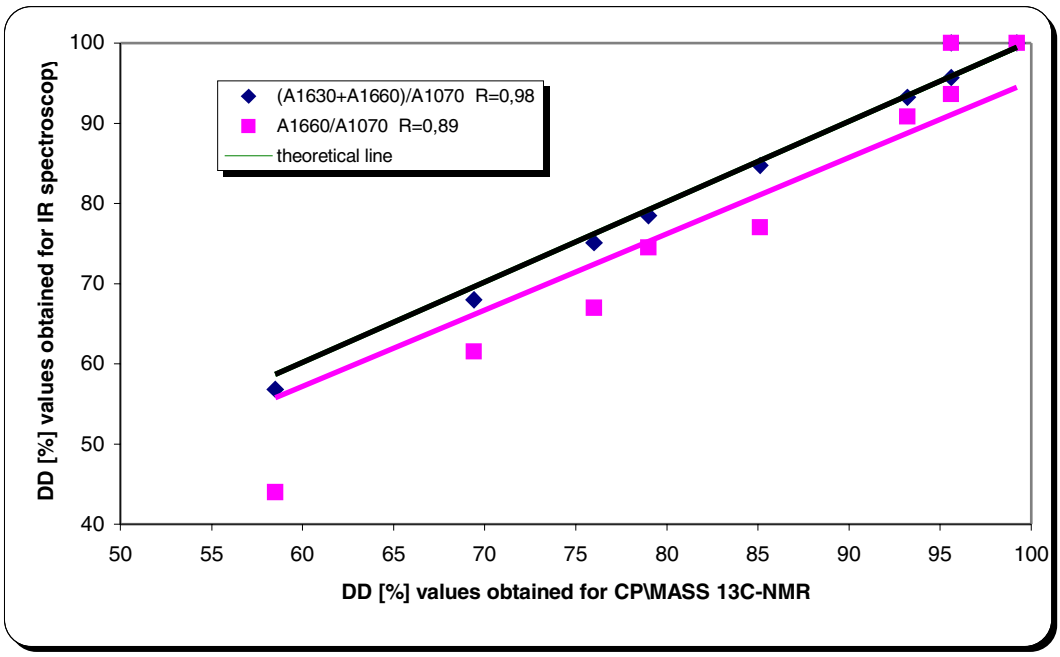



Figure 42. Correlation of DD values obtained by NMR and IR spectroscopy according to the method of Shigemasa's[7] (peak evaluation by PeakFit 4.0); R: correlation coefficient

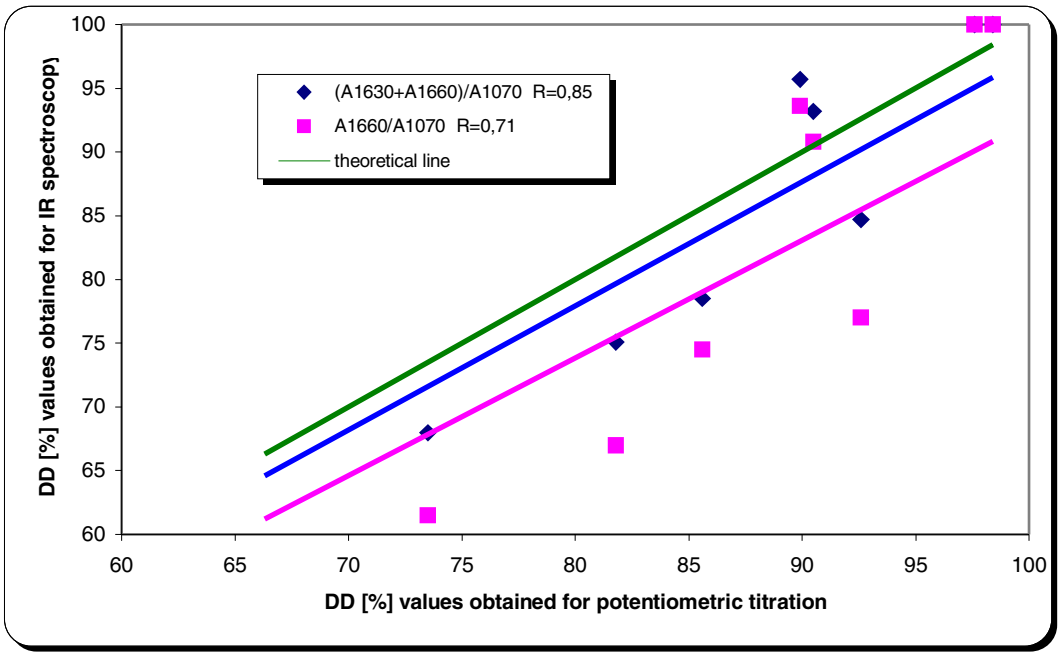



Figure 43. Correlation of DD values obtained by potentiometric titration and IR spectroscopy according to the method of Shigemasa[7] (peak evaluation by PeakFit 4.0; R: correlation coefficient)

## 4.5. Biodegradation of chitosan

### 4.5.1. Chitosan samples

Samples of chitosan were prepared by the deacetylation of chitin from shells of Antarctic krill (*Euphausia superba*) (Sample M, see Table 22; cf. [76]), or shrimp (*Pandalus borealis* – sample PB08 and PB10). Another commercial sample (P4) was obtained from Chemopol (Tada, India).

Table 22. Properties of chitosan used for biodegradation study

| Sample | Ash | Moisture (%) | $\bar{M}_V$ [(a)] | WRV [(b)] | DD (%)[(c)] | | |
|---|---|---|---|---|---|---|---|
| | | | | | $^{13}$C-NMR | Titration | IR |
| G3mA | 1.2 | 11.25 | 264,000 | 153.9 | 58.5 | 66.3 | 58.2 |
| M3A | 4.5 | 11.41 | 187,000 | 171.1 | 69.4 | 73.5 | 66.7 |
| G3mB | 1.1 | 10.41 | 135,000 | 99.7 | 76.0 | 81.8 | 76.9 |
| M3mB | 1.1 | 11.95 | 143,000 | 99.8 | 93.2 | 90.5 | 95.5 |
| M1mC | 1.1 | 11.18 | 111,000 | 135.9 | 79.0 | 85.6 | 82.5 |
| M6mC | 1.1 | 10.73 | 64,000 | 88.7 | 85.1 | 92.6 | 88.5 |
| PB08 | 2.6 | 11.05 | 248,000 | 65.2 | 95.6 | 89.9 | 95.9 |
| PB10 | 2.5 | 9.89 | 227,000 | 56.8 | 99.2 | 98.4 | 98.3 |
| P4 | 0.6 | 10.71 | 130,000 | 79.5 | ---- | 69.6 | 64.8 |

[(a)] determined by viscosimetry, [(b)] determined gravimetrically, [(c)] determination of DD according to Struszczyk et al.[227]

4.5.2. Biodegradation of chitosan of different DD

Incubation of chitosan samples with *Aspergillus fumigatus* reduced the viscosity of the solutions concomitant with an increase in the pH from 5.7 to 6.6. Figures 44 and 45 show the rate of degradation of various chitosan solutions with the concentration of 0.25 wt% or 0.5 wt% as a plot of $\Delta(1/[\eta]) = (1/[\eta_t]) - (1/[\eta_o])$[252] versus time, where $[\eta_t]$ = intrinsic viscosity of incubation mixture ($g \bullet dl^{-1}$) at time t and $[\eta_0]$ = intrinsic viscosity at t = 0 (h).

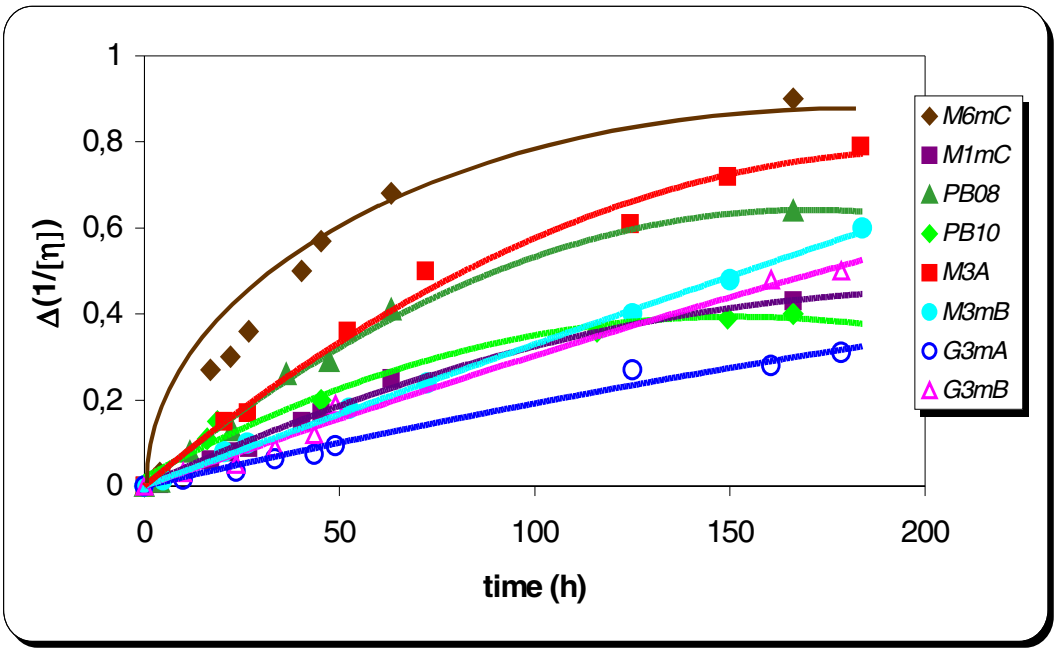



Figure 44. Changes in viscosity as a result of degradation of various chitosan samples by *Aspergillus fumigatus* (initial concentration of chitosan 5 $mg \bullet cm^{-3}$)

The shape of the curves indicates a random attack of hydrolytic enzymes. Viscosity decreased more slowly for samples with high $\bar{M}_v$ (>227,000 Da) and DD either >96% or <59%. Within these limits, there was no clear-cut correlation in the rate of degradation with $\bar{M}_v$ and DD. In the PB series, where both samples showed a similar $\bar{M}_v$, the degradation of the sample with higher DD (i.e. PB10) occurred at a much lower rate than that of PB08. This could be explained by preferential attack of an endochitinase for which a highly deacetylated sample (PB10: DD 98.3 %) seems to be a poor substrate, whereas a slightly higher number of acetyl groups (PB08: DD 95.5%) facilitated the attack and cleavage by the enzyme. However, at a chitosan concentration of 2.5 $mg \bullet ml^{-1}$ caused an increase in the degradation velocity as compared with chitosan sample PB10 (Figure 45).

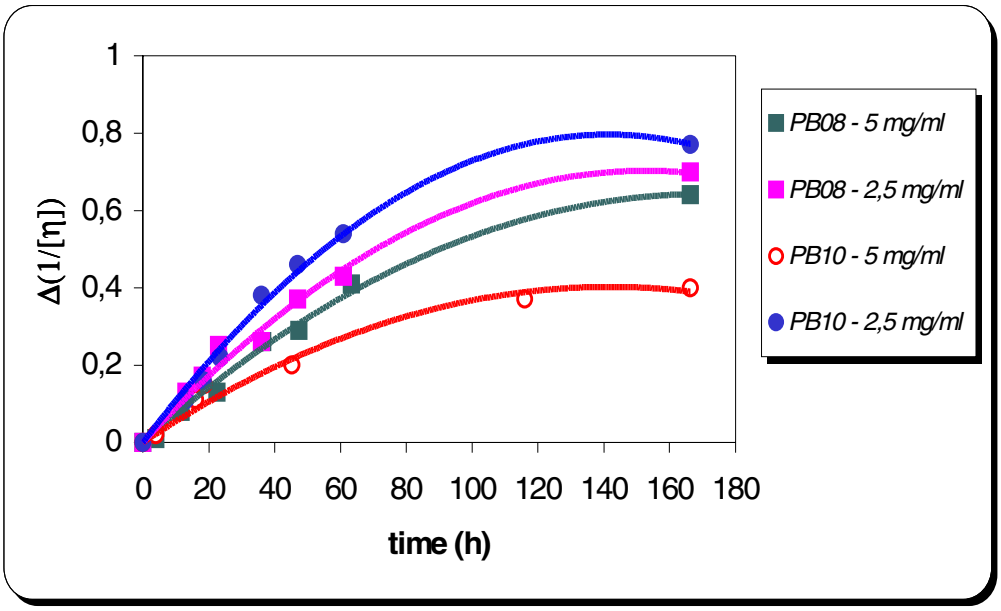



Figure 45. Changes in viscosity as a result of initial concentration of chitosan during degradation of two chitosan samples by *Aspergillus fumigatus*

These observations can be explained by the effect of medium viscosity influence on the fungus growth. More diluted chitosan solution used as a growth medium for plants pathogen fungi caused generally better growth.
Similar results were found in the literature. Fungus *Botrytis cinerea*, for example, was unable to grow on chitosan with high viscosity but grew when sucrose was added to the medium. *Fusarium oxysporum* growth was inhibited by a chitosan with high viscosity and high DD even in the addition of sucrose. The antagonist fungi of *Trichderma harzianum* and *T. viride* grew in chitosan media with a wide variation of DD and viscosities. However, it was significantly better on chitosan with low DD[253].
A chitosan with low DD induces high production of endochitinases (N-acetyl-β-D-glucosaminidase and chitobiosidase) for antagonists pathogens fungi. At the same time, the activity of exochitinase is usually detected after a longer period of fungal growth[253].
The results of determinations of the reducing sugars content are shown in Table 23.
In the M series, the highest concentrations were found with chitosans of high $\bar{M}_v$, low DD, or low $\bar{M}_v$ and high DD (G3mB, M3A and M6mC).

Table 23. Proportion of chitosan degradation as calculated from the amounts of reducing sugars [a)]

| Chitosan | Proportion of biodegradation [b)] for samples incubated for (days) | | | | |
|---|---|---|---|---|---|
| | 2 | 4 | 6 | 7 | 27 |
| G3mA | 19.3 | 18.6 | 20.7 | ---- | 28.0 |
| M3A | ---- | 20.5 | 31.3 | ---- | 34.8 |
| G3mB | ---- | 19.0 | 27.2 | ---- | 29.9 |
| M3mB | 24.0 | 18.7 | 23.8 | ---- | 27.8 |
| M1mC | ---- | 18.3 | 20.9 | ---- | 27.1 |
| M6mC | 20.2 | 18.9 | 25.2 | ---- | 31.9 |
| PB08 | ---- | 18.1 | 19.0 | ---- | 26.1 |
| PB10 | 22.3 | 15.7 | 17.3 | 28.2 | 26.3 |

[a)] - initial concentration of chitosan G3mA-PB10: 5 mg•ml$^{-1}$, [b)] - mol of reducing sugars / mol of monomer content (GlcN + GlcNAc) of the respective chitosan sample (%)

This result can be explained by the synergistic action of the various enzymes such as deacetylases, exo- and endochitinases and chitosanases. In the case of samples G3mA, M3mB, M6mC as well as PB10, it was observed that the amount of reducing sugar was higher after 2 days than after 4 days, probably due to the metabolism of the initially formed degradation products.

The differences in the amount of reducing sugars between samples with the highest DD (PB10, M6mC or M3mB) and low DD (G3mA) after 2 days of biodegradation suggests that during the first phase (phase of intensive growth) of fungus growth in the chitosan media with high DD, exochitinases and/or exochitosanases were produced.
These results are confirmed by data reported by Tronsmo et. al.[253].
Application of lower original concentration of chitosan (2.5 mg•ml$^{-1}$) resulted in a relative faster degradation (Table 24).

Table 24. Proportion of chitosan P4 degradation as calculated from the amounts of reducing sugars [a)]

| Chitosan | Proportion of biodegradation [b)] for samples incubated for (days) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| P4-0.25 | 19.3 | 16.6 | 26.2 | 26.7 | 28.9 | 43.3 |
| P4-0.5 | ---- | 14.7 | ---- | 25.3 | ---- | 25.0 |

[a)] - initial concentration of chitosan P4-0.25: 2.5 mg•ml$^{-1}$, chitosan P4-0.5: 5 mg•ml$^{-1}$, [b)] - mol of reducing sugars / mol of monomer content (GlcN + GlcNAc) of the respective chitosan sample (%)

The reducing sugars content decreased with an increase in chitosan DD. However, the high viscosity (chitosan G3mA) inhibited the growth of fungi.

The above results lead to the conclusion that the fungus *Asp. fumigatus* preferred a low viscosity medium containing chitosan with DD lower than 95%.

4.5.3. Composition of chito-oligosaccharides mixtures

Figure 46 shows the typical HPLC elution patterns of a standard mixture of oligosaccharides $(GlcNAc)_n$. n = 1-6, and of an analytical sample of M3mB.
HPLC samples analysis of fully *N*-acetylated reaction products present in the degradation mixtures of chitosan allowed the identification of chito-oligosaccharides which, except for DP1 and DP2, were confirmed by MALDI-TOF MS (Figure 47)[236].

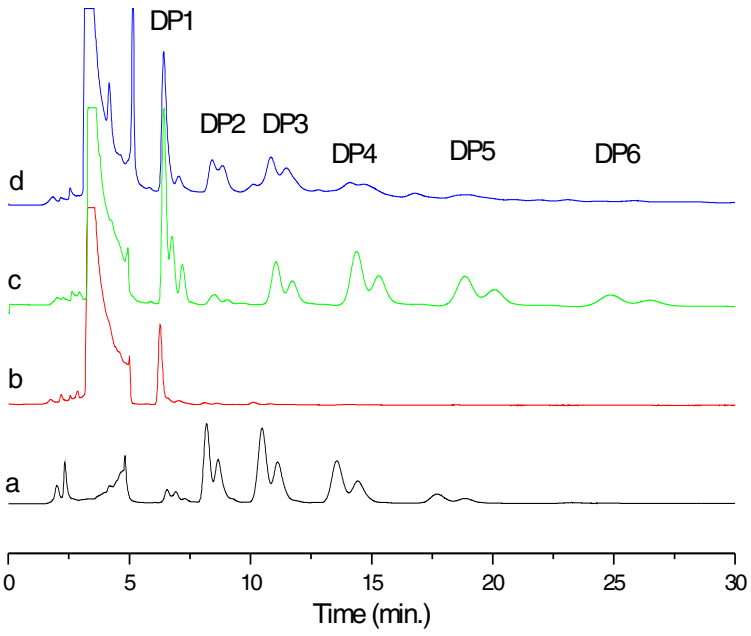



Figure 46. HPLC profiles of standard chito-oligosaccharides (a) of product mixture [(b) – sample M3mB after 2 days of degradation, (c) – M3mB after 4 days of degradation, (d) – M3mB after 6 days of degradation] after N-acetylation

The monosodiated ions $(M+23)^+$ detected in the mass spectra of oligomers isolated by HPLC of sample M1mC (after 4 days of incubation) are shown in Figure 47.
Mass spectra presented on Figure 48a and b show the monosodiated ions $(M+23)^+$ of $(GlcNAc)_3$ at m/z 650.1 and 650.2 respectively. The pseudomolecular ion $(M+H)^+$ slightly appeared at m/z = 628.1 (Figure 48a). The sodiated ion $(M+23)^+$ of $(GlcNAc)_4$ is detected at m/z 853.1 and 853.2 (Figures 48c and 48d). The protonated species $(M+H)^+$ was not found for both spectra. DP5 is confirmed on Figures 48e and 48f by the presence of sodiated ion $(M+23)^+$ at m/z 1056.1 and 1056.3, respectively.

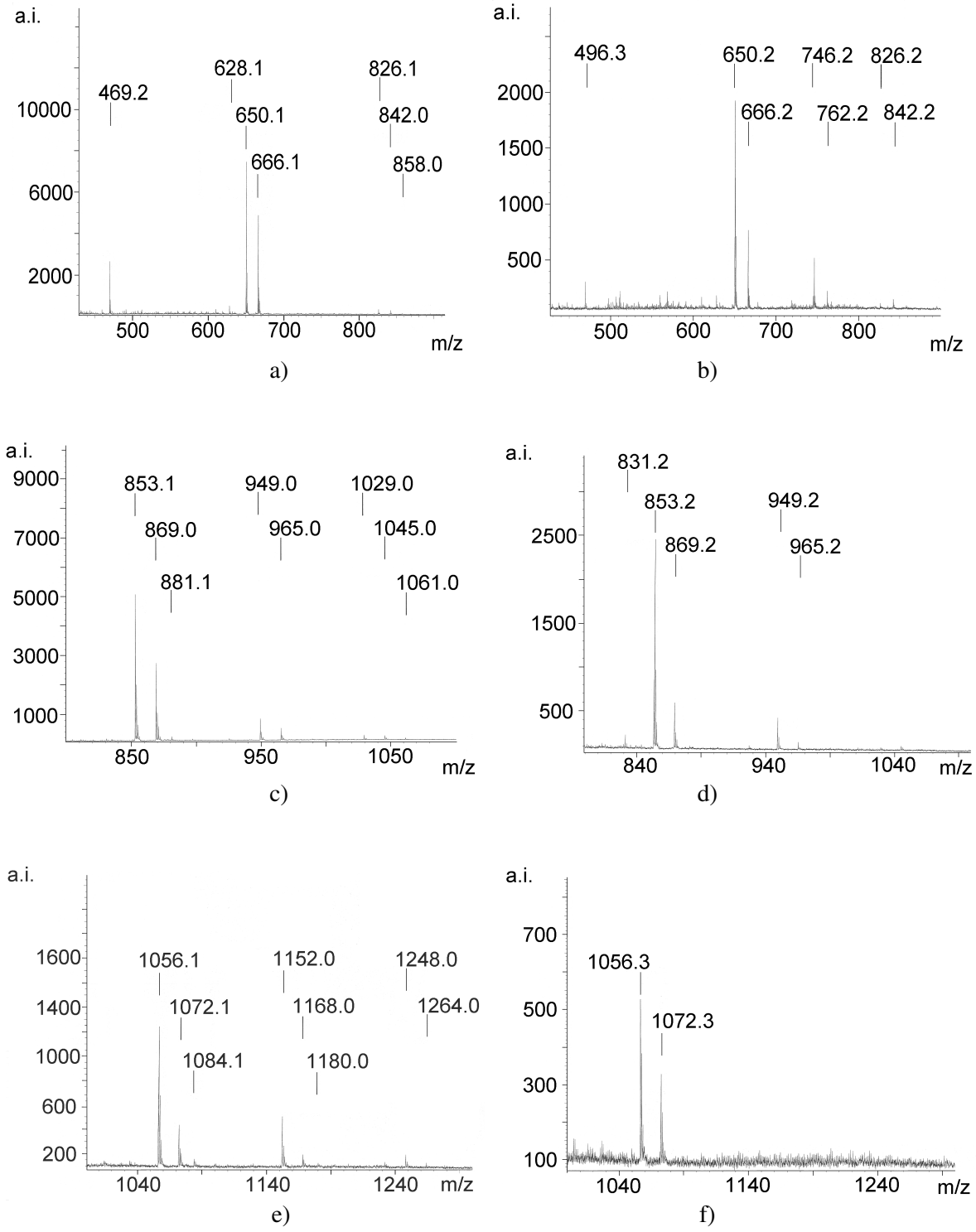



Figure 47. MALDI-TOF mass spectra of oligomers (DP3 - DP5) isolated by HPLC of sample M1mC (after 4 days of incubation); a), c) and e) - α-anomer, b), d) and f) - β-anomer

Table 25. Patterns of chito-oligosaccharides (rel. wt%) as determined by HPLC after degradation of chitosans by *Aspergillus fumigatus*

| Sample / days | DD* | Degree of polymerization | | | | | |
|---|---|---|---|---|---|---|---|
| | | *NAG* | *NAG-2* | *NAG-3* | *NAG-4* | *NAG-5* | *NAG-6* |
| Gm3A – 4 | 58.2 | 11.7% | 12.7% | 46.3% | 22.9% | 6.4% | Nd |
| Gm3A – 6 | | 13.4% | 20.1% | 42.1% | 20.3% | 2.0% | 2.2% |
| M3A - 2 | 66.7 | 98.0% | 2.0% | Nd | Nd | Nd | Nd |
| M3A - 4 | | 26.6% | 4.0% | 21.0% | 25.6% | 15.1% | 7.6% |
| M3A - 6 | | 9.7% | 18.2% | 43.0% | 21.6% | 6.4% | 1.1% |
| M3A - 27 | | 39.5% | 35.7% | 16.3% | 6.6% | 1.5% | 0.5% |
| GM3B - 2 | 76.9 | 93.3% | 5.6% | 1.1% | Nd | Nd | Nd |
| GM3B - 4 | | 33.1% | 6.2% | 21.1% | 24.5% | 13.0% | 2.1% |
| GM3B - 6 | | 20.0% | 27.2% | 35.9% | 14.2% | 2.6% | Nd |
| GM3B - 27 | | 38.8% | 33.0% | 17.9% | 7.6% | 2.8% | Nd |
| M3mB - 2 | 95.5 | 95.8% | 1.6% | 2.5% | Nd | Nd | Nd |
| M3mB - 4 | | 31.3% | 4.2% | 20.2% | 25.2% | 13.9% | 5.2% |
| M3mB – 6 | | 15.2% | 31.7% | 34.9% | 14.2% | 4.0% | Nd |
| M3mB – 27 | | 35.3% | 38.0% | 19.6% | 5.5% | 1.6% | Nd |
| M1mC – 4 | 82.5 | 17.4% | 22.9% | 41.4% | 15.2% | 3.1% | Nd |
| M1mC – 6 | | 15.8% | 14.1% | 44.5% | 19.9% | 5.6% | Nd |
| M6mC – 4 | 88.5 | 17.8% | 18.2% | 40.3% | 19.0% | 4.8% | Nd |
| M6mC – 6 | | 17.0% | 19.8% | 41.4% | 17.7% | 3.6% | 0.5% |
| PB08 – 4 | 95.9 | 4.4% | 14.0% | 19.5% | 47.2% | 14.9% | Nd |
| PB08 – 5 | | 21.5% | 21.2% | 26.1% | 16.7% | 14.5% | Nd |
| PB08 – 6 | | 13.4% | 17.0% | 42.3% | 21.4% | 6.0% | Nd |
| PB10 – 2 | 98.3 | 87.0% | 13.0% | Nd | Nd | Nd | Nd |
| PB10 – 4 | | 88.5% | 8.7% | 1.3% | 1.5% | Nd | Nd |
| PB10 – 6 | | 42.8% | 5.1% | 19.5% | 20.0% | 9.7% | 2.9% |
| PB10 – 7 | | 51.6% | 18.6% | 15.0% | 10.4% | 2.5% | 1.8% |
| PB10 – 27 | | 38.8% | 31.6% | 19.2% | 6.7% | 2.9% | 0.7% |
| P40.25 - 2 | 64.8 | 52.2% | 19.3% | 15.6% | 5.3% | 6.4% | 1.2% |
| P40.25 - 3 | | 34.4% | 8.1% | 30.6% | 1.2% | 18.8% | 6.9% |
| P40.25 - 4 | | 26.6% | 17.6% | 39.7% | 15.4% | 0.6% | Nd |
| P40.25 - 5 | | 22.8% | 18.9% | 41.1% | 13.5% | 3.7% | Nd |
| P4D - 3 | 64.8 | 54.8% | 5.2% | 21.6% | 12.4% | 4.7% | 1.2% |
| P4D - 5 | | 42.1% | 13.2% | 30.4% | 9.9% | 2.7% | 1.8% |
| P4D - 6 | | 43.0% | 19.3% | 27.9% | 7.9% | 1.2% | 0.7% |

Nd - not detected, * - DD was determined by FTIR spectroscopy, P40.25 – chitosan P4 was degraded at a concentration of 0.25 wt% solution, P4D – chitosan P4 was degraded at a concentration of 0.5 wt%. N-acetylation was carried out using $(D_3CCO)_2O$

As expected, the fraction absorbing at 205 nm corresponds mostly to mono- and disaccharides, which increased while DP3 to DP6 decreased during biodegradation time. Chitosan with higher DD yielded by relative higher amounts of mono- and disaccharide. The highest amount of the main product of DP3 was observed after 6 days of biodegradation. This phenomenon corresponds to the fast growing phase of the fungus, which is also evident from analysis of the reducing sugars (Table 23-24), especially in the case of chitosan with medium $\overline{M}_v$ and low DD. After 2 days of degradation, the main products of hydrolysis were mono- and dimers. Relatively higher amounts of dimers were found after the intensive growth phase of fungi on chitosan with highest DD (PB10). With the decreasing DD, the amount of disaccharides was reduced. This fact suggests the possibility of the action of various exohydrolases (i.e. exochitinases and/or exochitosanases). The amount of products with a higher DP increased with the reduction in DD (Table 25.). However, this relationship was strongly dependent on

the viscosity. The significant reduction in the amount of monomers and dimers in more diluted chitosan P4 media is resulted in faster and better metabolismus of fungus.

4.5.4. N-acetylation of oligosaccharides with $(D_3CCO)_2O$

The elution pattern of oligosaccharides N-acetylated with acetic anhydride-$d_6$ (Figure 48) shows a similarity to the standard chito-oligosaccharides HPLC chromatogram, as shown in Figure 46. The identification of chito-oligosaccharides from DP1 to DP6 was confirmed by MALDI-TOF MS.

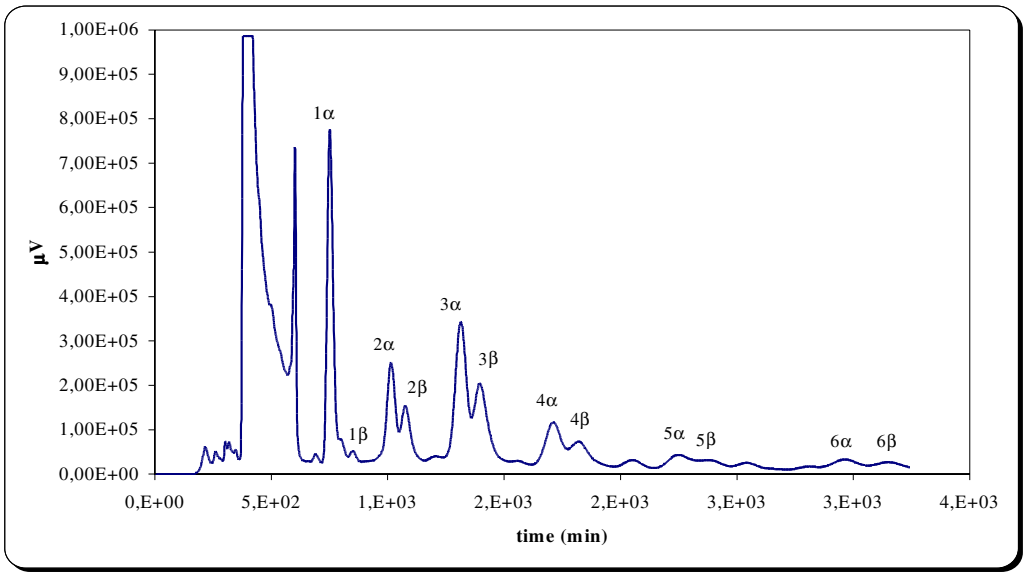



Figure 48. HPLC profile of a product mixture (sample P4 after 6 days of incubation) after N-acetylation with $(D_3CCO)_2O$

The presence of N-acetylglucosamine and glucosamine amounts suggests that the chitinases may play an important role during the biodegradation (Table 26).

Table 26. Type of the oligosaccharides produced by biodegradation of chitosan P4 (DD 69.4%) by *Aspergillus fumigatus* after 6 days*

| Type of oligosaccharides | | | | | |
|---|---|---|---|---|---|
| **Monomer** | **Dimer** | **Trimer** | **Tetramer** | **Pentamer** | **Hexamer** |
| GlcNAc | $(GlcNAc)_2$ | $(GlcNAc)_2$-$GlcNd_3$ | $(GlcNAc)_3$-$GlcNd_3$ | $(GlcNAc)_4$-$GlcNd_3$ | $(GlcNAc)_4$-$(GlcNd_3)_2$ |
| $GlcNd_3$ | GlcNAc-$GlcNd_3$ | GlcNAc-$(GlcNd_3)_2$ | $(GlcNAc)_2$-$(GlcNd_3)_2$ | $(GlcNAc)_3$-$(GlcNd_3)_2$ | $(GlcNAc)_3$-$(GlcNd_3)_3$ |
| | $(GlcNd_3)_2$ | $(GlcNd_3)_3$ | GlcNAc-$(GlcNd_3)_3$ | $(GlcNAc)_2$-$(GlcNd_3)_3$ | $(GlcNAc)_2$-$(GlcNd_3)_4$ |
| | | | $(GlcNd_3)_4$ | GlcNAc-$(GlcNd_3)_4$ | GlcNAc-$(GlcNd_3)_5$ |
| | | | | $(GlcNd_3)_5$ | $(GlcNd_3)_6$ |

* - detected by the MALDI-TOF MS

Moreover, the glucosamine may be more rapidly metabolized by the fungus.
Figures 49-60 show the MALDI-TOF mass spectra of oligomers (DP1 - DP6) isolated by HPLC of sample P4.
As shown in Figure 48 this method of oligosaccharide isolation resulted in a good separation pattern and it may support, following identification by MALDI-TOF mass spectrometry, the elution of degradation product at higher DP by column chromatography.
Figures 49-50 show the mass spectra of monomers, in which the peaks of monosodiated ion $(M+Na)^+$ of GlcNAc at m/z 244.2 is detected. The sodiated ion $(M+Na)^+$ of $GlcNAcd_3$ appeared at m/z 247.2 in both spectra.

The peak at m/z 460.2 are assigned for sodiated ion $(M+Na)^+$ of GlcNAc-$GlcNAcd_3$ and at m/z 463.2 for $(GlcNAcd_3)_2$ - (Figures 51-52).
The monosodiated ions $(M+Na)^+$ on Figures 53-54 were identified as $(GlcNAc)_2$-$GlcNAcd_3$ at m/z 653.3, GlcNAc-$(GlcNAcd_3)_2$ at m/z 656,7 and $(GlcNAcd_3)_3$ at m/z 659.3.
The weak peak at m/z 856.4 on spectrum shown in Figure 56 is assigned to sodiated ion of $(GlcNAc)_3$ GlcNAc. In both spectra (Figures 55-56) the sodiated ion of $(GlcNAc)_2(GlcNAcd_3)_2$ appeared at m/z 859.4, GlcNAc$(GlcNAcd_3)_3$ at m/z 862.4 and $(GlcNAcd_3)_4$ at 865.4.
The monosodiated ions appeared for mass spectra shown in Figure 57 is assigned for $(GlcNAc)_4$ $GlcNAcd_3$. The other sodiated species appeared in both spectra (Figures 57-58) are detected as $(GlcNAc)_3(GlcNAcd_3)_2$ at m/z 1062.6, $(GlcNAc)_2(GlcNAcd_3)_3$ at m/z 1065,7, GlcNAc$(GlcNAcd_3)_4$ at m/z 1068.7 and $(GlcNAcd_3)_5$ at m/z 1071.7.
The Figures 59 and 60 show mass spectra of α- and β- anomers with DP6. The sodiated ion of $(GlcNAc)_4(GlcNAcd_3)_2$ is detected at m/z 1265.6, $(GlcNAc)_3(GlcNAcd_3)_3$ at m/z 1268,7 in both spectra. Moreover, the monosodiated ion of $(GlcNAc)_2(GlcNAcd_3)_4$ appeared at m/z 1271.7 in mass spectrum of α-anomer (Figure 59.).

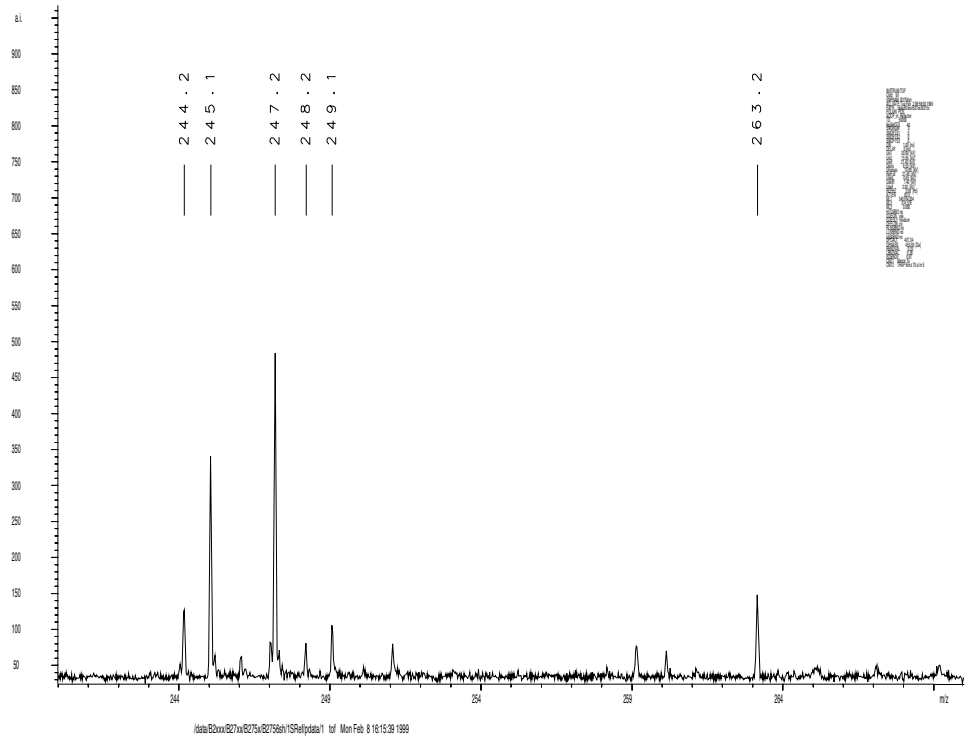



Figure 49. MALDI-TOF mass spectrum of DP1α - N-acetylated with $(D_3CCO)_2O$

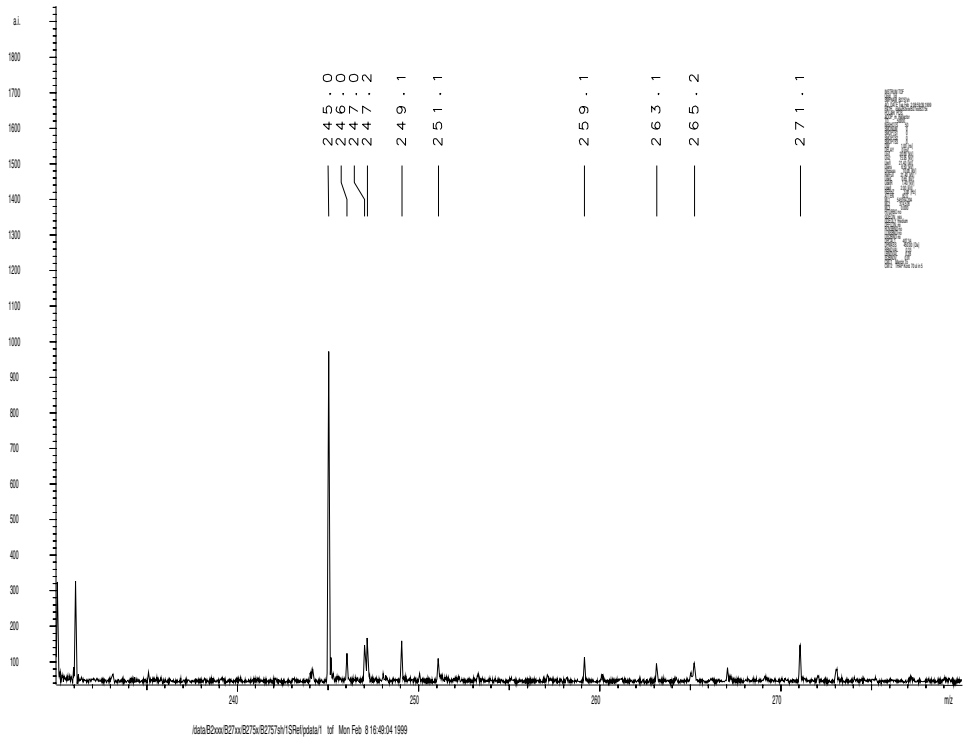



Figure 50. MALDI-TOF mass spectrum of DP1β - N-acetylated with $(D_3CCO)_2O$

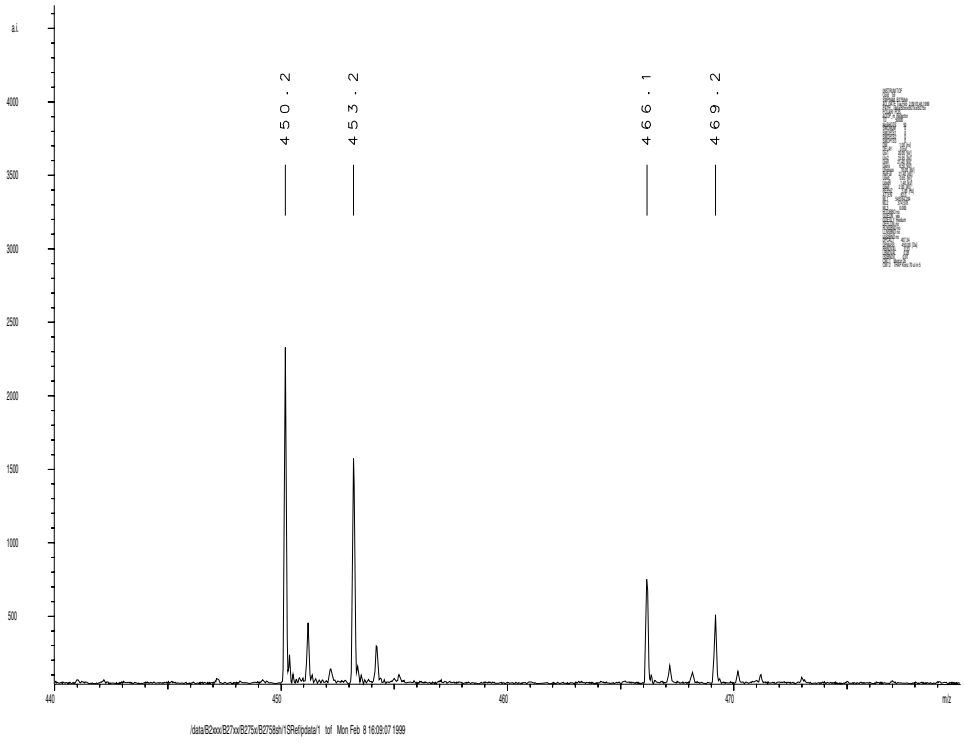



Figure 51. MALDI-TOF mass spectrum of DP2α - N-acetylated with $(D_3CCO)_2O$

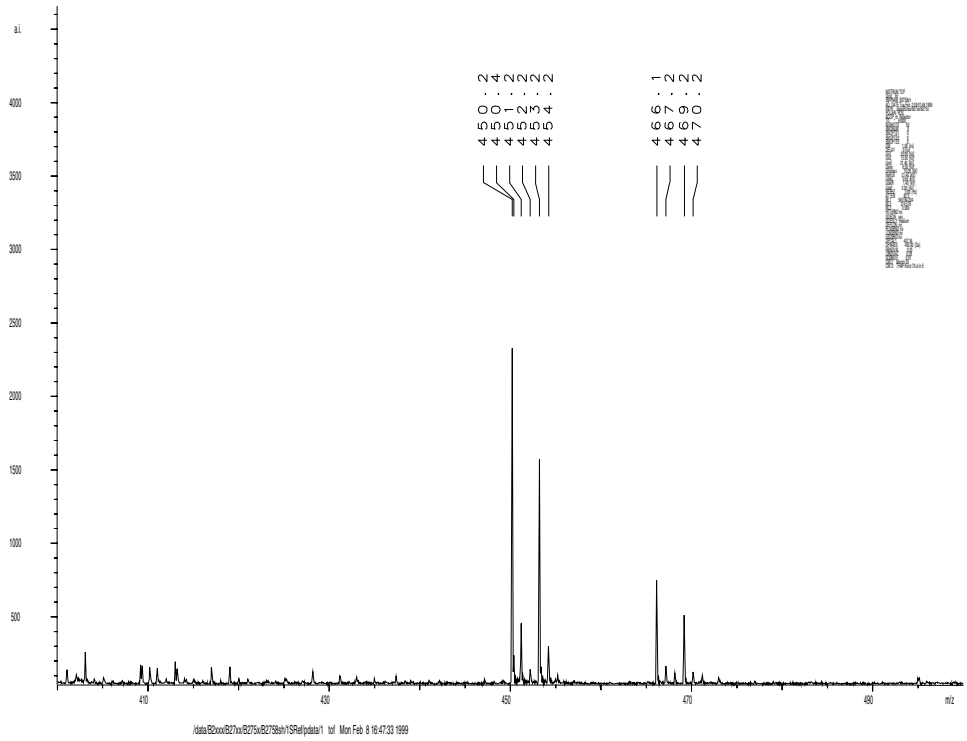



Figure 52. MALDI-TOF mass spectrum of DP2β - N-acetylated with $(D_3CCO)_2O$

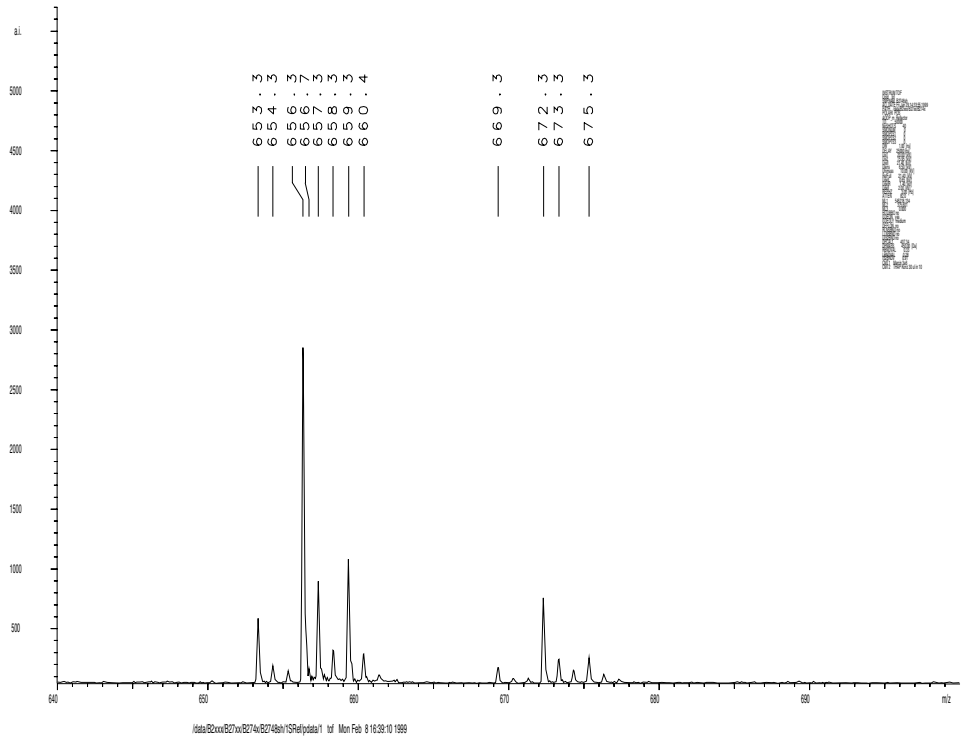



Figure 53. MALDI-TOF mass spectrum of DP3α - N-acetylated with $(D_3CCO)_2O$

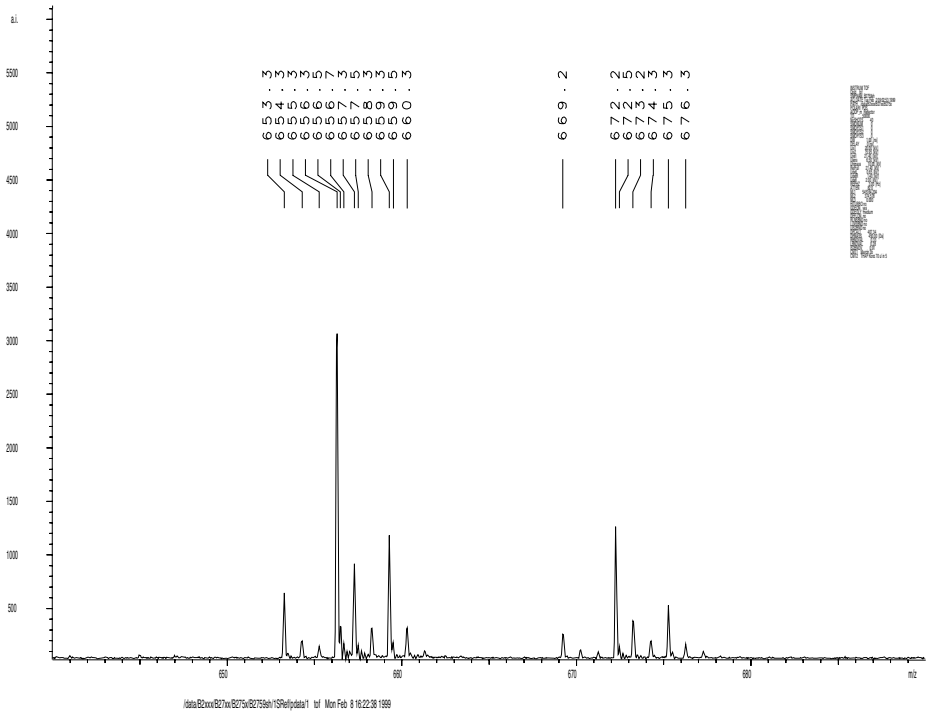



Figure 54. MALDI-TOF mass spectrum of DP3β - N-acetylated with $(D_3CCO)_2O$

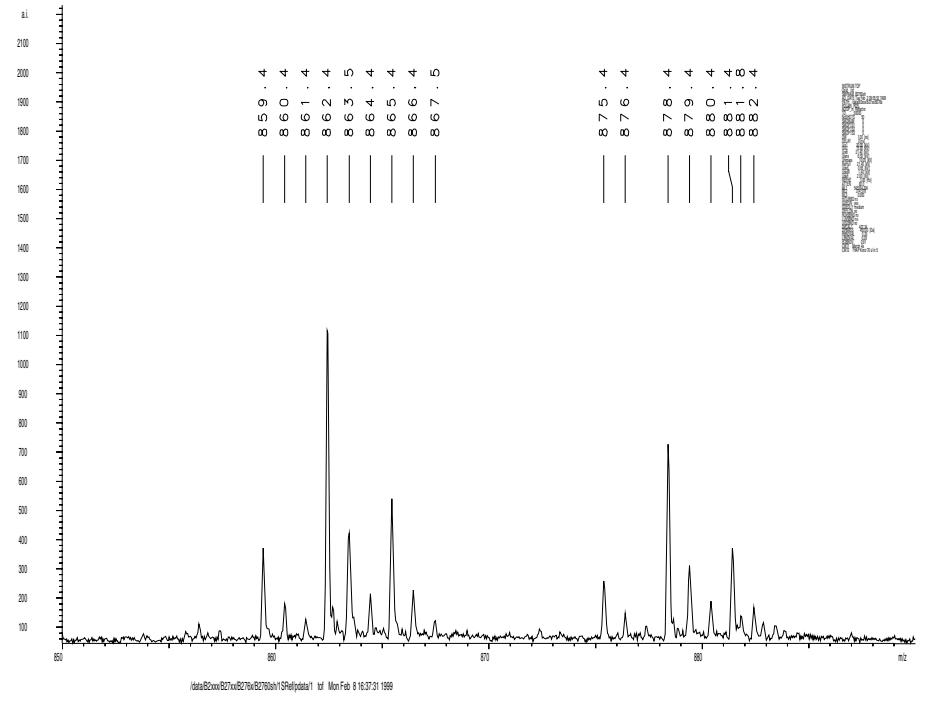



Figure 55. MALDI-TOF mass spectrum of DP4α - N-acetylated with $(D_3CCO)_2O$

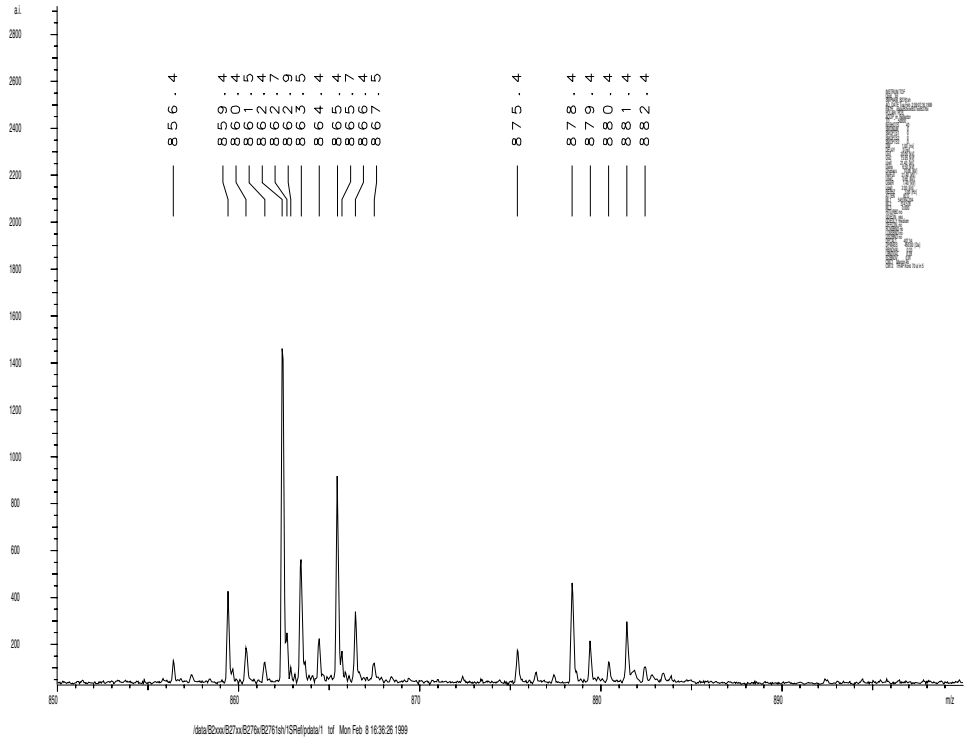



Figure 56. MALDI-TOF mass spectrum of DP4β - N-acetylated with $(D_3CCO)_2O$

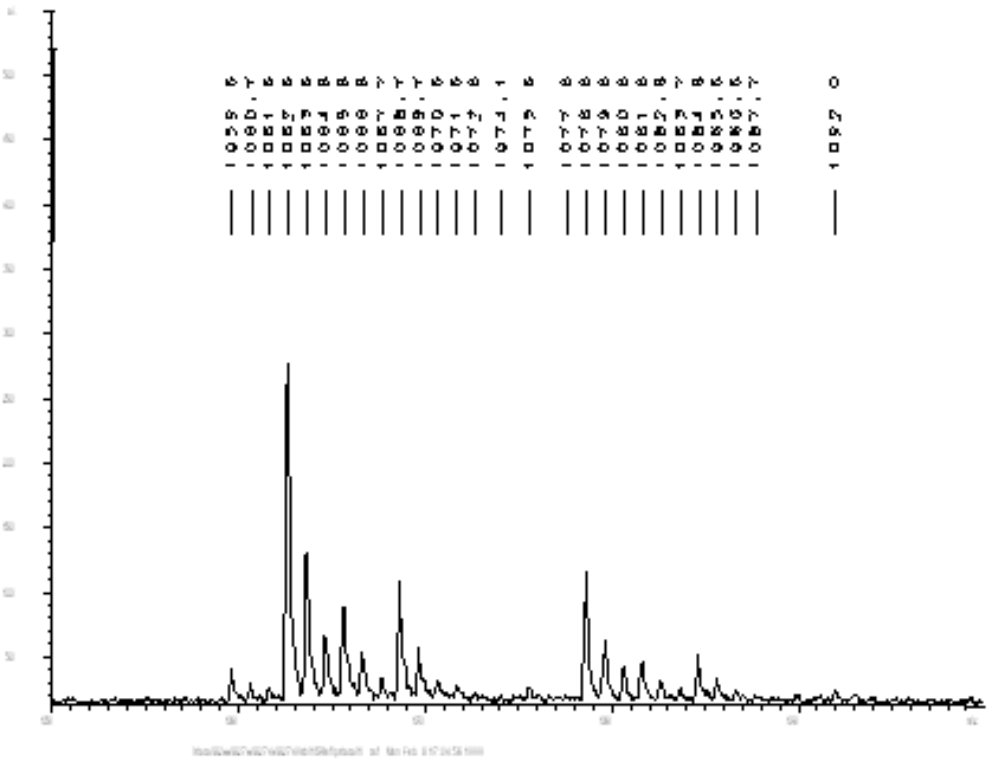


Figure 57. MALDI-TOF mass spectrum of DP5α - N-acetylated with $(D_3CCO)_2O$

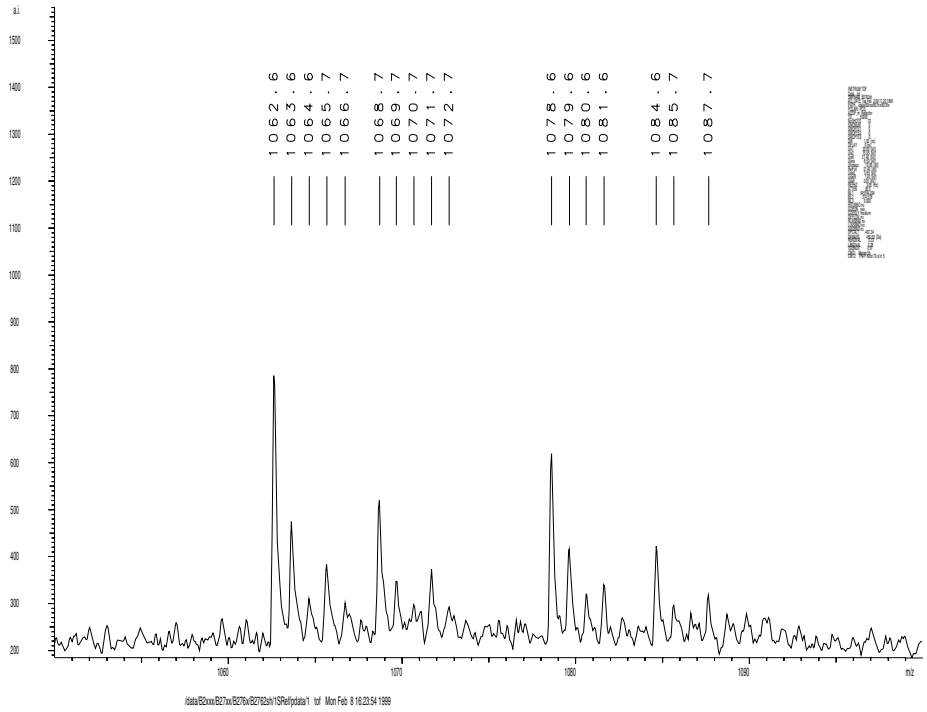



Figure 58. MALDI-TOF mass spectrum of DP5β - N-acetylated with $(D_3CCO)_2O$

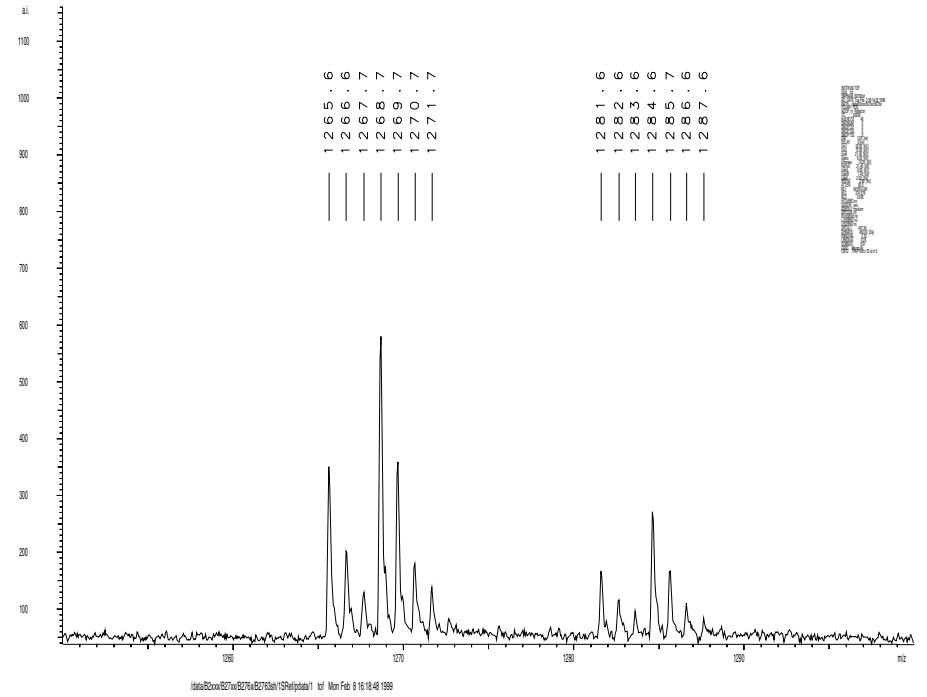



Figure 59. MALDI-TOF mass spectrum of DP6α - N-acetylated with $(D_3CCO)_2O$

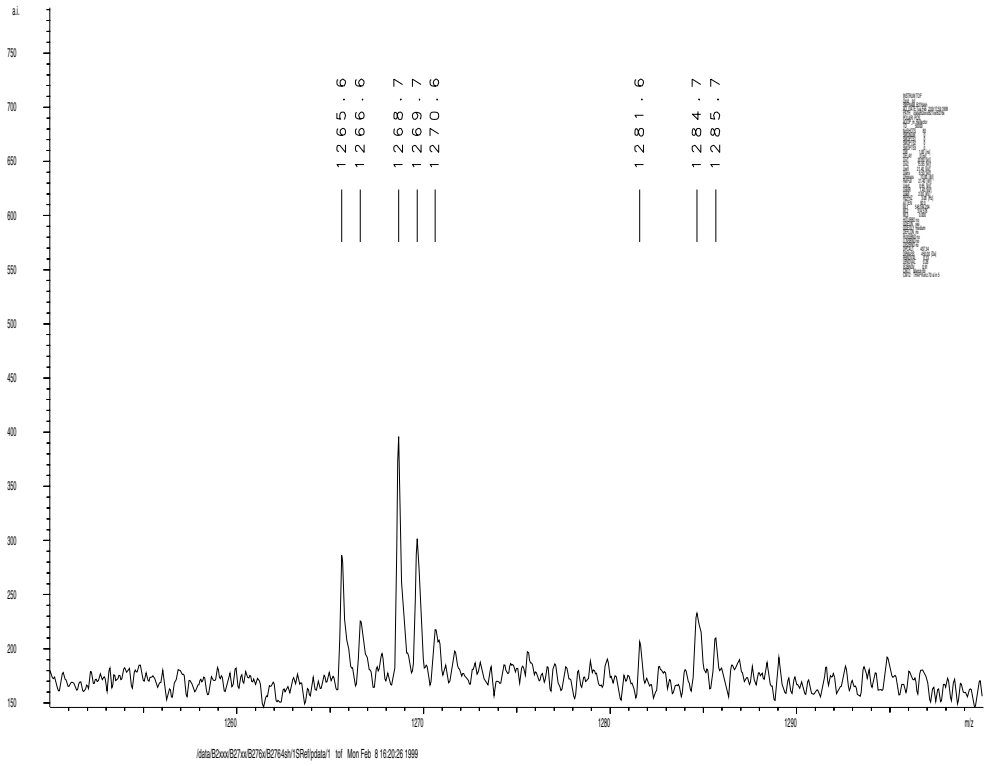



Figure 60. MALDI-TOF mass spectrum of DP6β - N-acetylated with $(D_3CCO)_2O$

4.5.5. GPC study

The result of SEC (GPC) of degraded mixture and initial chitosan is shown in Tables 27, 28, 29 and 30.

Table 27. Molecular weights of initial chitosan P4 by GPC

| | |
|---|---|
| $M_p$ | 43652 |
| $M_n$ | 15920 |
| $M_v$ | 56175 |
| $M_w$ | 63812 |
| Poldydispersity ($M_w/M_n$) | 4.008 |

Table 28. Elution pattern of initial chitosan P4 by GPC

| $M \times 10^{-3}$ | <5 | 5-50 | 50-100 | 100-200 | 200-400 | 400-800 | >800 |
|---|---|---|---|---|---|---|---|
| log M | <3.7 | 3.7-4.7 | 4.7-5.0 | 5.0-5.3 | 5.3-5.6 | 5.6-5.9 | >5.9 |
| % | 6 | 55 | 21 | 12 | 5 | <1 | <1 |

Table 29. Molecular weights of biodegraded chitosan P4 (after 6 days of degradation) by GPC

| | |
|---|---|
| $M_p$ | 528 |
| $M_n$ | 1300 |
| $M_v$ | 3996 |
| $M_w$ | 4877 |
| Poldydispersity ($M_w/M_n$) | 3.751 |

Table 30. Elution pattern of biodegraded chitosan P4 (after 6 days of degradation) by GPC

| M x $10^{-3}$ | <5 | 5-50 | 50-100 | 100-200 | 200-400 | 400-800 | >800 |
|---|---|---|---|---|---|---|---|
| log M | <3.7 | 3.7-4.7 | 4.7-5.0 | 5.0-5.3 | 5.3-5.6 | 5.6-5.9 | >5.9 |
| % | 75 | 24 | 1 | - | - | - | - |

Distributions of molecular weight of initial chitosan P4 and degraded mixture are shown in Figures 61 and 62 as well as the eluted profile of initial and biodegraded chitosan P4 is shown in Figures 61 and 62.

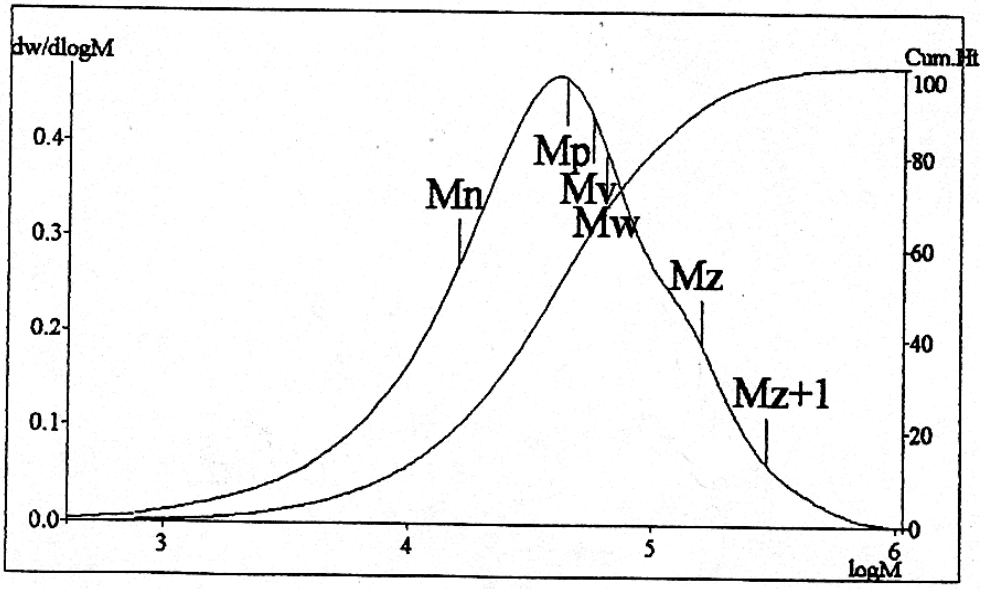



Figure 61. Distribution of molecular weight of initial chitosan P4

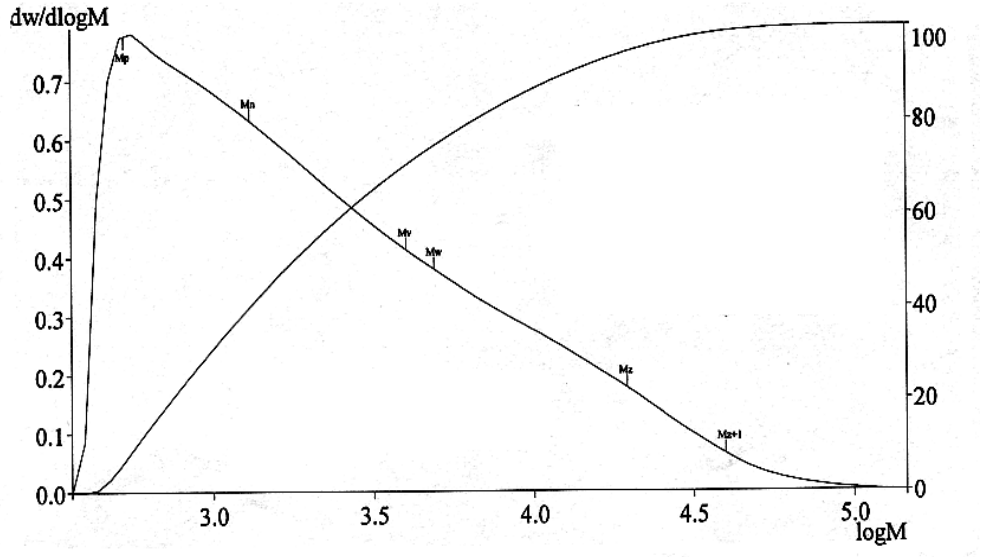



Figure 62. Distribution of molecular weight of degraded chitosan (after 6 days of degradation)

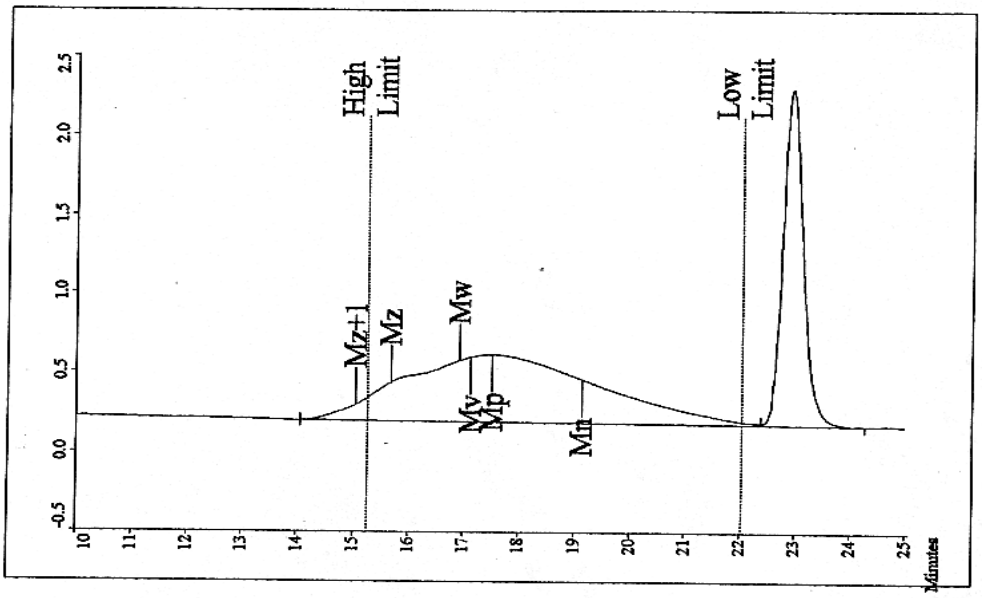



Figure 63. Elution profile (dw/dlogM) of initial chitosan compared with calibration curve

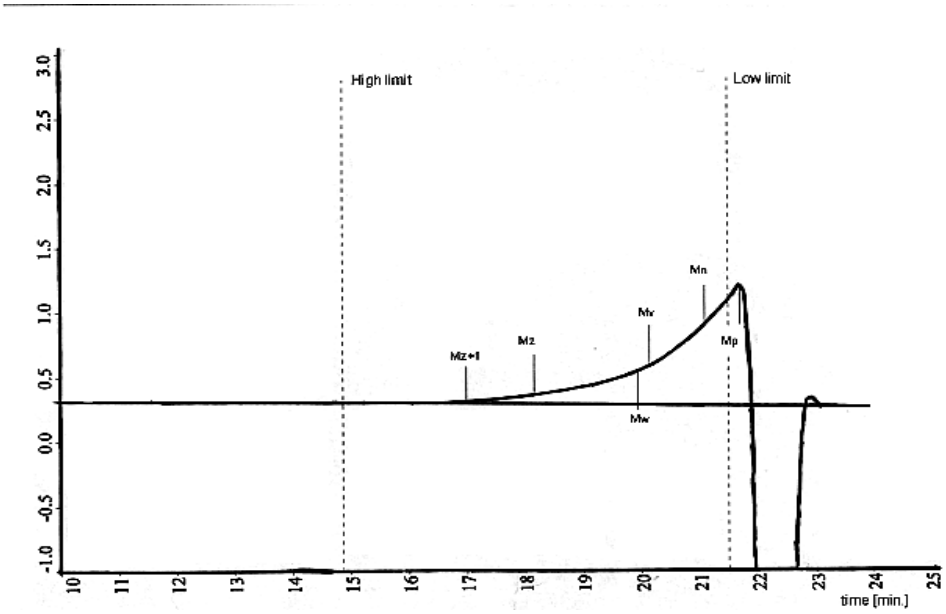



Figure 64. Elution profile (dw/dlogM) of chitosan oligosaccharides compared with calibration curve

The biodegradation of chitosan P4 resulted a decrease of the molecular weight to < 5000 Da. The appearance of low molecular fraction increased up to 75% for biodegraded chitosan, while the initial chitosan had ca. 6% of it. The absence of high molecular fraction, higher than 100 kDa for degraded chitosan was noted. The polydispersity of degraded sample little decreased.

4.6. Antiviral and antibacterial action of chitosan

It was found (Section 4.5.) that the chitinolytic activity of the fungus *Aspergillus fumigatus* is inhibited by high $\overline{M}_v$ chitosans when their concentration was 0.5 wt%. Therefore, antiviral and antibacterial activity was tested for the more diluted solutions obtained after the fermentation using above-mentioned fungus.

It was found that the inhibition rate decreased with an increase in the time of incubation of chitosan in the fermentation bath (Table 31).

Table 31. Effect of chitosan P4 and soluble products of degradation on the inhibition of **AlMV** virus*

| Time of the degradation (days) | Inhibition of virus infection (%). | |
|---|---|---|
| | Degraded chitosan[1)] | Original chitosan[2)] |
| 1 | 100 | 100 |
| 2 | 98.5 | 100 |
| 3 | 94.0 | 99.8 |
| 4 | 80.0 | 100 |
| 5 | 64.5 | 99.9 |

* Bean leaves were sprayed with 0.01 wt% solution of original and degraded chitosan 24 hours before the **AlMV** inoculation,
[1)] chitosan P4 was degraded for the time of 5 days using fungus *Aspergillus fumigatus*,
[2)] chitosan P4 without fungus *Aspergillus fumigatus* as a control was incubated at temperature of 30°C

The bean leaves sprayed with a 0.25. 0.1 and 0.01 wt% solution of chitosan sample P4 were completely protected against virus infections whereas no inhibition of virus infection was observed for tobacco plants (Table 32)[213].

Degradation of chitosan P4 leads to a decrease of antiviral activity in the case of the system of bean leaves/**A1MV**. Most remarkably, the degradation products of chitosan P4 provide a high antiviral activity on tobacco leaves.

The bean plants treated with biodegraded chitosan were protected against **AlMV** virus infection with reduced effectiveness according to the time of biodegradation as well as the concentration of initial chitosan (Table 33).

Table 32. Effect of chitosan P4 and its degradation mixture on plant virus infection[1)]

| Sample type | Inhibition (%) | |
|---|---|---|
| | bean / **AlMV**[(a)] | Tobacco / **TMV**[(b)] |
| 0.25 wt% chitosan solution | 100 | 25 |
| 0.1 wt% chitosan solution | 100 | 0 |
| 0.01 wt% chitosan solution | 100 | 0 |
| 0.25 wt% degraded chitosan solution | 95 | 90 |
| 0.1 wt% degraded chitosan solution | 87 | 75 |
| 0.01 wt% degraded chitosan solution | 45 | 50 |

[1)] Bean or tobacco *Xanthi nc* plants were sprayed with chitosan or soluble products of the degradation 24 hours before virus inoculation; Abbreviations:
(a) **AlMV -** Alfalfa mosaic virus,
(b) **TMV -** Tobacco mosaic virus,
All data are based on two tests series (16 half-leaves were used in each study)

Table 33. The **AlMV** virus infection inhibitions by chitosan and the soluble products of its degradation[1)]

| Concentration of original chitosan (wt %) | Inhibition (%) | | | |
|---|---|---|---|---|
| | Chitosan oligomers Days of biodegradation | | | Original chitosan |
| | 7 | 5 | 3 | |
| 0.1 | 85 | 94 | 99 | 100 |
| 0.01 | 56 | 75 | 80 | 100 |
| 0.001 | 42 | 39 | 55 | 100 |

[1)] Bean plants were sprayed with solutions of chitosan and soluble products of the degradation, 24 hours before **AlMV** infection

The inhibition of viral infection was dependent on the type of host-plant and virus species. For the **AlMV**-bean plant system, prolongation of the degradation of chitosan caused a reduction in the

inhibition effect. At the same time, the degradation of chitosan increased the inhibition of **TMV** growth on tobacco plants, while the influence of original chitosan was not observed.

The bacterial activity of chitosan was estimated by two methods:

⇒ inhibiting the growth *in vitro,*

⇒ decreasing the infection on plants;

The data presented in Table 34 indicate that the biodegradation of chitosan usually lowered biological activity of isolated product.

Table 34. Antibacterial activity of initial chitosan and *Aspergillus fumigatus* degraded chitosan[a)]

| Bacteria | Growth inhibition | | Inhibition of bacteria infection[2)] | |
|---|---|---|---|---|
| | Chitosan | Soluble products of | Chitosan | Soluble products of |
| Cmm | 0.05 | (1.0) | ------ | ------ |
| Psph | 0.20 | (1.0) | 70 | 65 |
| Ec | 0.25 | (1.0) | ------ | ------ |
| Pst | 0.20 | (1.0) | 65 | 70 |

[a)] The tomato or bean plants were sprayed with the solution of chitosan and soluble products of the degradation 24 h before the bacteria inoculation.

[1)] - the lowest concentration of chitosans that inhibited bacterial growth,

[2)] - the parentheses indicate ineffectiveness at concentration used,

Cmm – *Clavibacter michiganesis subsp. Michiganesis*, Psph – *Pseudomonas syringae pv. Phaseolicola*, Pst – *Pseudomonas syringae pv. Tomato*, Ec – *Escherichia coli*

Initial concentration of original chitosan of 0.1 wt %, after 5 days of chitosan biodegradation.

The **MIC** confirmed the fact that the degradation of the polymer affected a reduction in its biological activity. The results obtained suggested the possibility of the existence of the membrane-effect of chitosan activity against bacteria.

## 5. Conclusions

1. The results demonstrate lucidly that chitosan prepared from various chitin sources may differ considerably in $\overline{M}_v$. Highly deacetylated P*andalus borealis* chitosan (see e.g. sample PA10; DD 83.2%; $\overline{M}_v$ 543,000) showed at least a twice higher $\overline{M}_v$ than krill chitosan even, though the latter was prepared under much milder deacetylation conditions (cf. e.g. sample M3A; DD 66.3%; $\overline{M}_v$ 264,000) as well as a similar $\overline{M}_V$ for biopolymer preparations by the deacetylation of insect chitin (chitosan cuticle II –2A; DD 68.3%; $\overline{M}$v 490,000). The $\overline{M}_v$ of crustaceans chitosan decreases drastically with increases in the reaction temperature and time, though corresponding DD values are relatively much less affected. Insect chitosan prepared from the larvae of *Calliphora erythrocephala* shows an higher DD and lower depolymerization under conditions comparable to the deacetylation of crustacean. When, for example, a DD of ca. 90% is required, *P. borealis* chitin must be heated using multiple deacetylation in 50% NaOH for 8 h (7+1) at 120 °C which leads do a decrease in $\overline{M}_v$ by ca. 81 %, *E. superba* chitin requires at least 3 h at 120 °C and Mv decreases by ca. 86%, whereas *C. erythrocephala* chitin requires 3 h at 100 °C with a decrease in $\overline{M}$v by only ca. 67%. Thus, insect chitin seems to be a superior starting material when high $\overline{M}_v$, high DD is required. In conclusion, *Pandalus borealis* is a good source for the preparation of chitosan with high $\overline{M}_v$ and low DD, whereas chitosan of medium-to-high DD and medium-to-low $\overline{M}_v$ can be ideally prepared from krill chitin. However, the insect chitosans show similar properties as samples produced from *Pandalus borealis* at a relatively shorter time of deacetylation (Chapter I, section 4.2.).
2. The origins of chitin affect the final properties of deacetylated biopolymer. A low DD with high crystallinity of the starting chitin produces a low DD of chitosan with extensive depolymeriztaion. Moreover, the deacetylation of chitin with low crystallinity leads to a product with high DD and high $\overline{M}_v$ (Chapter I, sections 4.1., 4.2. and 4.3.).
3. A low concentration of sodium hydroxide is correlated with low degree of deacetylation as well as an increase in the viscosity. However, a wide variation between $\overline{M}_v$ and DD is found for chitosan deacetylated from the insects. In addition, the reduction in particle size of chitinous particles decreases the deacetylation time, resulting in a more soluble product distinguished by a higher DD (Chapter I, section 4.2.).
4. During deacetylation, an increase in WRV of low deacetylated samples is observed. Prolongation of the process causes a strong decrease of WRV, especially with samples of low crystallinity (Chapter I, section 4.2.).
5. The duplication for signal of C4 as observed by solid-state $^{13}$C-NMR for highly deacetylated chitosan suggests the presence of two polymorphic phases of biopolymer, confirmed by the increase in crystallinity determined by X-ray. During deacetylation, the lowering of the infrared absorbency of amide I band is observed, with an increase of amide II band intensity responsible for amine groups. The absence of duplication of amide I band resulting from two possibilities of hydrogen-bonding amide groups for chitosan with DD > 70% is also noted (Chapter I, section 4.3.).
6. Determination of the DD of chitosan carried out by IR according to Muzzarelli with baseline separation based on group of peaks and chitosan samples prepared as KBr disks fine most reliable results. However, the accuracy of DD depends on various factors such as the method of baseline separation; in IR selection of the reference method (i.e. potentiometric titration or NMR), etc. It is recommended to calibrate the method in each new case (Chapter I, section 4.4.).
7. The liquefication and saccharification of chitosan by *Aspergillus fumigatus* proceded significantly better in the more diluted solution of biopolymer. At the same time, the inhibition of the growth of fungus by a highly viscous solution of chitosan is observed. The highly deacetylated chitosan also inhibits fungus growth (Chapter I, section 4.5.).
8. The main product of the degradation of chitosan by *Aspergillus fumigatus* are oligomers with DP of 3. An increase in the excess of monomers and dimers is observed during the first two days of the process with a decrease in DD of initial chitosan (Chapter I, section 4.5.).

9. The mass spectrometry investigation of fermentation mixtures of chitosan with *Aspergillus fumigatus* confirms the presence of chito- and chitosano-oligosaccharides (Chapter I, section 4.5.).
10. The bioactivity of chitosan depends on the $\overline{M}_v$ as well as on the species of the host-plant and pathogens. The controlled biodegradation of chitosan effects an increase in the inhibition for the *Tabbacco mosaic virus* (**TMV**) on tobacco plants and a reduction in inhibition for *Alfalfa mosaic virus* (**AlMV**) on bean plants. The inhibition of bacterial plant pathogens is also observed. The reduction in bacteria growth inhibition with the decrease in $\overline{M}_v$ suggests the membrane-effect possibility of non-degraded chitosan action (Chapter I, section 4.6.).

# Chapter II: Chitosan films and paper sheets containing microcrystalline chitosan (MCCh)

## 1. Materials

### 1.1. Chitosan

Chitosan samples were prepared by heterogeneous deacetylation of chitin from shells of the Antarctic krill (*Euphausia superba* – "M" and "GM", see Table 35; cf.[76]), shrimps (*Pandalus borealis* – "PB") or from insect cuticles of *Calliphora erythrocephala*. Another sample (P4) purchased from Chemopol Co. (Tada, India) was used as a reference.

Table 35. Properties of chitosans

| Symbol of sample | Ash content [%] | Moisture content [%] | $\bar{M}_v$ [(a)] | WRV [(b)] (%) | DD (%)[(c)] by $^{13}$C-NMR | DD (%)[(c)] by Titration | DD (%)[(c)] by IR |
|---|---|---|---|---|---|---|---|
| G3mA | 1.2 | 11.25 | 264,000 | 153.9 | 58.5 | 66.3 | 58.2 |
| M3A | 4.5 | 11.41 | 187,000 | 171.1 | 69.4 | 73.5 | 66.7 |
| G3mB | 1.1 | 10.41 | 135,000 | 99.7 | 76.0 | 81.8 | 76.9 |
| M3mB | 1.1 | 11.95 | 143,000 | 99.8 | 93.2 | 90.5 | 95.5 |
| M1mC | 1.1 | 11.18 | 111,000 | 135.9 | 79.0 | 85.6 | 82.5 |
| M6mC | 1.1 | 10.73 | 64,000 | 88.7 | 85.1 | 92.6 | 88.5 |
| PB8 | 2.6 | 11.05 | 248,000 | 65.2 | 95.6 | 89.9 | 95.9 |
| PB10 | 2.5 | 9.89 | 227,000 | 56.8 | 99.2 | 98.4 | 98.3 |
| MCCh I-3/1/A | 0.0 | 7.16 | 237,000 | 74.5 | 100 | 97.6 | 100 |
| P4 | 0.6 | 10.71 | 130,000 | 79.5 | ---- | 69.6 | 64.8 |

[(a)] determined by viscometry, [(b)] determined gravimetrically, [(c)] DD determined according to the method described previously [227]

### 1.2. Proteins

Casein and keratin were used for the preparation of chitosan-protein modified films.

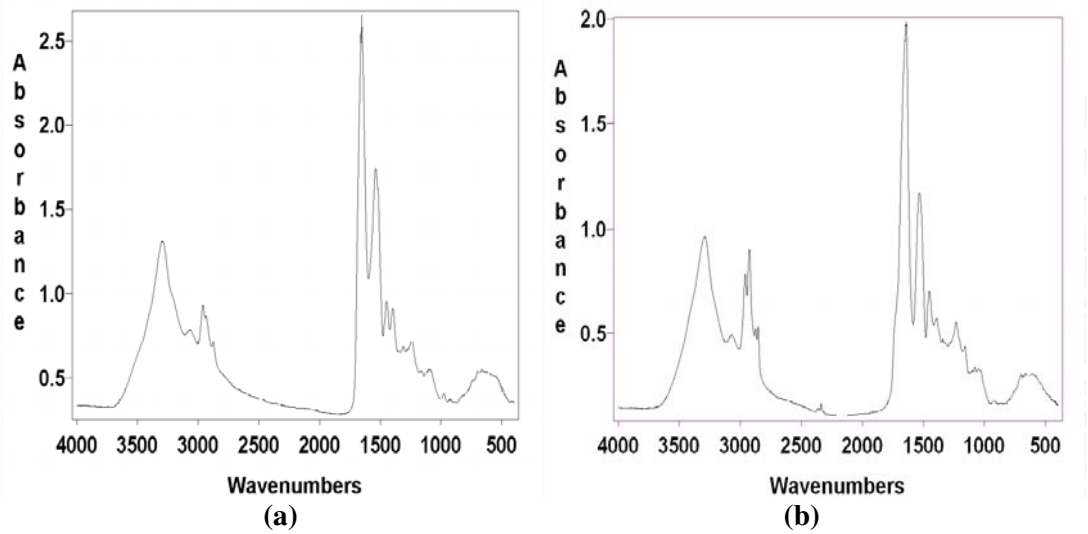



Figure 65. FTIR spectra of casein **(a)** and keratin **(b)**

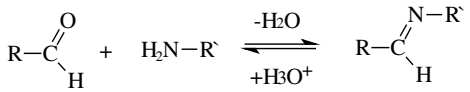
Casein from bovine milk was obtained from Sigma (Figure 65a). Keratin (Figure 65b) from chicken feathers was prepared as described bellow:
The finely ground chicken feathers were stirred with 10 wt% sodium hydroxide solution (aq.), for 3 h at a temperature of 40°C. The feathers-NaOH solution weight ratio ranged from 1:50 to 1:25. The resulting mixture was cooled and the keratin was precipitated using 10 wt% aqueous hydrochloric acid solution till pH ca. 4.5. The precipitate was washed by suspending in distilled water and centrifugation

at 4,000 rpm for 10 min (four times) using Cryofuge 6000 centrifuge (Hearaeus Sepatech, Germany) and finally air-dried.
Sodium hydroxide (pellets), acetic acid (glacial), 1,4-dioxane, hydrochloric acid (fuming), sodium chloride (extra pure), perchloric acid (70 wt%), potassium hydrogen phthalate were obtained from Fluka. Urea and glycerol were from Merck. Cellulose was obtained from filter paper circles of Schleicher & Schuell GmbH (Germany) by suspending 24g of filter in 500 $cm^3$ distilled water overnight and then homogenization in homogenizer (type 240A, Krups, Germany) for 30 s at 80 rpm.

**2. Analytical methods**

2.1. Determination of water retention value of modified chitosan film

Determination of the water retention value (WRV) of chitosan films, microcrystalline chitosan films, and paper sheets were carried out similarly as described before (see Chapter I, p. 2.5.). Microcrystalline gel-like dispersion directly after preparation, without soaking in distilled water was also tested. The sample weight was determined after centrifugation ($m_1$) and after drying at 105°C to a constant weight ($m_0$). The water retention value was calculated according to the equation mentioned in Chapter I, equation [7].
The determination of WRV of microcrystalline films cross-linked by glutaraldehyde was carried out with 1 wt% aqueous solution of acetic acid. However, the hydrolysis of Schiff base in acidic condition as shown below it is necessary to take into account.




2.2. Determination of the water content (Wc)

The procedure for determination of Wc of chitosan films, gels, or paper was the same as described previously in the case of determination of Wc (see Chapter I, p. 2.6.). The coefficient of Wc was defined using the equation described in Chapter I, equation [8].
The determination of Wc of microcrystalline films cross-linked by glutaraldehyde was also carried out with 1 wt% aqueous solution of acetic acid.

2.3. Determination of swelling coefficient

Coefficient of swelling (Cs) of chitosan films and paper sheets were determined and defined as described previously in Chapter I, p. 2.7., equation [8].

2.4. Determination of polymer content

A solution of chitosan in 1% aqueous acetic acid or MCCh gel-like dispersion was evaporated and the residue dried at a temperature of 105°C to constant weight. The polymer content (Pc) was calculated according to the equation:

$$P_C = \frac{m_1}{m_0} \times 100\% \qquad [10]$$

where :

$m_1$ - weight of chitosan solution or MCCh gel-like dispersion,
$m_0$ - weight of sample after drying

2.5. Determination of the crystallinity index

The crystallinity index (CrI) of chitosan-protein films was determined by X-ray as described previously (Chapter I, 2.10.).

2.6. FTIR spectroscopy

FTIR spectra were measured as described above (see Chapter I, p. 2.3.)

2.7. Determination of the energy of hydrogen bonds

The energy of hydrogen bonds ($E_H$) was determined by FTIR[254] of MCCh-protein complexes obtained from microcrystalline gel-like dispersion by ethanol exchanged process as described in Chapter II, p. 2.11. The values of $E_H$ were calculated from FTIR spectra according to the equation:

$$E_H = \frac{1}{k} \times \frac{v_0 - v}{v_0} \text{ [kJ/mol]} \qquad [11]$$

where:

$v_0$ - standard frequency corresponding to OH stretch (at 3650 $cm^{-1}$),
$v$ - frequency corresponding to bound OH groups,
k - constant equal to 6.7 x $10^{-2}$ $kJ^{-1}$

2.8. Determination of insoluble residues in chitosan films

Chitosan films (approx. 0.2 g) were dissolved in 1% (v/v) aqueous acetic acid (25 $cm^3$) at room temperature with vigorous agitation. The insoluble particles were separated using a glass-sintered filter (Schott No.3.) and washed two times with 1% (v/v) aqueous acetic acid (2 x 50 $cm^3$) and distilled water (50 $cm^3$) till the presence of precipitant was not detected by dropping of 2% (w/v) NaOH (aq.) in the supernatant. The residues were dried at a temperature of 105°C to a constant weight. The protein content ($C_p$) was calculated according to the equation:

$$C_p = \frac{m_n}{M} \times 100\% \qquad [12]$$

where:

$m_n$ - weight of the insoluble particles [g].
M - initial weight of sample [g].

2.9. Determination of insoluble particles in microcrystalline chitosan-casein powders

Chitosan-casein powder (approx. 0.1g) was dissolved in 50 $cm^3$ of 1% (v/v) aqueous acetic acid for 24 h at room temperature with vigorous agitation. The procedure of separation and calculation proceeded by the method described in Chapter II section 2.8.

2.10. Determination of protein content in the microcrystalline chitosan-keratin powders

A chitosan-keratin powder (approx. 0.1g) was dissolved in 50 $cm^3$ of 1% (v/v) aqueous acetic acid for 24 h at room temperature with vigorous agitation. The insoluble particles were separated using centrifugation at 4,000 rpm for 15 min (3 times with exchange of the solvent using 1% (v/v) acetic acid (2 x 50 $cm^3$) and distilled water (50 $cm^3$)) until the precipitant was not detected by dropping of 2% (w/v) NaOH (aq.) in the supernatant. The residues were transferred to a measuring vessel and dried at a temperature of 105°C until constant weight. The protein content ($C_p$) was calculated according to the equation:

$$C_p = \frac{(m_1 - m_n)}{M} \times 100\% \qquad [13]$$

where:

$m_n$ - weight of empty measuring vessel [g],
$m_1$ - weight of the measuring vessel after drying [g],
M - initial weight of sample [g].

2.11. Determination of degree of deacetylation of microcrystalline chitosan

The degree of deacetylation was determined by a potentiometric titration as described in the Chapter I. p. 2.2, except that the microcrystalline chitosan gel like-dispersion was dehydrated 2 times by means of ethanol (50 $cm^3$ per 1g of dry chitosan) before the measurements. Then the suspension was filtered off on a cheese fabric and the filtration residue was air-dried.

2.12. Determination of microcrystalline chitosan $\bar{M}_v$

The microcrystalline chitosan gel-like dispersion was exchanged two times with ethanol (50 $cm^3$ per 1g of dry chitosan) and air-dried before measurement of the $\bar{M}_v$ by viscometry (see Chapter I. p 2.1.).

2.13. Determination of the mechanical properties of chitosan films and paper sheets

The mechanical properties of films and paper sheets were determined on a tensile-strength-testing machine Zwick Z020. The Young's modulus [MPa], elongation at break [%] and tensile strength [MPa] analysis were determined in accordance with the DIN/EN ISO 527 standard, with the length of samples of 100 mm., (60 mm. for films), width of 10 mm, at a temperature of 19.3°C - 20.7°C, at a speed of 100 mm/min and at relative humidity of 50% (dry conditions) or 90% (wet conditions).

Tensile strength is the maximum tensile stress, which a material can sustain and is taken to be the maximum load exerted on the films sample during the test, divided by the original cross-section of the sample. Elongation at break is usually measured at the point where the sample breaks and is expressed as the percentage of change in the original length of the sample between the grips of the testing machine. Its importance is as a measure of the ability of sample to stretch. Young's modulus is the ratio of stress (the ratio of the force exerted on a body to its cross-section area) to strain (the change in the dimension of the body when the force is applied to it) over the range for which this ratio is constant. It is a measure of the force that is required to deform the sample by a given amount and so it is a measure of the intrinsic stiffness of the sample.
The thickness of all films was determined using a micrometer. All data are from 6-10 different films specimens or paper sheets of the same chitosan charge.

2.14. Determination of MCChB content in paper sheet

Dried MCChB paper (0.3 g) was added to 50 $cm^3$ of 1% (v/v) aqueous acetic acid and agitated overnight at room temperature. Then the suspension was homogenized for 1 min at 80 rpm and separated through a glass-sintered filter (Schott No.1.). The cellulose fibres were washed with two portions of 1% (v/v) aqueous acetic acid solution (50 $cm^3$) till the precipitant was not detected by dropping of 2% (w/v) NaOH (aq.) in the supernatant and then with distilled water until neutrality. Chitosan retention was calculated according to the equation below:

$$C_p = [1 - \frac{(m_1 - m_n)}{M}] \times 100\% \qquad [14]$$

where:

$m_n$ - weight of empty a glass-sintered filter (Schott No.1.) [g],
$m_1$ - weight of a glass-sintered filter (Schott No.1.) after drying at 105°C [g],
M - initial weight of sample [g]

In the case of paper containing MCChB modified by protein, the suspension was washed until neutrality and three portions of 25 $cm^3$ solution of 2% (w/v) aqueous sodium hydroxide were added. Then the residue was washed with distilled water until neutrality and dried at 105°C.

2.15. Determination of reducing sugar content

Reducing sugar was determined by the method described before (see Chapter I, p. 2.11.).

2.16. Determination of chitosan film solution viscosity

Chitosan films (approx. 250 mg) were dissolved in 25 $cm^3$ of 1% (v/v) aqueous acetic acid. After the dissolution, the insoluble particles were removed by filtration through a glass-sintered filter (Schott No.1.). The solution viscosity was determined using an Ubbelohde's viscometer Type 53110/I and calculated according to the equation:

$$\eta_i = \frac{(t_1 - t_0)/t_0}{C_{\%}} \qquad [15]$$

where:

$\mathbf{C_{\%}}$ - concentration of chitosan in the solvent system (calculated as a difference between the original weight of sample and the weight of insoluble particles),
$\mathbf{t_1}$ - flow time for the chitosan solution,
$\mathbf{t_0}$ - flow time for the solvent system,
$\eta_i$ - viscosity.

**3. Methods**

3.1. Preparation of chitosan films

All chitosan films were prepared using 1.0% or 2.0% (w/v) chitosan solution in 1% (v/v) aqueous acetic acid.

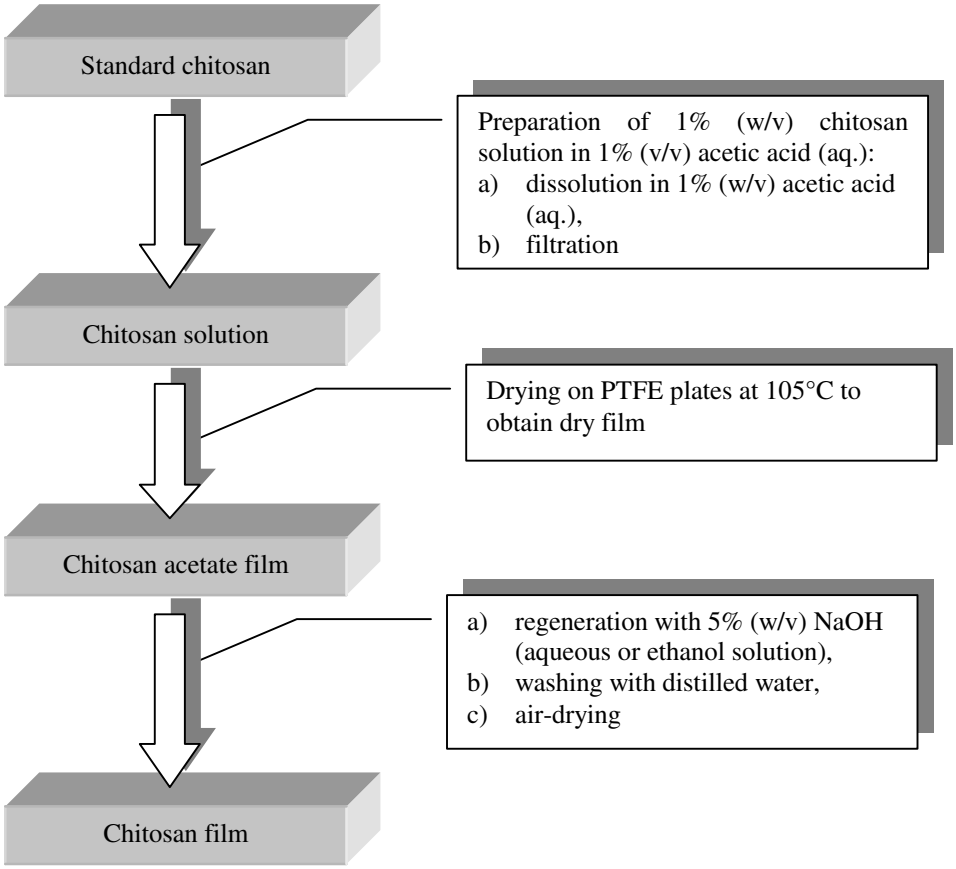



Figure 66. Preparation of chitosan films from solution of chitosan in 1% (v/v) acetic acid

The solutions were mechanically stirred during 24 h at room temperature and filtered using a glass-sintered filter (Schott No.2.) for removal of the insoluble particles. Then 10 $cm^3$ of chitosan solution was poured onto tetrafluoroethylene plates (50 mm x 60 mm) and dried for 3h at a temperature of 105°C. The conversion of chitosan acetate films to chitosan films (regeneration) was carried out by soaking with 25 $cm^3$ of a 5% (w/v) solution of sodium hydroxide in water or in ethanol for 24 h at room temperature. Then chitosan films were washed with distilled water and finally air-dried. The procedure for the regeneration of chitosan films modified by casein was similar to that described above. However, 2% (w/v) solution of casein in 5% (w/v) aqueous NaOH was used in this experiment (Figure 66).

3.2. Preparation of microcrystalline chitosan powder (MCChA, method A, standard procedure)

1% (w/v) solution of chitosan (100 $cm^3$) in 1% (v/v) aqueous acetic acid, with or without 0.3g sodium chloride, was added to 1 L round bottom flask containing 2% (w/v) aqueous solution of sodium hydroxide (50 $cm^3$) using a peristaltic pump[167,255], under intensive stirring at room temperature. The pH of the reaction medium was kept below 10.0 by means of a pH–stat connected with a peristaltic pump, which passed 2% (w/v) aqueous solution of sodium hydroxide. After 1h, the dispersion was adjusted to pH=8.0 by slow addition of a 1% (w/v) aqueous solution of acetic acid and than agitated again for 0.5 h. The MCCh gel-like dispersion was separated on cheese-cloth and washed with distilled water until neutrality. Then the MCCh gel-like dispersion was dehydrated with ethanol (2 x 50 $cm^3$) and separated by filtration over chasse cloth. The homogenized (2 min. at 80 rpm) dispersion of MCCh, with polymer content ranging from 0.5 to 2.0 wt%, was poured onto polytetrafluoroethylene plates (PTFE) and air-dried overnight. The product was then ground to a powder.

3.2.1. Preparation of microcrystalline chitosan-protein powder with protein (MCChA-Ca or MCChA-Kr)

1% (w/v) solution of chitosan (100 $cm^3$) in 1% (v/v) aqueous acetic acid, with or without 0.3g sodium chloride, was passed to a 0.25, 0.5, 1.0, 2.0 or 3.0% (w/v) protein solution (casein or keratin) in 2% (w/v) aqueous sodium hydroxide with or without 0.3g of sodium chloride. The MCCh-protein powder was formed as described previously (see Chapter II, p. 3.2.).

3.3. Preparation of microcrystalline chitosan powder (MCChB, method B, modified procedure)

50 $cm^3$ of 2% (w/v) aqueous sodium hydroxide solution was slowly dropped under intensive stirring to 1 $dm^3$ three-arms flask containing 1% (w/v) chitosan solution in 1% (v/v) aqueous acetic acid (100 $cm^3$), with or without 0.3 g. 0.6g or 0.9g sodium chloride. The procedures for microcrystalline gel-like dispersion and powder preparation were similar to those described in Chapter II. p. 3.2.

3.3.1. Preparation of MCChB-protein powder (MCChB-Ca or MCChB-Kr)

0.12g, 0.25g, 0.5g, 1.0g or 1.5g casein or keratin was swollen overnight in a chitosan solution (1% (w/v) in 1% (v/v) aqueous acetic acid) under the conditions described in Chapter II, p. 3.3. Then the modified powders (MCChB-protein) were prepared as above (see Chapter II, p. 3.2).

3.4. Preparation of microcrystalline chitosan films with or without proteins

An aqueous MCChB gel-like dispersion, separated from a coagulation bath for the preparation of MCChB-protein films with addition of glycerol, was used according to the procedure presented in Figure 67.
The mixture, containing 1g dry weight of MCChB-protein gel-like dispersion, was filled to 70 $cm^3$ using distilled water and homogenized for 2 min at 50 rpm. Glycerol (0.15, 0.3, 0.7 or 0.9 g) was added and the mixture was again homogenized for 2 min. Films were prepared on polytetrafluoroethylene (PTFE) plates overnight at room temperature. MCChB film prepared without addition of glycerol was used as a reference.

3.5 MCChB films cross-linked by glutaraldehyde

The MCChB films prepared as previously on the basis of chitosan M3A or M3mB were added to a solution of glutaraldehyde (GA) with a molar ratio GA:DD of 6, 3, 1, 0.5, 0.25 or 0.11 in a citrate buffer at pH=6.0 or 7.0 The films were soaked at room temperature for 0.12, 0.24, 0.5, 4 or 24h. Then the films were separated, washed with distilled water, and air-dried.

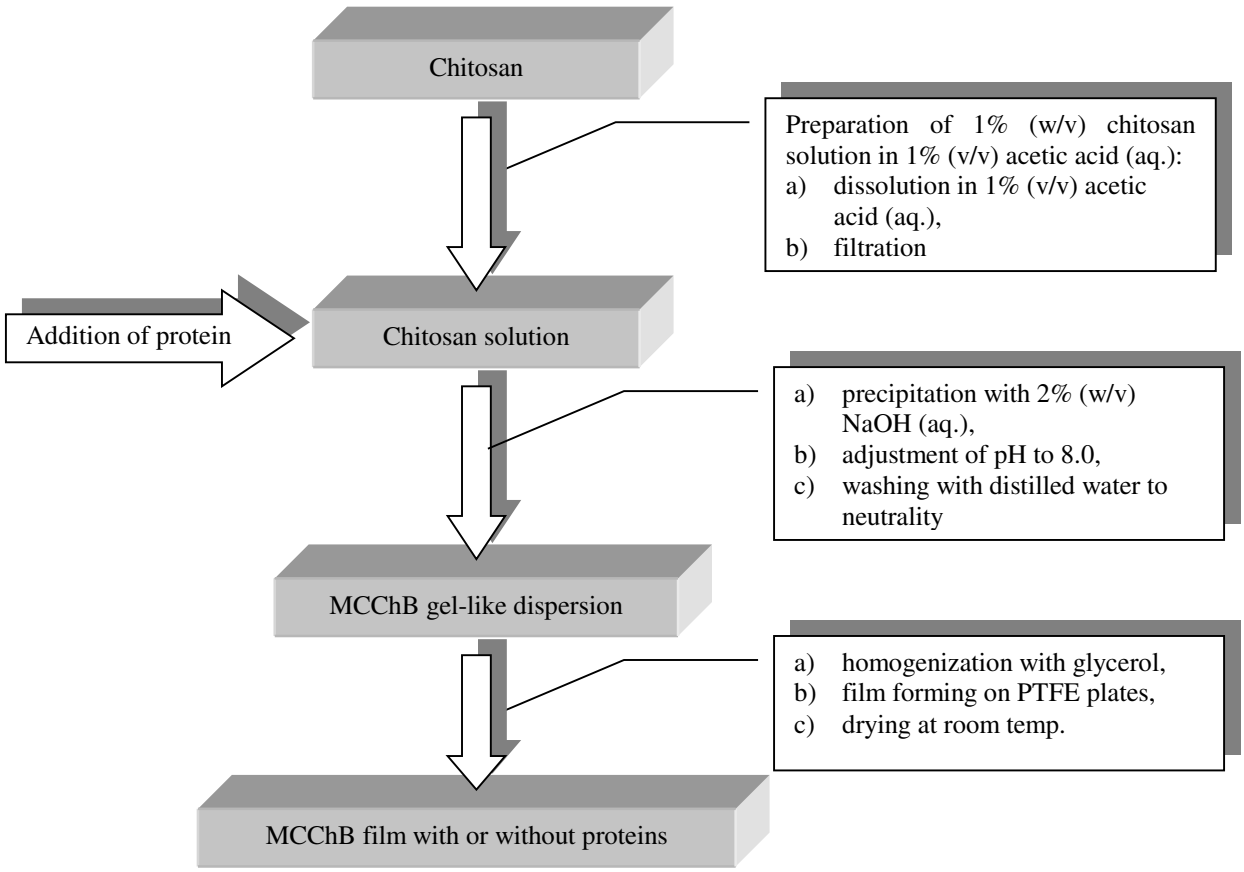



Figure 67. Preparation of MCChB films containing protein

3.6 MCChB gel-like dispersion cross-linked by glutaraldehyde

MCChB gel-like dispersion (0.5 g of dry weight) prepared on the basis of chitosan M3A was added to GA solution with a molar ratio GA:DD of 4, 2, 0.5 or 0.11 in a citrate buffer (at pH 5.0, 6.0 or 7.0) and stirred for 0.12, 0.24, 0.5, 4 or 24h. Than the gel-like dispersion was separated by the filtration through cheese cloth and washed by distilled water. The MCChB film was formed according to the procedure described previously (see Chapter II, p. 3.4.).

3.7. Preparation of paper modified by MCChB with or without proteins

A MCChB gel-like dispersion prepared by the method described before (see Chapter II, p. 3.3.) was added to 1 $dm^3$ cellulose fibre suspension (2.4 g•$dm^{-3}$, equilibrated to 75 ± 2 g•$m^{-3}$ of dry paper - according to DIN 54358 standard). The amount of MCChB 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 the Rapid Homogenizer PTI-31 (Paper Testing Instruments GmbH, Austria) according to the German standard of ISO 5263 for 10 min. at 3,000 rpm. Paper sheets were formed using paper-forming machine V/8/76 (Paper Testing Instruments GmbH. Austria) based on the ISO 5269/2 and the DIN 54358 standards (Figure 68). 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 the DIN 50014 standard. A MCChB gel-like dispersion with 20 wt% proteins (casein or keratin) was also used for paper modification.

3.8. Preparation of paper modified by precipitation of MCChB

50 $cm^3$ distilled water was added to a cellulose-fibre dispersion (2.4 g of dry weight) and the mixture was adjusted to pH=5.0 by adding 1% (v/v) of aqueous acetic acid during agitation. Then 1.0% (w/v) 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 sodium hydroxide (2 wt%) was adding dropwise until pH=10.0. Cellulose-MCChB pulp was stirred for 1 h and pH was lowered to 8.0 with 1 wt% aqueous acetic acid solution. The suspension was filled to 1 $dm^3$. Homogenization and paper-forming procedures were carried out according to the conditions described before (see Chapter II, p. 3.7.).

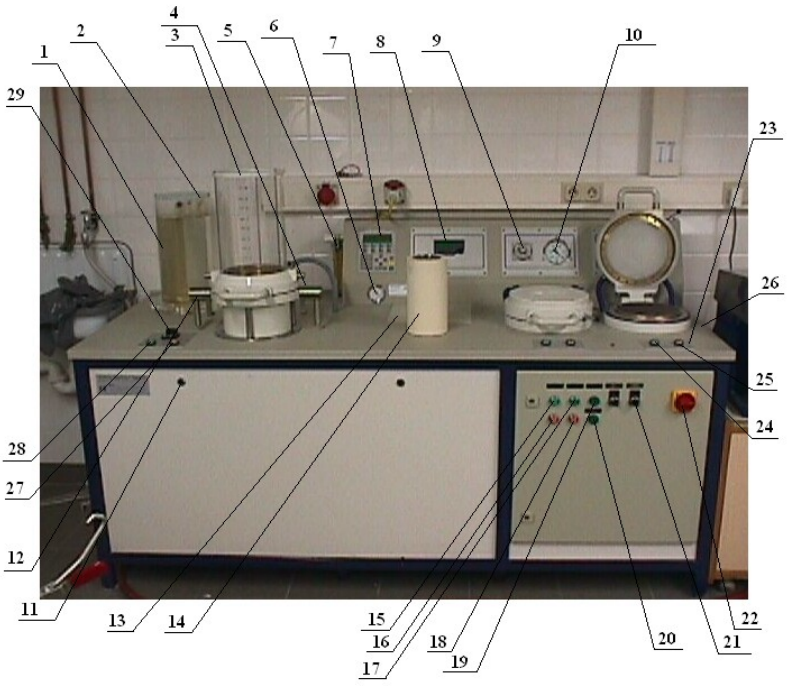



Figure 68. Paper-forming machine V/8/76 (Paper Testing Instruments GmbH. Austria):
*1) water container, 2) water heater, 3) forming cistern (10 $dm^3$), 4) vacuum valve, 5) vacuum indicator, 6) water valve indicator, 7) SPS console, 8) temperature indicator, 9) drying range indicator, 10) drying time indicator, 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) run option (0 –*

*"Automatic", 1 – "Manual"), 21) water course option (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*

3.9. Estimation of biodegradability of chitosan films

Estimation of the biodegradability of the chitosan films prepared according to the procedures described above was carried out in the presence of the fungus *Aspergillus fumigatus* in a physiological saline (50 $cm^3$).
Chitosan films (approx. 0.2 g) were introduced into a physiological saline and sterilized in an autoclave at a temperature of 121°C for 15 min. Macrospores of *A. fumigatus* in a ratio of 15 $mg \bullet g^{-1}$ chitosan film were used. The biodegradation test was carried out in a Julaba shaker SW20 at a temperature of 30°C using a shaking speed of 140 rpm for 28 days. The remaining films were removed from the fermentation bath after 7, 14, 21 and 28 days, washed with hot water (approx. 80°C) and placed in 70% ethanol for 10 – 15 min. Then the residues of chitosan films were air-dried. The biodegradation was estimated by:

- ⇒ Percentage of weight loss,
- ⇒ Changes in $\overline{M}_v$ of remaining polymer by viscometry,
- ⇒ Amount of reducing sugars present in the liquid phase of the culture,
- ⇒ Changes of WRV as well as Wc.

3.10. Biological decomposition of MCChB films and paper sheets with or without proteins in communal waste (mineralization test)

The experimental procedure is based on the fact that MCChB films containing also casein and paper sheets have prepared by direct addition or by precipitation of MCChB (with or without proteins). As a reference, a cellulose sheet prepared by analogous procedure was used[256].
The samples were milled using the Analytical Mill (IKA-WERK GmbH – Germany) at 20,000 rpm for 2 min. samples (25mg) were kept with the inoculum in the reaction vessel (containing manometer) in the sapromat containing phosphate buffer. Then the medium was filled to 250 $cm^3$ with distilled. As inoculum, 2.5 $cm^3$ fresh, unclear slime obtained from the sewage-treatment plant Stahnsdorf was added per vessel. The decomposition was carried out during 24 days at a temperature of 25°C.
Organic substances are decomposed by aerobic microorganisms. $CO_2$ is absorbed with soda. The consumption of oxygen causes a reduction of the gas volume in the reaction vessel that through which the electrolytic production of oxygen is balanced, so that the volume of gas in the vessel is constant. The demand for current during the electrolysis corresponds to the biological demand on the oxygen and 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 reference sample without addition of other substances containing carbon. The difference between biological and theoretical demand for oxygen corresponds to the degree of biological decomposition of films or paper sheets.

## 4. Results and discussion

4.1. Properties of chitosan films prepared from acetic solution

The influence of the type of coagulation bath and concentration of chitosan on the properties of the films prepared from its acidic solution was assessed during this stage of research. The results of polymer content as well as WRV, Wc, and Cs of chitosan films prepared from chitosan solution in aqueous acetic acid by regeneration in the coagulation bath is shown in Tables 36-39.
During formation of chitosan films, especially under drying stage, the modifications of the polymer structure increased the viscosity of the solution of films in aqueous acetic acid[181]. This can be explained by two phenomena: firstly, by the self-condensation of biopolymer during prolonged drying[257]; secondly, during this film forming, chitosan loses acetic acid and is thereby converted into the insoluble free amine form[178]. Taking into account this aspect, it was not possible to determine the $\overline{M}_v$ of films by the viscometry (the absence of K and a constant characterizing this solution type of aggregated polymer). Thus, in order to determine the influence of $\overline{M}_v$ and DD on the swelling behaviour of films the parameters of the starting polymer were used.

WRV, Wc, as well as Cs increased with concentration of chitosan, except for films regenerated using ethanol-containing baths. However, this improvement in the swelling behaviour of films was not so significant. The high values of the coefficients were recorded for chitosan films showed a high $\overline{M}_v$ and coagulated with aqueous sodium hydroxide (Figure 69a. 69b.).

Table 36. Properties of films prepared from aqueous acetic acid solution of chitosan from Antarctic krill using various coagulation baths*

| Sample | Chitosan content [%] | Coagulation bath | $W_c$ [%] | WRV [%] | Cs [%] | Insoluble particles [%] |
|---|---|---|---|---|---|---|
| G3mA | 1.13 | NaOH (aq.) | 57.6 | 136.1 | 149.4 | 13.1 |
| | 2.08 | NaOH (aq.) | 65.8 | 192.4 | 190.3 | 13.5 |
| | 1.13 | NaOH (eth.) | 63.9 | 176.6 | 178.8 | 4.5 |
| | 2.08 | NaOH (eth.) | 65.1 | 186.4 | 185.3 | 5.3 |
| | 1.13 | NaOH + casein | 28.8 | 40.4 | 77.6 | 12.1 |
| | 2.08 | NaOH + casein | 50.9 | 103.4 | 124.3 | 27.4 |
| M3A | 1.2 | NaOH (aq.) | 66.7 | 145.0 | 154.7 | 3.5 |
| | 2.08 | NaOH (aq.) | 63.8 | 176.3 | 178.6 | 4.6 |
| | 1.2 | NaOH (eth.) | 56.1 | 127.9 | 143.4 | 9.6 |
| | 2.08 | NaOH (eth.) | 57.8 | 132.0 | 146.3 | 7.2 |
| | 1.2 | NaOH + casein | 50.5 | 102.1 | 124.3 | 7.5 |
| | 2.08 | NaOH + casein | 62.0 | 163.3 | 167.9 | 9.2 |
| G3mB | 1.14 | NaOH (aq.) | 53.3 | 114.2 | 132.5 | 0.0 |
| | 2.11 | NaOH (aq.) | 55.1 | 122.9 | 138.3 | 0.0 |
| | 1.14 | NaOH (eth.) | 51.3 | 105.4 | 126.3 | 0.0 |
| | 2.11 | NaOH (eth.) | 53.4 | 115.4 | 132.8 | 0.0 |
| | 1.14 | NaOH + casein | 45.6 | 83.9 | 110.3 | 0.6 |
| | 2.11 | NaOH + casein | 52.8 | 111.9 | 130.7 | 0.0 |
| M3mB | 1.11 | NaOH (aq.) | 61.9 | 162.5 | 167.8 | 0.0 |
| | 2.14 | NaOH (aq.) | 70.6 | 198.3 | 194.4 | 0.0 |
| | 1.11 | NaOH (eth.) | 53.7 | 118.3 | 135.0 | 3.9 |
| | 2.14 | NaOH (eth.) | 53.7 | 116.1 | 134.3 | 0.8 |
| | 1.11 | NaOH + casein | 48.6 | 90.4 | 115.6 | 0.3 |
| | 2.14 | NaOH + casein | 53.4 | 112.1 | 131.1 | 0.0 |
| M1mC | 1.11 | NaOH (aq.) | 46.8 | 87.9 | 113.0 | 0.0 |
| | 1.97 | NaOH (aq.) | 55.4 | 121.4 | 138.4 | 0.0 |
| | 1.11 | NaOH (eth.) | 51.1 | 105.4 | 126.2 | 0.0 |
| | 1.97 | NaOH (eth.) | 52.4 | 109.1 | 129.1 | 5.7 |
| | 1.11 | NaOH + casein | 54.3 | 118.6 | 135.9 | 2.6 |
| | 1.97 | NaOH + casein | 52.6 | 111.2 | 129.6 | 4.3 |
| M6mC | 1.12 | NaOH (aq.) | 46.8 | 87.9 | 112.8 | 0.0 |
| | 2.19 | NaOH (aq.) | 53.9 | 116.9 | 135.4 | 0.0 |
| | 1.12 | NaOH (eth.) | 48.7 | 98.0 | 121.3 | 0.0 |
| | 2.19 | NaOH (eth.) | 50.4 | 101.4 | 123.3 | 19.7 |
| | 1.12 | NaOH + casein | 51.5 | 106.0 | 126.6 | 0.2 |
| | 2.19 | NaOH + casein | 54.2 | 114.0 | 132.1 | 0.2 |

M- chitosan produced from non demineralized chitin, Mm- chitosan produced from demineralized chitin,
* the coagulation bath: NaOH (aq.) – 5% (w/v) aqueous solution of sodium hydroxide, NaOH (eth.) – 5% (w/v) solution of sodium hydroxide in ethanol, NaOH + casein – 2% (w/v) solution of casein in 5% (w/v) aqueous solution of sodium hydroxide.

Table 37. Properties of films prepared from aqueous acetic acid solution of chitosan using various coagulation baths*

| Sample | Content of chitosan [%] | Coagulation bath | $W_c$ [%] | WRV [%] | Cs [%] | Insoluble particles [%] |
|---|---|---|---|---|---|---|
| P4 | 1.09 | NaOH (aq.) | 56.4 | 132.2 | 146.4 | 0.0 |
| | 2.0 | NaOH (aq.) | 68.8 | 168.8 | 173.2 | 0.0 |
| | 1.09 | NaOH (eth.) | 55.1 | 121.7 | 138.0 | 0.0 |
| | 2.0 | NaOH (eth.) | 56.2 | 128.9 | 142.7 | 0.0 |
| | 1.09 | NaOH + casein | 52.4 | 110.2 | 129.2 | 0.0 |
| | 2.0 | NaOH + casein | 63.1 | 165.3 | 169.4 | 0.0 |

* the coagulation bath: NaOH (aq.) – 5% (w/v) aqueous solution of sodium hydroxide, NaOH (eth.) – 5% (w/v) solution of sodium hydroxide in ethanol, NaOH + casein – 2% (w/v) solution of casein in 5% (w/v) aqueous solution of sodium hydroxide.

Table 38. Properties of films prepared from aqueous acetic acid solution of chitosan from *Pandalus borealis* chitin using various coagulation baths*

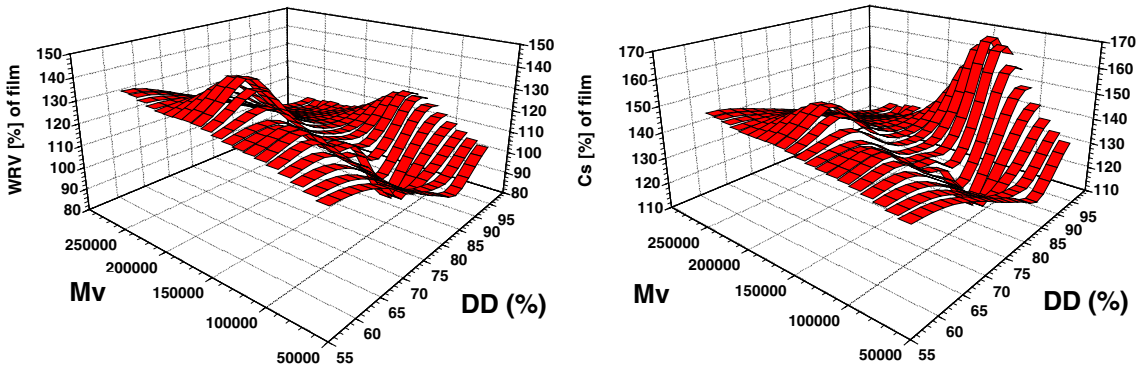
| Sample | Chitosan content [%] | Coagulation bath | $W_c$ [%] | WRV [%] | Cs [%] | Insoluble particles [%] |
|---|---|---|---|---|---|---|
| PB8 | 1.03 | NaOH (aq.) | 50.3 | 101.2 | 123.4 | 11.6 |
| | 2.03 | NaOH (aq.) | 52.1 | 108.7 | 128.9 | 9.1 |
| | 1.03 | NaOH (eth.) | 58.1 | 136.6 | 148.6 | 0.0 |
| | 2.03 | NaOH (eth.) | 58.1 | 138.8 | 151.4 | 5.3 |
| | 1.03 | NaOH + casein | 38.9 | 63.5 | 94.7 | 11.8 |
| | 2.03 | NaOH + casein | 44.1 | 78.8 | 105.6 | 23.7 |
| PB10 | 1.18 | NaOH (aq.) | 51.0 | 104.2 | 125.4 | 9.9 |
| | 2.03 | NaOH (aq.) | 60.6 | 153.7 | 161.1 | 32.3 |
| | 1.18 | NaOH (eth.) | 52.7 | 111.3 | 129.9 | 15.1 |
| | 2.03 | NaOH (eth.) | 49.2 | 96.9 | 120.4 | 9.8 |
| | 1.18 | NaOH + casein | 28.8 | 40.4 | 78.3 | 27.6 |
| | 2.03 | NaOH + casein | 46.3 | 86.3 | 112.1 | 65.9 |

* the coagulation bath: NaOH (aq.) – 5% (w/v) aqueous solution of sodium hydroxide, NaOH (eth.) – 5% (w/v) solution of sodium hydroxide in ethanol, NaOH + casein – 2% (w/v) solution of casein in 5% (w/v) aqueous solution of sodium hydroxide.

Table 39. Properties of films prepared from aqueous acetic acid solution of chitosan from insect larvaes *C. erythrocephala* using various coagulation baths*

| Sample | Chitosan content (%) | Coagulation bath | $W_c$ (%) | WRV (%) | Cs (%) | Insoluble particles (%) |
|---|---|---|---|---|---|---|
| MCCh I-3/1/A | 0.98 | NaOH (aq.) | 51.4 | 105.4 | 129.0 | 0.0 |
| | 1.97 | NaOH (aq.) | 39.8 | 41.4 | 111.6 | 0.0 |
| | 0.98 | NaOH (eth.) | 52.5 | 116.6 | 135.1 | 0.0 |
| | 1.97 | NaOH (eth.) | 51.2 | 101.1 | 124.3 | 0.0 |
| | 0.98 | NaOH + casein | 39.9 | 44.6 | 81.1 | 0.0 |
| | 1.97 | NaOH + casein | 46.3 | 84.4 | 106.0 | 0.0 |

* the coagulation bath: NaOH (aq.) – 5% (w/v) aqueous solution of sodium hydroxide, NaOH (eth.) – 5% (w/v) solution of sodium hydroxide in ethanol, NaOH + casein – 2% (w/v) solution of casein in 5% (w/v) aqueous solution of sodium hydroxide.



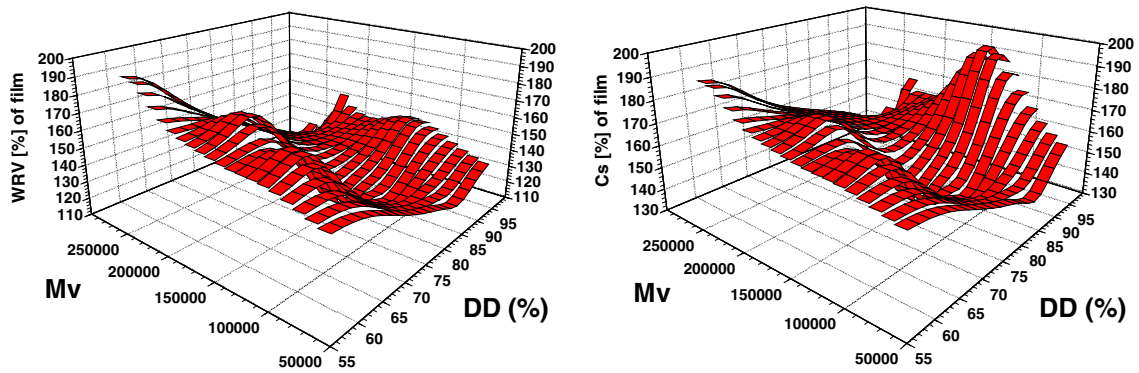

(a) (b)

Figure 69. The effect of DD and $\overline{M}v$ of chitosan on WRV (a) and Cs (b) of chitosan films prepared from 1% (w/v) chitosan solution, coagulated with an aqueous solution of sodium hydroxide

A similar phenomenon for films prepared from 2% (w/v) chitosan solution was observed. Nevertheless, the value of the above-mentioned coefficients increased with the increase in the concentration of chitosan solution used for film formation (

(a) (b)

Figure 70a, 70b).The films prepared from highly deacetylated chitosans prepared from *Pandalus borealis* showed lower swelling properties. This aspect was detected more clearly for the change in swelling coefficients (Figure 69a, 69b).




(a) (b)

Figure 70. The effect of DD and $\overline{M}_v$ of initial chitosan on WRV (a) and Cs (b) of chitosan films prepared from 2% (w/v) chitosan solution, coagulated with an aqueous solution of sodium hydroxide

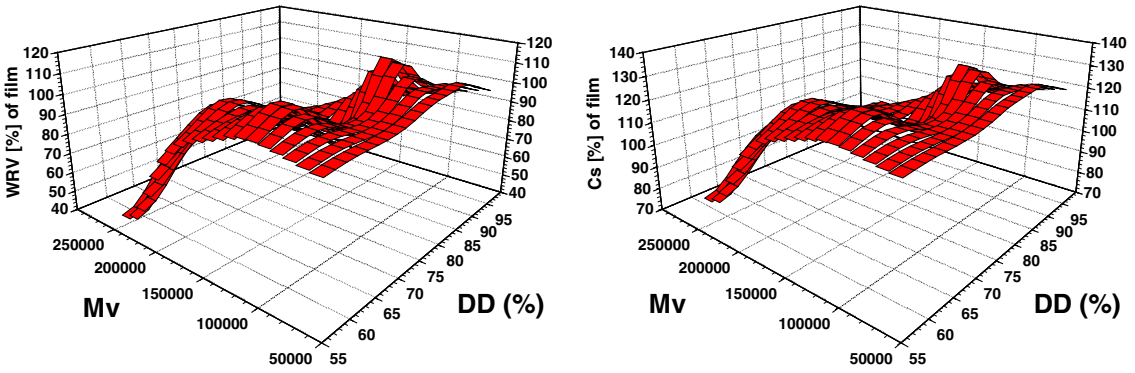
In the case of films produced from krill chitosan, an increase in amount of retained water for higher DD with the increase in $\overline{M}_v$ was observed.

WRV, Wc, and Cs of chitosan films prepared in an aqueous sodium hydroxide bath containing casein were dramatically reduced with the increase in the initial $\overline{M}_v$ (Figures 71a, 71b). This observation showed a resemblance to the solubility of formed films resulting from the high amount of insoluble particles for chitosan with high $\overline{M}_v$.

Films regenerated in a presence of casein possessed opposite swelling properties, with the existence of a higher amount of insoluble residues, as compared with films coagulated under the same conditions from protein-free baths. This fact confirmed the effect of a protein-containing bath on the alteration of the structure of chitosan film (surface, porosity) during the regeneration process. Films prepared from higher concentrated of chitosan solution showed lower swelling when casein was present in coagulation bath then films made from solution of chitosan at lower concentration (Figures 72a, 72b).

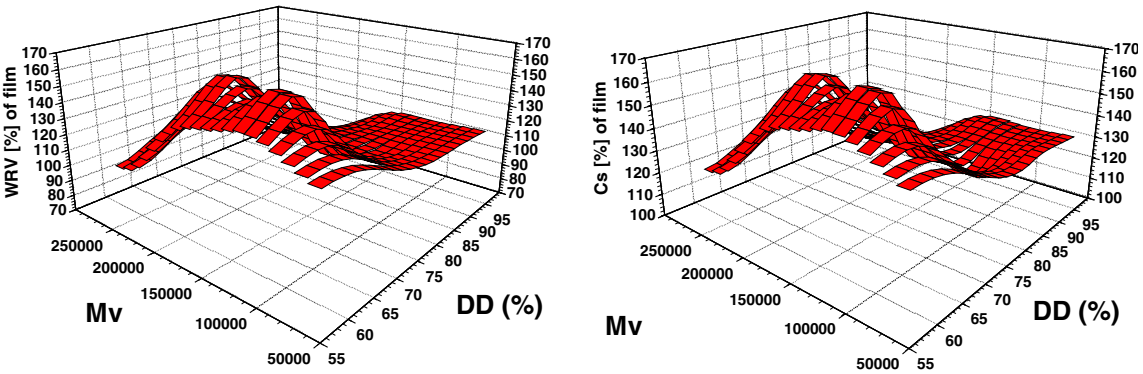
Films prepared by coagulation with ethanolic NaOH solution showed better solubility in aqueous acetic acid than those prepared in aqueous NaOH solution (Tables 36-39). A decreasing tendency was found for WRV, Wc and Cs coefficients with lower $\overline{M}_v$ and an increase in DD of the original chitosan

(Figures 73a, 73b and 74a, 74b). The coagulation in the presence of ethanol affected the swelling behaviour of films by decreasing their value considerably.



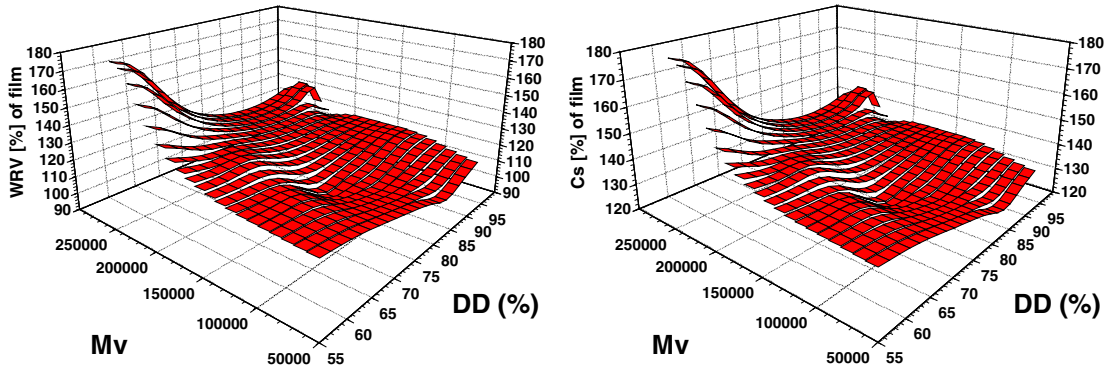

(a) (b)

Figure 71. The effect of DD and $\overline{M}_v$ of initial chitosan on WRV (a) and Cs (b) of chitosan films prepared from 1% (w/v) chitosan solution, coagulated with an aqueous solution of sodium hydroxide + casein



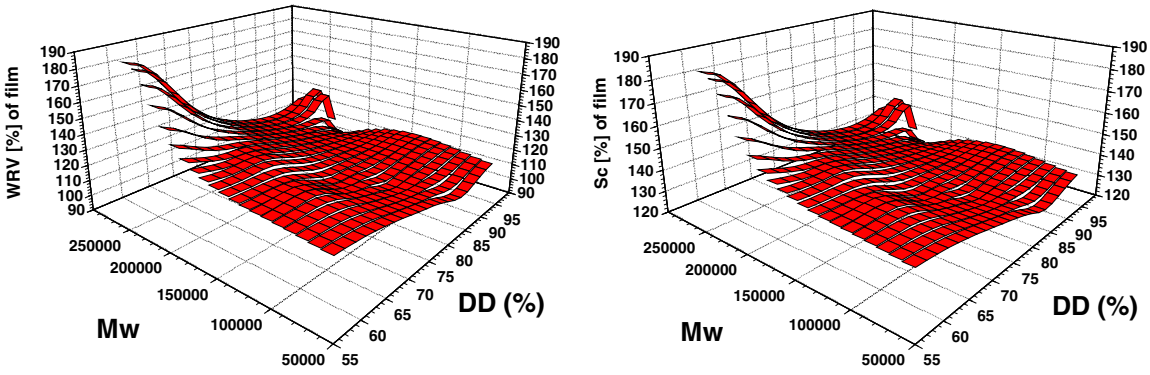

(a) (b)

Figure 72. The effect of DD and $\overline{M}_v$ of initial chitosan on WRV (a) and Cs (b) of chitosan films prepared from 2% (w/v) chitosan solution, coagulated with an aqueous solution of sodium hydroxide + casein




(a) (b)

Figure 73. The effect of degree of deacetylation and $\overline{M}_v$ of initial chitosan on WRV and Cs of chitosan films prepared from 1% (w/v) chitosan solution, coagulated with a solution of sodium hydroxide with ethanol




(a) (b)

Figure 74. The effect of DD and $\bar{M}_v$ of initial chitosan on WRV and Cs of chitosan films prepared from 2% (w/v) chitosan solution, coagulated with a solution of sodium hydroxide with ethanol

It is concluded that the solvent used for coagulation as well the concentration of the chitosan solution significantly affects the swelling parameters of the films. The films coagulated in the presence of casein showed lower swelling. However, no significant change in the amounts of insoluble particles was found, except for films prepared from highly deacetylated shrimp chitosan.
In addition, the swelling parameters were connected with the original $\bar{M}_v$ of chitosan. Higher WRV, Wc, and Cs were obtained for films derived from high-molecular chitosan, often by a low DD.

4.1.1. Mechanical properties of chitosan films

Mechanical properties of films prepared from chitosan samples M3A, PB10 and MCCh I-3/1/100 were determined.

Table 40. Mechanical properties of chitosan films

| Film prepared from chitosan: | Tensile strength (MPa) | Elongation at break (%) | Thickness (mm) |
|---|---|---|---|
| M3A [1)] | 10.30±2.34 | 11.00±2.56 | 0.061 |
| PB10 [1)] | 5.45±1.21 | 6.00±1.10 | 0.104 |
| MCCh I-3/1/100 [1)] | 15.70±3.31 | 16.50±3.20 | 0.084 |
| M3A [2)] | 9.53±2.10 | 8.10±1.90 | 0.068 |
| PB10 [2)] | 9.61±2.22 | 9.80±1.10 | 0.104 |
| MCCh I-3/1/100 [2)] | 18.10±4.21 | 27.80±8.53 | 0.085 |

[1)] - regeneration with ethanol containing alkali,
[2)] - regeneration with aqueous alkali

The tensile strength and elongation at break of formed films showed a wide variation because the process of drying, formation and regeneration of films in aqueous sodium hydroxide solution negatively affected the surface of the films caused their shrunk (Table 40.).
Better mechanical parameters were found for the films regenerated using aqueous sodium hydroxide. The same observation was found for the change in the elongation at break. The difference between the mechanical properties of films prepared from chitosan PB10 and MCCh I-3/1/100 (chitosans deacetylated from various sources but possessing similar $\bar{M}_v$ and DD) leads to the conclusion that method of chitosan preparation (standard chitosan from crustacean and microcrystalline one from insects) important factors affecting the mechanical properties of film (Figure 75). The thickness of films regenerated with ethanolic NaOH was similar or lower than that of films coagulated in aqueous solution of sodium hydroxide.
Films prepared from aqueous alkali showed less smoothness of surface. This fact emphasizes the important role played by water molecules during conversion to chitosan from its acetate salt.

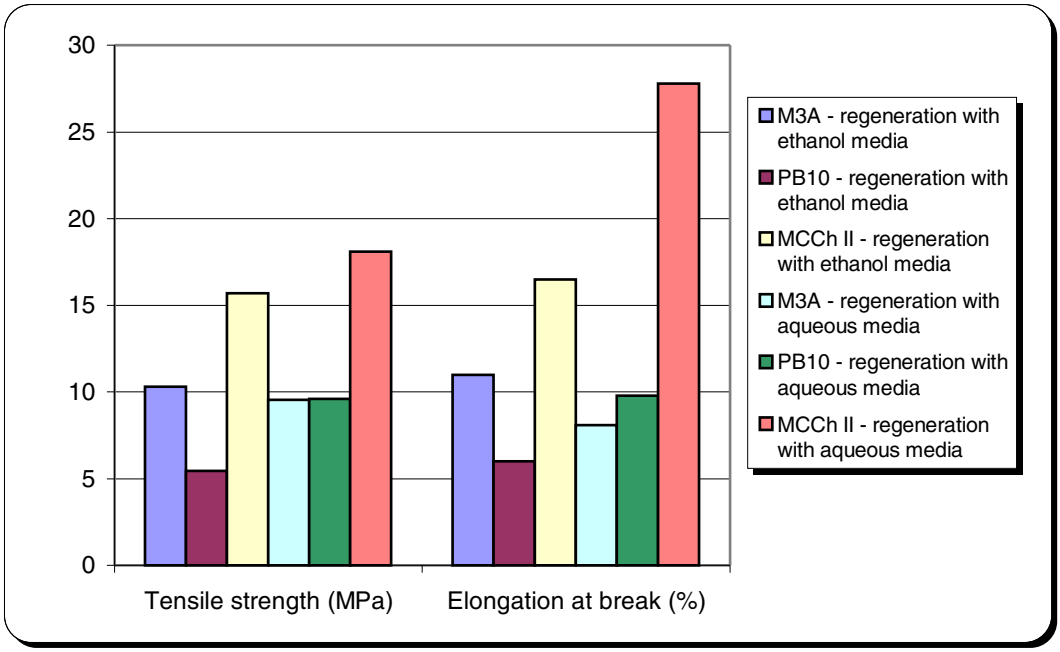



Figure 75. Mechanical properties of films prepared from various chitosans

4.2. Influence of the preparation method of microcrystalline chitosan on changes in its $\overline{M}_v$

For evaluation of the optimal conditions of the preparation of the microcrystalline chitosan and film with chitosan P4 was used.

The microcrystalline chitosan gel-like dispersion was produced according to the standard (MCChA) or modified procedures (MCChB) and the changes in the $\overline{M}_v$ were determined. The effect of the presence of sodium chloride in the coagulation bath was also investigated.

$\overline{M}_v$ was lowered when sodium chloride was not present during the coagulation process (28.6%) in contrast to the starting $\overline{M}_v$ (Table 41, Figure 76). Higher $\overline{M}_v$ was found when the coagulation was done in the presence of NaCl. In the presence of NaCl, also degradation of MCChA was lower as revealed by $\overline{M}_v$ measurements.

Table 41. Changes in $\overline{M}_v$ of chitosan prepared by different methods as microcrystalline chitosan gel-like dispersions

| Method of MCCh gel-like dispersion preparation. | Amount of NaCl (wt%) | $\overline{M}_v$ (Da) |
|---|---|---|
| MCChB | 0 | 93,000 |
| MCChB | 23 | 123,000 |
| MCChA | 23 | 105,000 |

The MCChB procedure for the preparation of microcrystalline chitosan gel-like dispersion was more effective, producing a less-degraded product. The MCChA procedure was carried out under more drastic conditions because the chitosan-acetate solution added to an alkali solution caused a stronger degradation. In the contrast, the MCChB procedure was carried out by a slow increase in pH, resulting in a lower rate of aggregation and degradation.

The results shown in Table 41 reveal the protective action of sodium chloride on polymers during aggregation. Moreover, the MCChA procedure of microcrystalline chitosan powder preparation produced a stronger degradation effect compared to $\overline{M}_v$ obtained by the MCChB procedure.

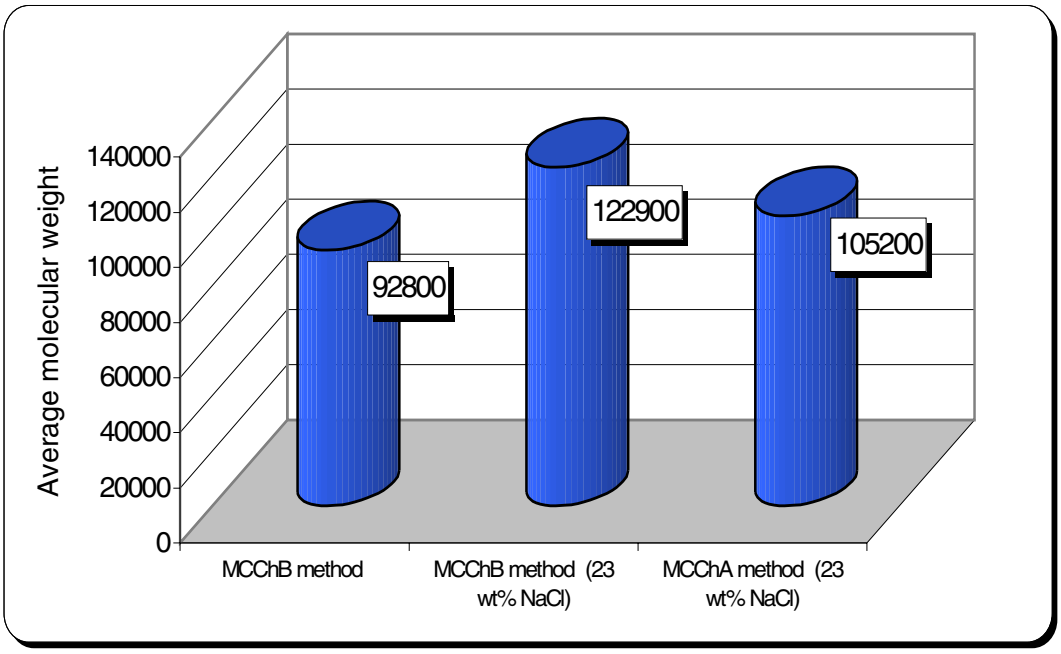



Figure 76. Effect of the method of preparation of MCCh gel-like dispersion on the changes in $\overline{M}_v$

4.3. Influence of the amount of sodium chloride on the change in WRV and Wc of microcrystalline chitosan gel-like dispersion during its preparation

Microcrystalline chitosan gel-like dispersion was prepared according to the MCChB procedure with a suitable amount of sodium chloride. The changes in some swelling properties were assessed.
A strong increase in WRV and Wc of microcrystalline chitosan gel-like dispersion for the sample prepared in the presence of 0.3 g (23 wt%) of sodium chloride was obtained. However, further increase in sodium chloride concentration resulted in lower swelling of microcrystalline chitosan (Table 42).

Table 42. The swelling parameters of microcrystalline chitosan gel-like dispersion (MCChB) prepared in the presence of sodium chloride

| Amount of NaCl (wt%) | WRV of gel (%) | Wc of gel (%) |
|---|---|---|
| 0 | 417.8 | 80.7 |
| 23 | 449.3 | 81.8 |
| 38 | 429.5 | 81.1 |
| 47 | 386.0 | 79.4 |

This phenomenon can be considered to arise from changes in the molecular conformation during the period of microcrystalline chitosan gel-like dispersion preparation. Chitosan has a relatively more flexible backbone than other polysaccharides, which allows it to occupy a compact quasiglobular or a typical random coil conformation. The conformations of chitosan chains depend on the conditions of solution. The neutralization of the charges on the polyelectrolyte by salt ions causes a break of Van der Waals interactions between the chains of chitosan. Some amount of salt ions can be used to stabilize the chains. At the same time, they support a better aggregation of microcrystalline chitosan gel-like dispersion. The lower amount of hydrogen intra- as well as intermolecular interaction increases the sensibility to degradation of polymer chains in aqueous solutions of bases.
It was necessary to determine the optimum concentration of sodium chloride for better aggregation of microcrystalline chitosan gel-like dispersion.
Highest swelling parameters were found when the MCChB gel-like dispersion was prepared in a coagulation bath containing 23 wt% sodium chloride. A relatively low degradation rate was also observed (Figure 77).

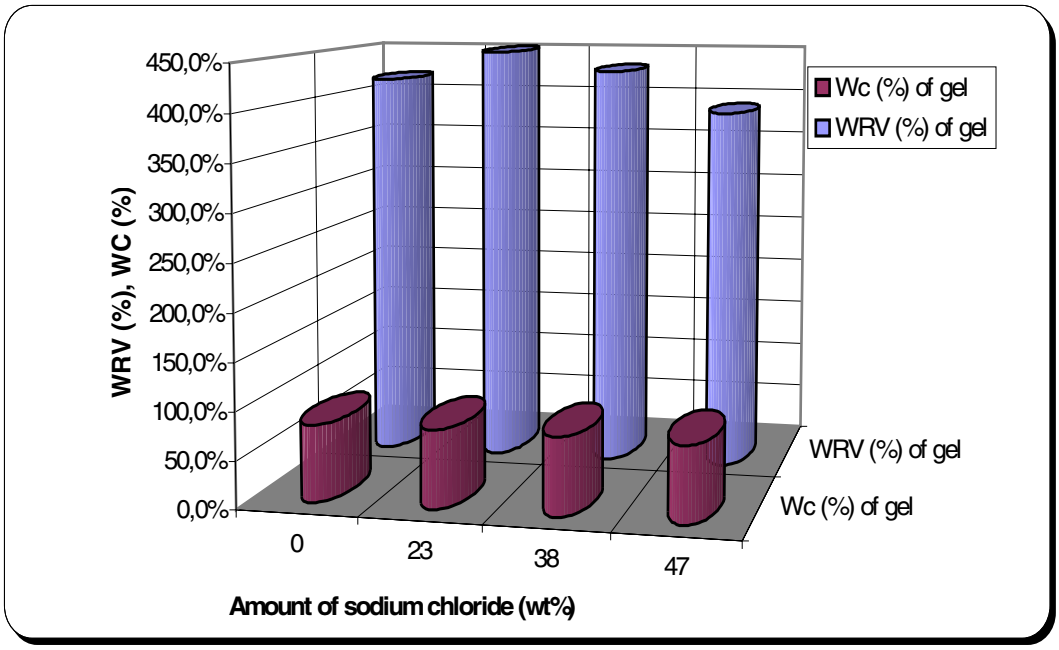



Figure 77. The influence of presence of the sodium chloride in the coagulation bath on the changes in the swelling properties of the microcrystalline chitosan gel-like dispersion produced using MCChB procedure

It may be concluded that application of 23 wt% of NaCl for microcrystalline chitosan preparation protects the chitosan against strong degradation.

4.4. Assessment of some properties of microcrystalline chitosan powder prepared by the ethanol exchange procedure

The idea for these studies was derived from an evaluation of the protein retention in a microcrystalline gel-like dispersion prepared by the MCChA or MCChB method in the presence of proteins (casein or keratin). In addition, the dependence of swelling behaviour on protein content was assessed.

4.4.1. Swelling properties of MCCh powder

MCCh gel-like dispersion was prepared from 1% (w/v) solution of chitosan in 1% (v/v) aqueous acetic acid containing 23 wt% sodium chloride with or without casein.
MCChA and MCChB gel-like dispersion showed a higher WRV in a contrast to the original chitosan. The WRV of gels slowly reduced swelling with the increase in casein content compared to the pure microcrystalline chitosan gel-like dispersion (Table 43).

Table 43. Swelling properties of chitosan powder prepared from microcrystalline chitosan gel-like dispersion on the basis of the MCChA procedure

| Sample | Initial protein content (wt%) | Insoluble particles (wt%) | NaCl content [wt%] | Properties of MCChA gel-like dispersion | | Properties of MCChA powder | |
|---|---|---|---|---|---|---|---|
| | | | | WRV (%) | Wc (%) | WRV (%) | Wc (%) |
| MCChA | 0 | 0 | 23 | 454.5 | 81.8 | 71.0 | 42.0 |
| Ca0.12 | 11 | 1.9 | 23 | 401.2 | 79.9 | 65.0 | 40.0 |
| Ca0.25 | 20 | 2.3 | 23 | 397.2 | 79.8 | 71.0 | 42.0 |
| Ca0.50 | 33 | 3.4 | 23 | 394.5 | 79.8 | 86.0 | 46.0 |
| Ca1.00 | 50 | 3.9 | 23 | 346.3 | 77.6 | 145.0 | 59.0 |
| Ca1.50 | 60 | 4.1 | 23 | 321.1 | 76.4 | 194.0 | 66.0 |
| Ca1.00 | 50 | 3.4 | 38 | 330.6 | 76.8 | 144.0 | 59.0 |

CaX - microcrystalline chitosan gel-like dispersion and powders containing the indicated amount of casein

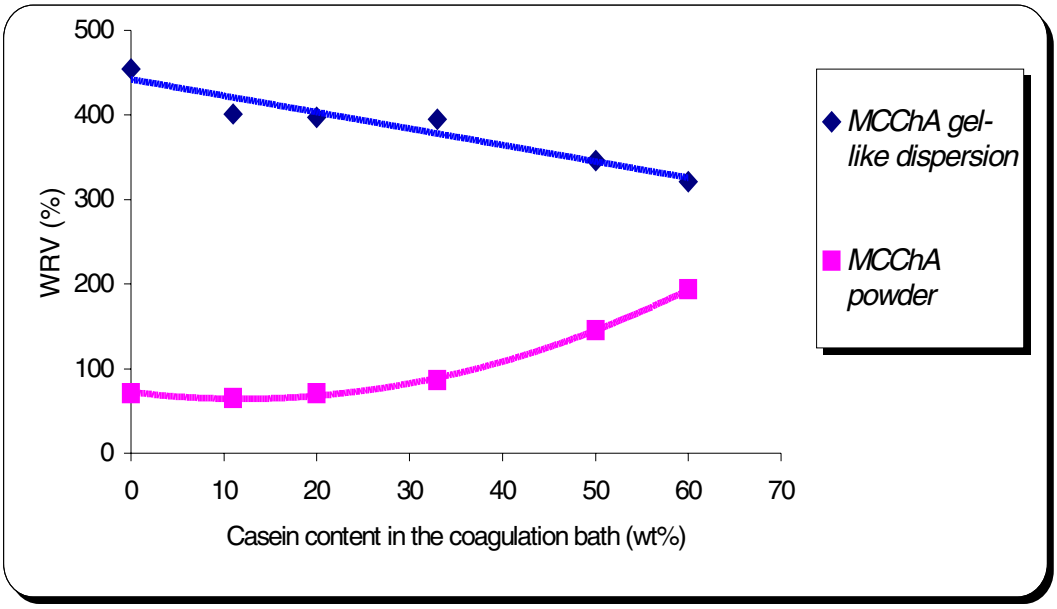



Figure 78. Correlation of the casein content WRV of MCChA gel-like dispersion and MCChA powder

The WRV values increased for MCChA powder containing casein with an initial concentration in the coagulation bath higher than 33 wt%. The same phenomenon was observed for the water content coefficient of microcrystalline gel-like dispersions and formed powders (Figures 78-79.). The acid resistance of microcrystalline chitosan powders modified by proteins was much higher than the MCChA film itself.

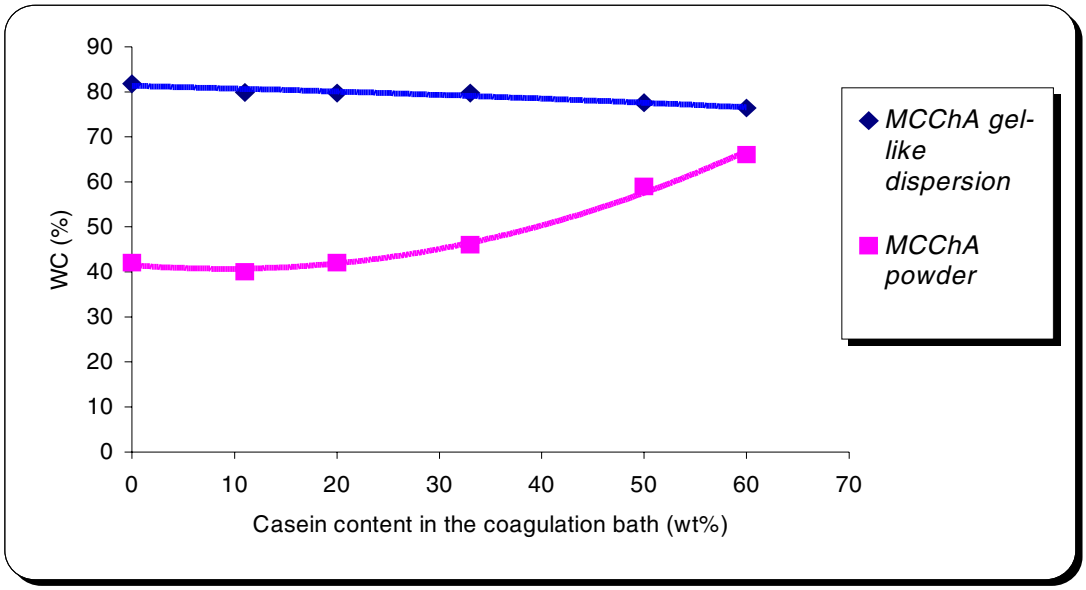



Figure 79. Correlation of casein content Wc of MCChA gel-like dispersion and MCChA powder

MCChA powder was completely soluble in 1% (v/v) aqueous acetic acid, whereas MCChA powder containing casein left insoluble particles, which were soluble in 1% (w/v) aqueous sodium hydroxide solution.

4.4.2. Swelling properties of MCChB powder

Microcrystalline chitosan gel-like dispersions produced according to the MCChB method described before (see Chapter II., p. 3.3) had similar WRV and Wc compared with MCChA gel-like dispersion (Table 44).

Table 44. Swelling properties of microcrystalline chitosan powder prepared on the basis of the MCChB gel-like dispersions

| Sample | Initial protein content (wt%) | Insoluble particles (wt%) | NaCl content (wt%) | Properties of MCChB gel-like dispersion | | Properties of powder formed with ethanol exchanged MCChB | |
|---|---|---|---|---|---|---|---|
| | | | | WRV (%) | Wc (%) | WRV (%) | Wc (%) |
| MCChB | 0 | 0.0 | 0 | 417.8 | 80.7 | 156.4 | 61.0 |
| MCChB | 0 | 0.0 | 23 | 449.3 | 81.8 | 211.8 | 69.3 |
| Ca0.12 | 11 | 7.6 | 23 | 443.5 | 81.6 | 97.2 | 47.1 |
| Ca0.25 | 20 | 19.6 | 23 | 604.6 | 85.1 | 141.4 | 60.6 |
| Ca0.50 | 33 | 28.3 | 23 | 750.1 | 87.7 | 238.7 | 69.6 |
| Ca1.00 | 50 | 43.5 | 23 | 834.0 | 89.1 | 484.2 | 83.4 |
| Ca1.50 | 60 | 51.3 | 23 | 901.2 | 90.2 | 921.6 | 90.5 |
| Kr0.12 | 11 | 6.8 | 23 | 340.6 | 77.3 | 66.3 | 38.0 |
| Kr0.25 | 20 | 15.1 | 23 | 353.9 | 78.0 | 72.6 | 39.9 |
| Kr0.50 | 33 | 21.3 | 23 | 412.2 | 80.5 | 87.3 | 44.4 |
| Kr1.00 | 50 | 30.8 | 23 | 311.7 | 75.7 | 80.2 | 42.2 |
| Kr1.50 | 60 | 36.2 | 23 | 258.4 | 72.1 | 55.6 | 34.7 |

*MCChB – microcrystalline chitosan gel-like dispersion prepared on the basis of the modified method, CaX - modified microcrystalline chitosan gel or powder containing casein, KrX - modified microcrystalline chitosan gel or powder containing keratin.

Addition of sodium chloride to coagulation bath resulted in an increase of both WRV and Wc of the powders. MCChB gel-like dispersion and powders showed a higher WRV and Wc as a function of casein content. The addition of casein changed the properties of MCChB gel-like dispersion. WRV of MCChB gel-like dispersion was at least 3 times and of MCChB powder 4.5 times as compared with MCChA gel or powder (Figure 80.).

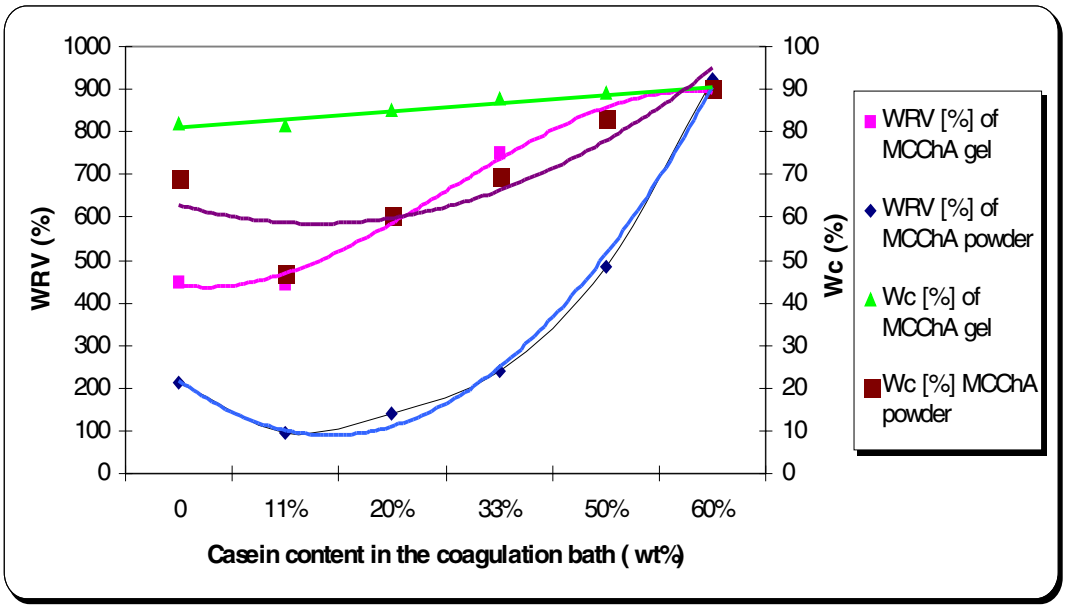



Figure 80. The influence of casein on the values of Wc and WRV of MCChB gel and powder

The globular structure of casein caused an increase in the swelling behaviour of MCChB gels. It was observed in both MCChB gel-like dispersions and powders modified by the addition of the least 23 wt% of casein. The introduction of casein of 11 wt% in the coagulation bath resulted in the reduction of the swelling behaviour of MCChB gel-like dispersion as well as powder. At the same time, the modification by keratin showed less progress of WRV and Wc during the addition of lower amounts of keratin. The WRV of gel prepared in the presence of keratin slowly increased where the content of keratin in the coagulation bath was increased to 33 wt% and then drastically reduced. Further increases in protein content decreased WRV and Wc (Figure 81).

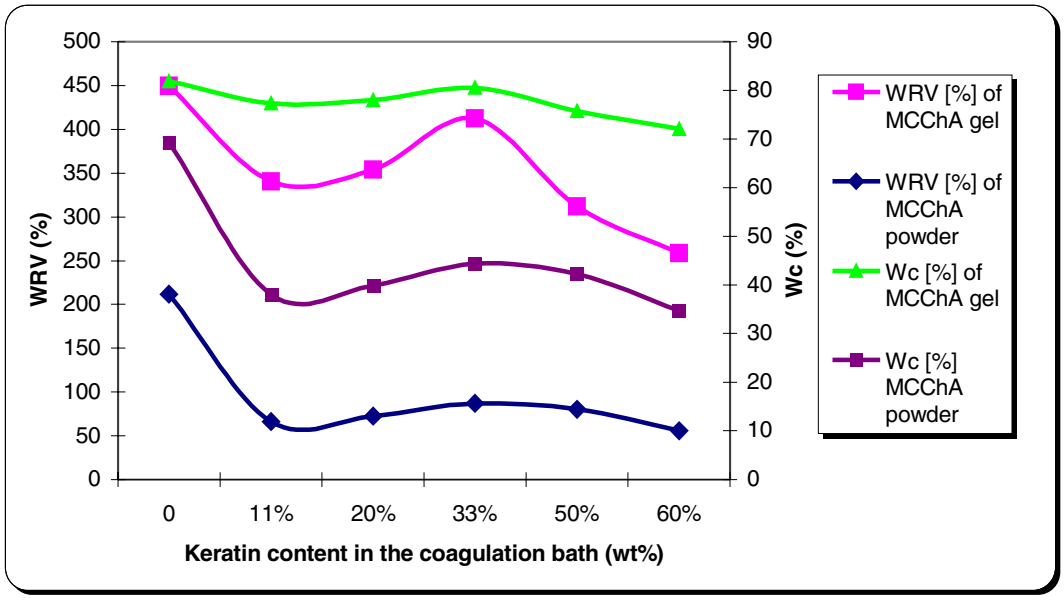



Figure 81. The influence of keratin on the Wc and WRV of MCChB gel and powder

This phenomenon was caused by the presence of the amount of aliphatic compounds, which keratin contains. The supermolecular structure of keratin has an affect on the properties of MCChB gel and powder as was investigated for change in swelling properties. The resulting dependence was observed in the WRV and Wc coefficients determined for dried powder of MCChB with keratin.
The solubility in 1% (v/v) aqueous acetic acid of MCChB powders modified by proteins increased when the protein content decreased.

4.5. Swelling properties of films prepared directly from aqueous MCChB gel-like dispersion

MCChB film prepared from aqueous MCChB gel-like dispersion had similar properties as MCChB powder produced by the ethanol-exchanged process (Table 45, Figure 82).

Table 45. Swelling properties of MCChB-casein gel-like dispersion and films

| Sample | Protein content[a)] (wt%) | Insoluble particles (wt%) | NaCl content (wt%) | Properties of MCChB gel-like dispersion | | Properties of film | |
|---|---|---|---|---|---|---|---|
| | | | | WRV (%) | Wc (%) | WRV (%) | Wc (%) |
| MCChB | 0 | 0 | 0 | 417.8 | 80.7 | 160.2 | 62.0 |
| MCChB | 0 | 0 | 23 | 449.3 | 81.8 | 182.2 | 64.5 |
| Ca0.12 | 11 | 10.3 | 23 | 443.5 | 81.6 | 202.8 | 67.0 |
| Ca0.25 | 20 | 22.1 | 23 | 604.6 | 85.8 | 229.5 | 69.6 |
| Ca0.50 | 33 | 32.4 | 23 | 750.1 | 90.1 | 258.2 | 72.0 |
| Ca1.00 | 50 | 46.5 | 23 | 834.0 | 93.7 | 463.7 | 82.3 |
| Ca1.50 | 60 | 59.0 | 23 | 901.2 | 97.6 | 918.7 | 90.2 |

[a)] – concentration of the casein in coagulation bath, CaX - MCChB gel or powder containing casein,

The films prepared from MCChB gel-like dispersion showed higher WRV and Wc than powders obtained from MCChA gel-like dispersion. However, the increase in WRV for films containing lower amounts of casein lets to the conclusion that better swelling behaviour showed films dried from aqueous dispersion of MCChB (Figure 82).
Drying from the aqueous dispersion caused better swelling properties of MCChB-produced films. Otherwise, the MCChB films prepared from the aqueous suspension were fragile. A high amount of casein than 33 wt% especially disturbed the process of the film formation, therefore it was necessary to add a plasticizer in the form of glycerol.

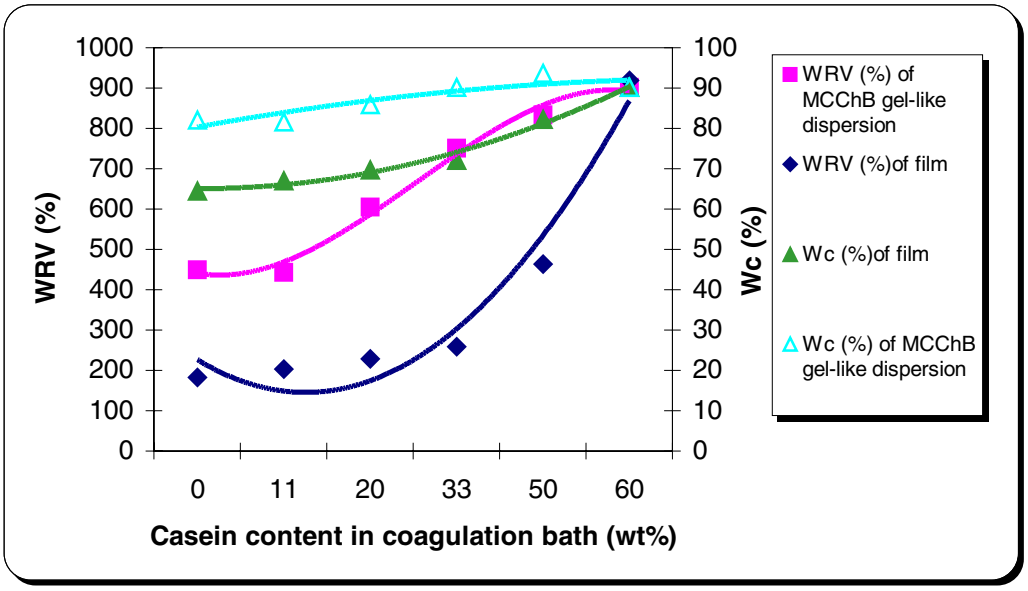



Figure 82. Effect of the forms of MCChB on their water retention values

4.5.1. Evaluation of the optimal MCChB content for film preparation

Chitosan films were formed using aqueous MCChB gel-like dispersion with various polymer contents. Some properties of dried films are presented in Table 46 and Figure 83.

Table 46. Influence of polymer content at initial gel-like dispersion on some properties of formed films.

| Polymer content (wt%) | Film properties | |
|---|---|---|
| | WRV (%) | Wc (%) |
| 0.5 | 132.2 | 58.2 |
| 0.7 | 155.3 | 62.3 |
| 1.4 | 182.2 | 64.0 |

Both the WRV and Wc parameters increased with increase in polymer content in MCChB gel-like dispersion (Figure 83).

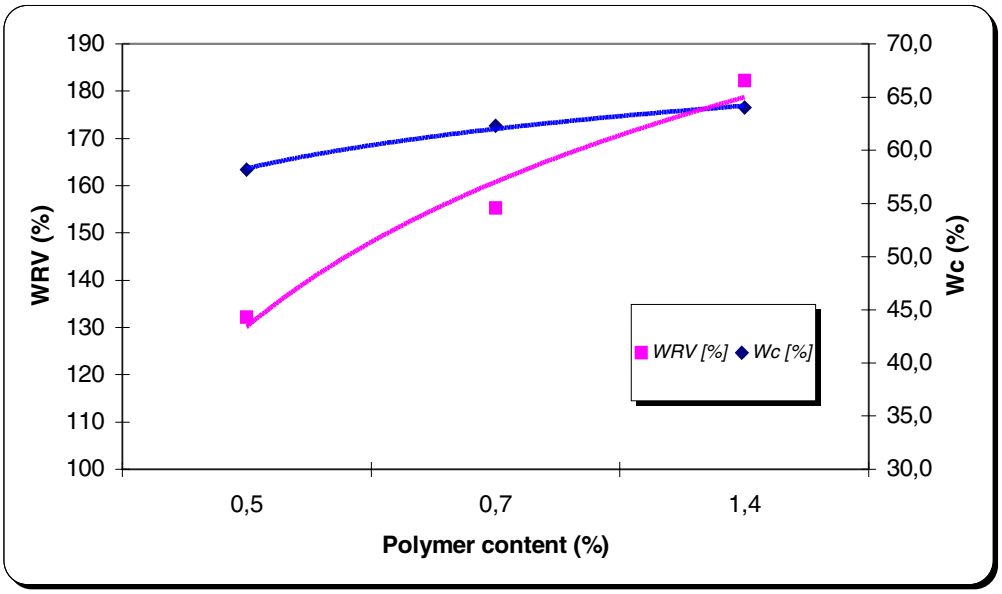



Figure 83. Correlation of polymer content on WRV and Wc of MCChB films

The increase in WRV is most rapid in the range of polymer content from 0.5 wt% to 0.75 wt%. The films using above chitosan content showed considerable fragility and they shrunk during film

formation. The highest WRV and Wc were determined for film prepared from suspension containing 1.4 wt% of chitosan. Moreover, the lowest fragility of this film was obtained. This concentration of MCChB was used for the preparation of MCChB powder and film.

4.5.2. Assessment of the optimal concentration of the plasticizer in MCChB film

The optimal concentration of the plasticizer (glycerol) introduced during MCChB film formation was determined.
The films were formed using a homogenized MCChB gel-like dispersion containing 0.13 g, 0.23 g, 0.41 g or 0.47 g of glycerol and their swelling behaviour was assessed.
Addition of glycerol before MCChB film formation caused reduction in swelling parameters (Table 47, Figure 84). However, the highest values of the above-mentioned parameters were observed for films containing 0.13 wt% of glycerol. However, the addition of glycerol with concentration not higher than 23 wt% yielded shrunk of films.

Table 47. The swelling properties of MCChB films containing various contents of glycerol

| Concentration of glycerol in MCChB film (wt%) | WRV (%) | Wc (%) | Cs (%) |
|---|---|---|---|
| 0 | 182.2 | 64.5 | 211.8 |
| 13 | 181.3 | 64.4 | 207.4 |
| 23 | 173.1 | 63.4 | 187.3 |
| 41 | 154.6 | 61.1 | 185.2 |
| 47 | 143.2 | 58.7 | 168.9 |

The addition of a plasticizer with a concentration of 0.47 wt% caused problems connected with film separation because of their strong adhesion to the PTFE plates.

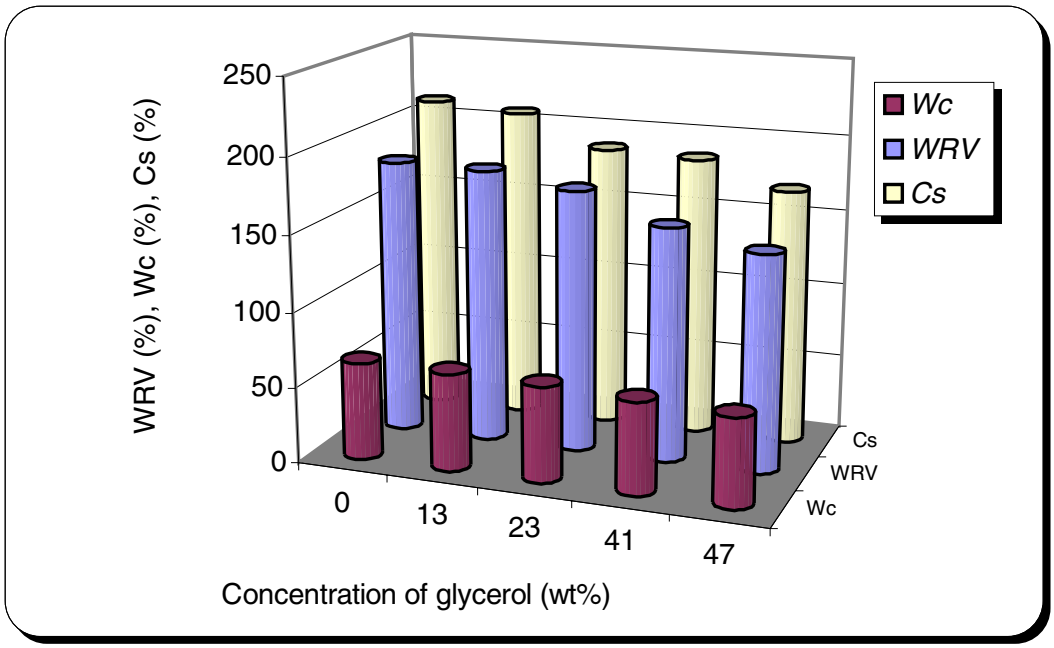



Figure 84. Effect of glycerol content on WRV, Wc and Cs of MCChB films

The above phenomena suggests that the optimal concentration of glycerol in MCChB gel-like dispersion is 41 wt%. The films produced by addition of 41 wt% plasticizer showed good surface form, lack of shrink behaviour, high swelling properties, and significantly low adhesion to the PTFE plates.

4.6. Determination of protein retention in MCChA and MCChB powder

MCChA or MCChB powders were prepared from an acidic solution of chitosan containing 23 wt% sodium chloride and dried according to the ethanol-exchange procedure.

The lowest value of protein retention was obtained for MCCh powders dried prepared from MCChA gel-like dispersion. This observation confirmed the absence of the probability for complexation with chitosan during precipitation by adding aqueous solution of sodium hydroxide with dissolved protein (casein). The low level of casein retention affects the swelling behaviour of MCCh gel-like dispersion (Figure 85, Table 48).

Table 48. Protein retention in MCChA and MCChB powder

| Initial protein content (%wt) | Protein retention (% of MCChACa powder) | Protein retention (% of MCChBCa powder) | Protein retention (% of MCChBKr powder) |
|---|---|---|---|
| 11% | 1.9 | 7.6 | 6.8 |
| 20% | 2.3 | 19.6 | 15.1 |
| 33% | 3.4 | 28.3 | 21.3 |
| 50% | 3.9 | 43.5 | 30.8 |
| 60% | 4.1 | 51.3 | 36.2 |

The application of the MCChB method for the microcrystalline gel-like dispersion preparation affects a of considerable higher protein retention in comparison to the MCChA procedure.

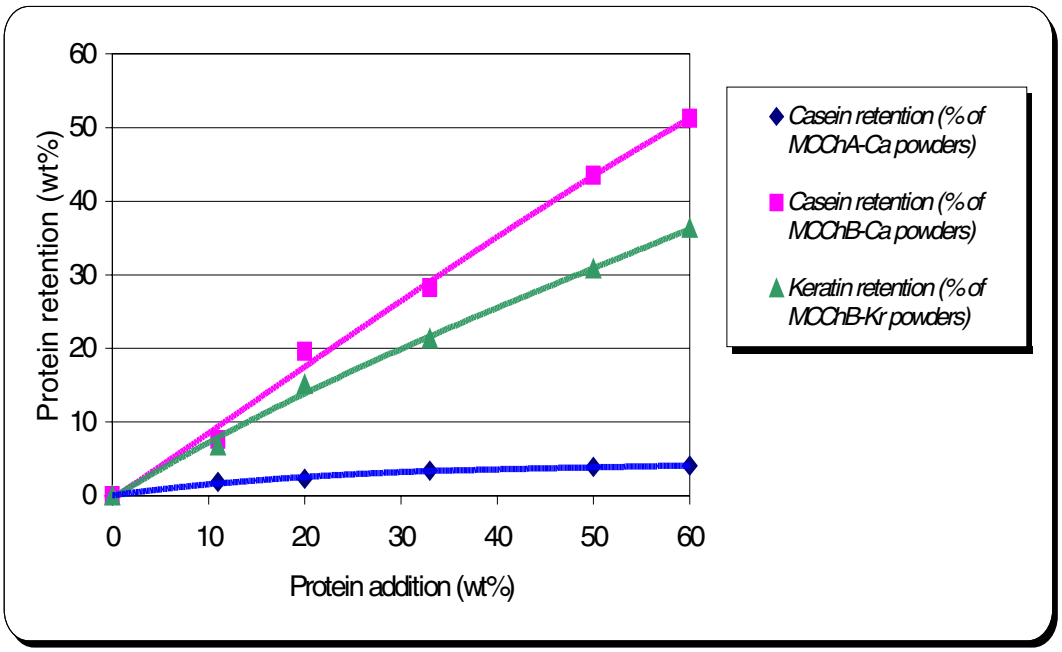



Figure 85. Protein content vs. retention for MCCh powders prepared according to the standard or modified procedure

4.7. Preparation of MCChB-casein/keratin films with addition of glycerol.

The MCChB film-containing proteins (keratin or casein) were plasticized by addition of glycerol according to the method as described in section 3.4.

WRV of chitosan-casein films increased with an increase in the casein content in the coagulation bath (Table 49, Figure 86). However, WRV, Wc and Cs increased in the presence of casein with a concentration higher than 20 wt%. A concentration in 33 wt% of casein in the coagulation bath limited the film-forming behaviour. A reduction in WRV and Wc was observed in all ranges of keratin concentration introduced to the coagulation bath (Table 49, Figure 86.). Films containing keratin possessed a lower value Cs than was noted for films with casein. This fact may be explained by the globular structure of casein and lower amount of aliphatic aminoacids. Films containing this type of protein have an affinity to water, higher as the content of casein increases.

The increase in Cs of MCChB films containing keratin was lower than films with casein. The addition of casein significantly increased the Cs value of MCChB films. The difference in WRV between the casein- and keratin-MCChB films ranged from at least 20% (film prepared in the coagulation bath containing 33 wt% protein) to higher than 100% (film prepared in the coagulation bath containing 60 wt% protein) (Figure 87).

Table 49. Some properties of chitosan-protein films with glycerol (41 wt%)

| Symbol of sample | Protein content[a)] (wt%) | NaCl content (wt%) | Properties of MCChB films | | |
|---|---|---|---|---|---|
| | | | WRV (%) | Cs (%) | Wc (%) |
| MCChB | 0 | 23 | 154.6 | 185.2 | 61.1 |
| Ca0.12 | 11 | 23 | 142.7 | 188.1 | 58.6 |
| Ca0.25 | 20 | 23 | 186.7 | 193.1 | 65.1 |
| Ca0.50 | 33 | 23 | 210.0 | 214.1 | 67.7 |
| Ca1.00* | 50 | 23 | 337.2 | 334.4 | 77.1 |
| Ca1.50* | 60 | 23 | 431.0 | 465.7 | 81.2 |
| Kr0.12 | 11 | 23 | 166.7 | 183.9 | 62.5 |
| Kr0.25 | 20 | 23 | 167.4 | 178.3 | 62.6 |
| Kr0.5 | 33 | 23 | 143.0 | 170.7 | 58.8 |
| Kr1.0 | 50 | 23 | 133.5 | 155.8 | 57.0 |
| Kr1.5 | 60 | 23 | 139.8 | 143.1 | 58.3 |

Ca - MCChB with casein,
Kr - MCChB with keratin,
[a)] - concentration of casein in the coagulation bath,
* - perfect film was not formed

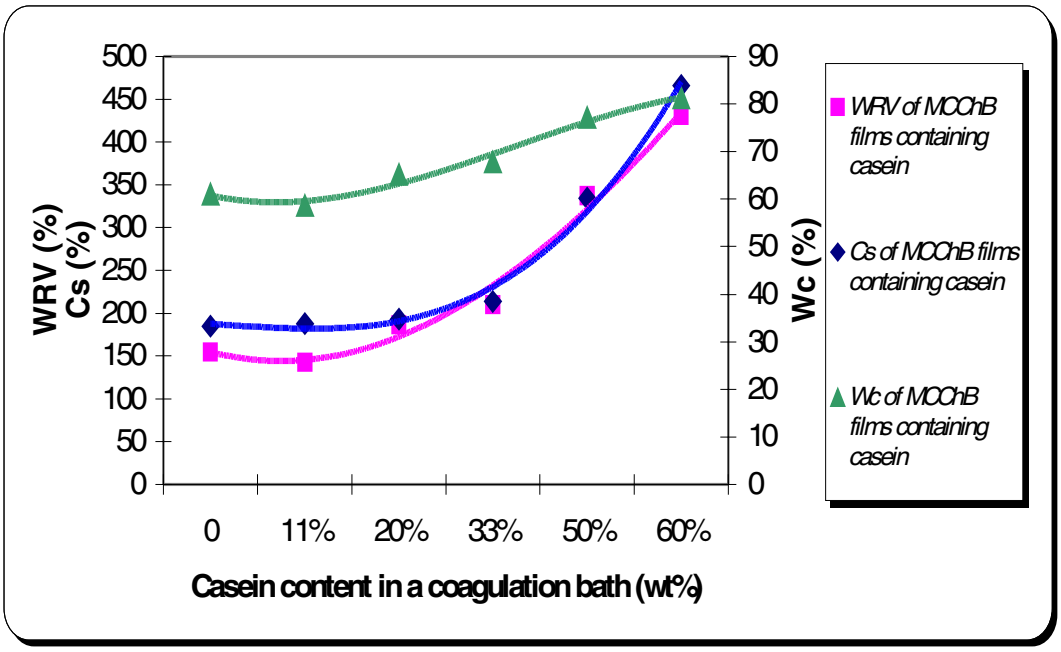



Figure 86. The effect of the addition of glycerol on WRV, Cs, and Wc of MCChB films containing casein

A decreasing tendency was found in films swelling behaviour with the increase in the content of keratin.
However, the introduction of higher amounts of keratin during coagulation caused local increases of WRV/Wc coefficients.
This observation is supported by the keratin-like structure of this protein, containing a high amount of hydrophobic aminoacids.

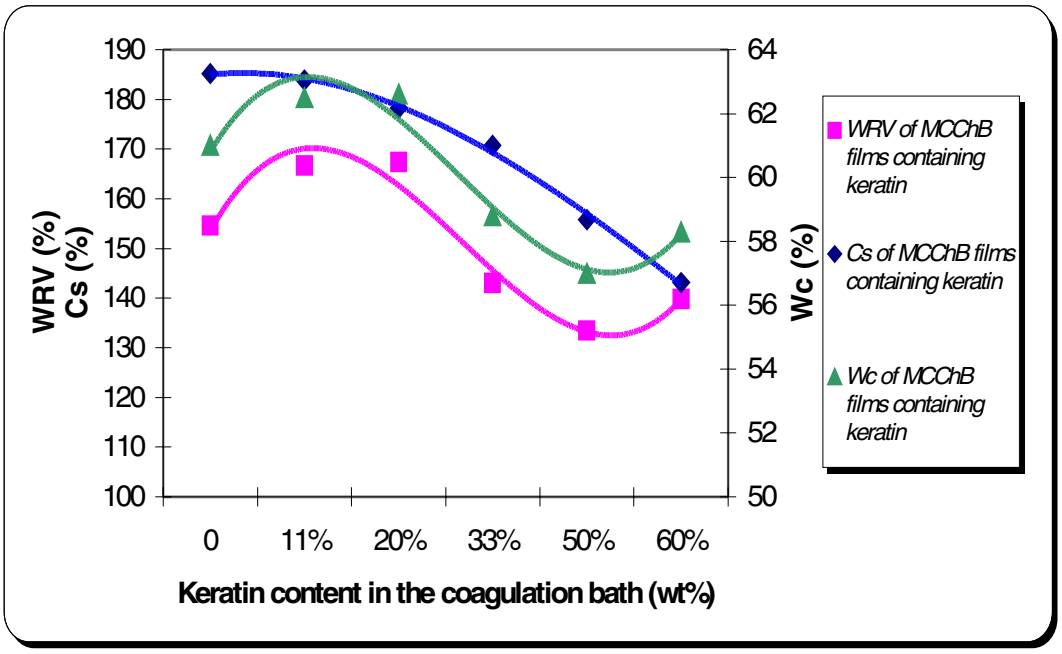



Figure 87. The effect of the addition of glycerol on WRV, Cs and Wc of MCChB films containing keratin

4.7.1. Influence of the presence of sodium chloride during the preparation of MCChB gel-like dispersion on swelling properties of MCChB films

The aim of this stage of the research was to determine the influence of the presence of sodium chloride in the coagulation bath during the preparation of MCChB gel-like dispersions on the swelling behaviour of films.
In the case of MCChB films, the same observation was found as determined for WRV and Wc of MCChB gel-like dispersion. A concentration of sodium chloride higher than 23 wt% limited the increase of WRV, Wc, as well as Cs.
It may be concluded that the swelling behaviour of films resulted from the conditions of the MCChB gel-like dispersion coagulation and the presence of electrolytes.

Table 50. The change in WRV, Wc and Cs of MCChB gel-like dispersion and MCChB film in the presence of sodium chloride

| Amount of NaCl (wt%) | WRV (%) of film | Wc (%) of film | Cs (%) of film |
|---|---|---|---|
| 0 | 154.6 | 61.0 | 185.2 |
| 23 | 211.8 | 69.3 | 233.5 |
| 38 | 118.1 | 54.1 | 176.5 |
| 47 | 111.9 | 52.8 | 177.0 |

The high WRV and Wc of MCChB gel-like dispersions (Tables 41-42) increased their swelling coefficient for films (Table 50, Figure 88).

The optimal concentration of sodium chloride (23 wt%) in the coagulation bath resulted in the lowest degradation (see section 4.2.) with the highest swelling behaviour of MCChB films.

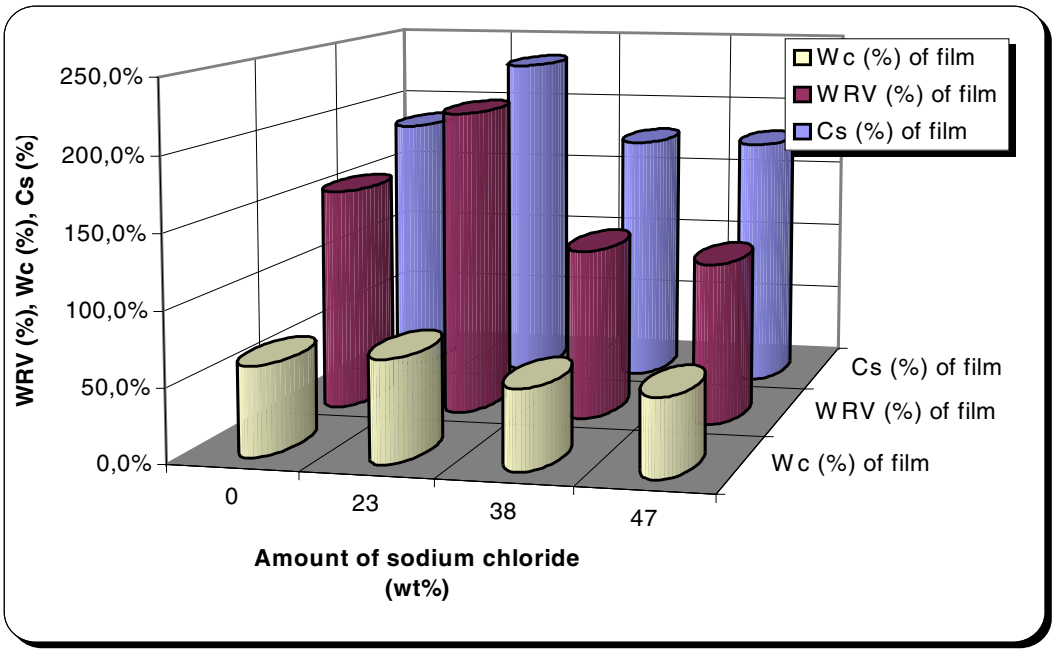



Figure 88. The alterations in WRV, Wc, and Cs for films produced in coagulation baths containing different amounts of sodium chloride

4.8. Mechanical properties of chitosan-protein films

The aim of this study was to compare the mechanical properties of MCChB films with proteins (casein or keratin) in the presence of glycerol with MCChA and MCChB film without protein, as well as to find the optimum concentration of protein resulting in the best mechanical properties of films.

4.8.1. Determination of the mechanical properties of microcrystalline chitosan-casein or chitosan-keratin films

The tensile strength of tested MCChB-casein films increased with the increase in casein concentration in the coagulation bath up to 20 wt%. After that these parameters rapidly decreased (Table 51, Figure 89).

Table 51. Mechanical properties of chitosan-casein/keratin films formed from MCChB gel-like dispersion

| Symbol of sample | Protein content[a)] (wt%) | Tensile strength (MPa) | Breaking force (N) | Elongation at break (%) |
|---|---|---|---|---|
| MCChA | 0 | 3.45 | 4.44 ± 0.61 | 17.6 ± 3.5 |
| MCChB | 0 | 5.45 | 4.53 ± 0.26 | 14.6 ± 3.9 |
| Ca0.12 | 11 | 6.04 | 4.92 ± 1.49 | 35.7± 4.6 |
| Ca0.25 | 20 | 6.39 | 5.15 ± 0.93 | 41.6 ± 9.6 |
| Ca0.50 | 33 | 2.06 | 2.41 ± 1.15 | 48.3 ± 9.0 |
| Kr0.12 | 11 | 5.34 | 5.86 ± 1.49 | 14.3 ± 4.1 |
| Kr0.25 | 20 | 4.50 | 5.52 ± 1.47 | 24.8 ± 3.3 |
| Kr0.50 | 33 | 3.95 | 5.55 ± 1.16 | 31.1 ± 10.5 |
| Kr1.00 | 50 | 3.20 | 5.71 ± 2.79 | 31.6 ± 10.9 |
| Kr1.50 | 60 | 3.08 | 3.35 ± 1.11 | 40.2 ±7.2 |

[a)] – concentration of casein in the coagulation bath

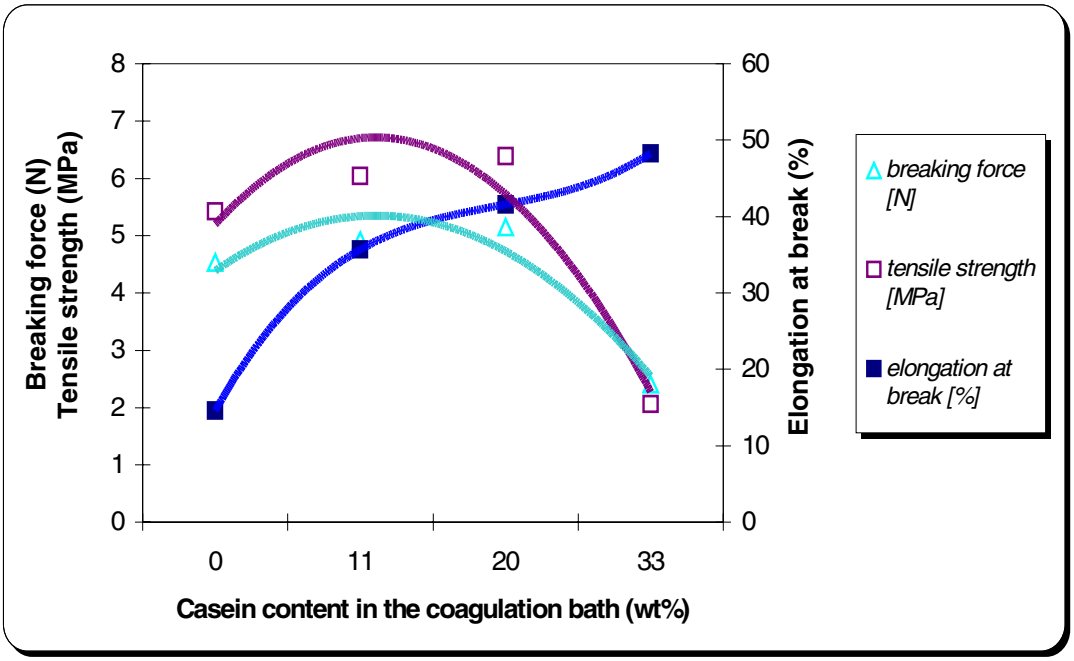



Figure 89. The effect of the casein content in the coagulation bath on the mechanical properties of MCChB film with glycerol

This observation is responsible for the tendency of the WRV to increase for microcrystalline MCChB films prepared in the presence of a high amount of casein, as well as the reduction in the crystallinity regions. The elongation at the break increased with the increase in the casein content.
The relation of the breaking force dependence of MCChB films on the keratin content showed a similar character as was found in the case of MCChB films containing casein (Figure 90). A reduction in applied breaking force was observed for films prepared in the presence of keratin with content higher than 33 wt%. The maximum value of the above parameter (5.9 N) was higher than that obtained for the MCChB films containing casein.

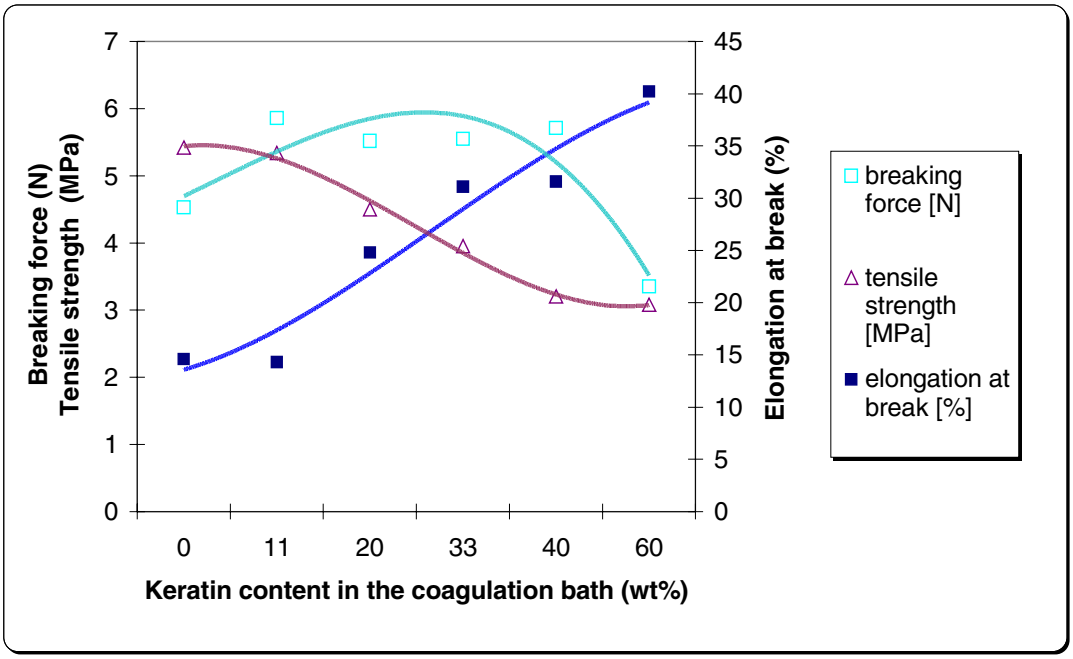



Figure 90. The effect of keratin content in the coagulation bath on the mechanical properties of MCChB film with glycerol

The tensile strength decreased with the increase in keratin content in the modified MCChB films. At the same time, the elongation at break increases with protein content, but this was lower that with MCChB film containing casein. The optimum mechanical properties, such as high value of breaking

force, elongation at break and tensile strength, were found for MCChB films prepared in a coagulation bath containing 20 wt% of casein. At the same time, the most considerable increase in mechanical properties was determined for MCChB films prepared in the presence of 20 wt% keratin.
The method of preparation of microcrystalline gel-like dispersion affected the mechanical parameters of prepared films, which was especially apparent in the case of tensile strength. However, for MCChB films modified by each protein a reduction in the elongation at break parameter was observed.

4.9. Morphological properties of MCChB gel-like dispersion

The purpose of this study was to find the morphological properties of microcrystalline chitosan gel-like dispersion modified by proteins (keratin or casein).

The change in MCChB structure was investigated by:

⇒ FTIR spectroscopy (IR spectra and calculation of energy of hydrogen bonds) of powdered MCChA and MCChB,
⇒ X-ray investigation of powdered MCChB,
⇒ Optical microscopy investigation of MCChA and MCChB gel-like dispersion.

4.9.1. Determination of the energy of hydrogen bonds of chitosan-casein or chitosan-keratin complexes.

The energy of hydrogen bonds was calculated according to the method usually used for cellulose[254]. The peaks were separated within the frequency range of 2700 $cm^{-1}$ - 3800 $cm^{-1}$ using the PeakFit programme. The frequencies of OH stretching of MCChA powders were shifted to a range of wavenumbers that showed intermolecular hydrogen bonds (Figure 91.). The method used for the preparation of the microcrystalline gel-like dispersion affected the supermolecular parameters of the powders. The increase in protein concentration caused the increase in energy of hydrogen bonds of MCChB-casein complex.

Table 52. Influence of the method of microcrystalline gel-like dispersion preparation on the energy of hydrogen bonds in microcrystalline powders

| Sample | Initial casein content[a] (%wt) | Preparation of MCChB powders* | | | | Preparation of MCChA powders* | |
|---|---|---|---|---|---|---|---|
| | | Energy of hydrogen bonds (kJ/mol) | | | | Energy of hydrogen bonds (kJ/mol) | |
| MCCh | 0 | 15.6 | 15.3 | 4.9 | ---- | 14.81 | 14.51 |
| Ca0.12 | 11 | 16.79 | 16.30 | 15.27 | 13.36 | Nd | Nd |
| Ca0.25 | 20 | 16.45 | 14.33 | 13.64 | ---- | Nd | Nd |
| Ca0.5 | 33 | 16.51 | 16.30 | 13.36 | 12.95 | 15.31 | Nd |
| Ca1.0 | 50 | 16.38 | 13.36 | ---- | ---- | 16.08 | 15.72 |
| Ca1.5 | 60 | 16.38 | 13.50 | ---- | ---- | Nd | Nd |
| Original chitosan | -- | 19.21 | 18.42 | 17.85 | 10.25 | -- | -- |

* - IR determination was carried out with microcrystalline powder in a dry form,
[a] – concentration of casein in the coagulation bath,
Nd – IR spectrum was not recorded.

The energy of hydrogen bonds of the powders prepared from MCChA standard procedure with added casein (with concentrations in the coagulation bath ranging from 33 wt% to 50 wt%) was lower than for films formed from MCChB gel-like dispersion (Table 52). The increase in the frequency of the hydrogen bonded hydroxyl groups to the higher wavenumber (increased energy of hydrogen bonds) with the increase in the concentration of keratin was observed. The appearance of new bands corresponding to hydroxyl stretching (inter- as well as intramolecular) was observed in the case of MCChB-keratin IR spectra. This can be explained by the presence of more possibility for intermolecular interactions between polymers (chitosan-chitosan, chitosan-protein and protein-protein). Most of the O-H stretching was distinguished by intermolecular interactions ranging from 3200 $cm^{-1}$-3400 $cm^{-1}$ (Table 53).

Table 53. Energy of hydrogen bonds as well as the frequencies of hydrogen bonds absorption for MCChB powders containing keratin

| Sample | Keratin content[a] (wt%) | Energy of hydrogen bonds (kJ/mol) | | | | | | Frequencies of hydrogen bonded hydroxyl groups ($cm^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MCChB | 0 | 28.2* | 15.6 | 15.3 | 4.9 | --- | --- | 3284* | 3430 | 3437 | 3558 | --- | --- |
| Kr0.12 | 11 | 25.1 | 19.7 | 19.1 | 18.3 | 17.7 | 16.9 | 3300 | 3375 | 3384 | 3395 | 3403 | 3414 |
| Kr0.25 | 20 | 23.2 | 19.0 | 18.3 | 17.7 | 16.9 | 16.5 | 3327 | 3386 | 3394 | 3404 | 3414 | 3420 |
| Kr0.5 | 33 | 24.8 | 19.6 | 19.3 | 18.4 | 17.8 | 13.4 | 3303 | 3377 | 3381 | 3394 | 3402 | 3464 |
| Kr1.0 | 50 | 24.6 | 19.8 | 19.6 | 18.9 | 18.5 | 17.8 | 3307 | 3374 | 3377 | 3386 | 3392 | 3402 |
| Kr1.5 | 60 | 30.8* | 25.2* | 19.9 | 19.6 | 19.1 | 18.4 | 3221* | 3298* | 3372 | 3377 | 3384 | 3394 |
| Keratin | 100 | 30.9* | 25.5* | 19.9 | --- | --- | --- | 3219* | 3294* | 3373 | --- | --- | --- |

* - existence of NH stretching (usually observed at wavenumber of 3270 $cm^{-1}$),
[a] – concentration of casein in the coagulation bath,

The results shown in Tables 52 and 53 lead to the conclusion that the preparation of microcrystalline chitosan with proteins directly from the acidic solution of microcrystalline chitosan by a neutralization and regeneration in mild conditions makes it possible to obtain a product of high crystallinity.

4.9.2. FTIR spectroscopy of microcrystalline chitosan powder containing protein.

4.9.2.1. Chitosan and MCChB powder

The spectrum of microcrystalline chitosan powder shows the characteristic peaks of amide I (C-O) absorption at 1652 $cm^{-1}$, amide II (N-H) at 1560 $cm^{-1}$ and CH stretching band at 2871 $cm^{-1}$and 2921 $cm^{-1}$. Other peaks at wavenumbers near 1150 $cm^{-1}$ and 1050 $cm^{-1}$ are from C-O-C. The red line in Figure 91a shows intensities of the O-H stretching band at 3450 $cm^{-1}$ of chitosan.
MCChB absorption bands at the frequency range from 3600 to 3300 $cm^{-1}$ displayed the broad band of N-H stretches shifts to higher frequencies resulting from intra- and intermolecular hydrogen bonds. There are also resolved peaks at 3284 $cm^{-1}$ describing N-H stretching band (intermolecular hydrogen bonding) superimposed with OH stretching due to the presence of crystalline regions (Figure 91b.).

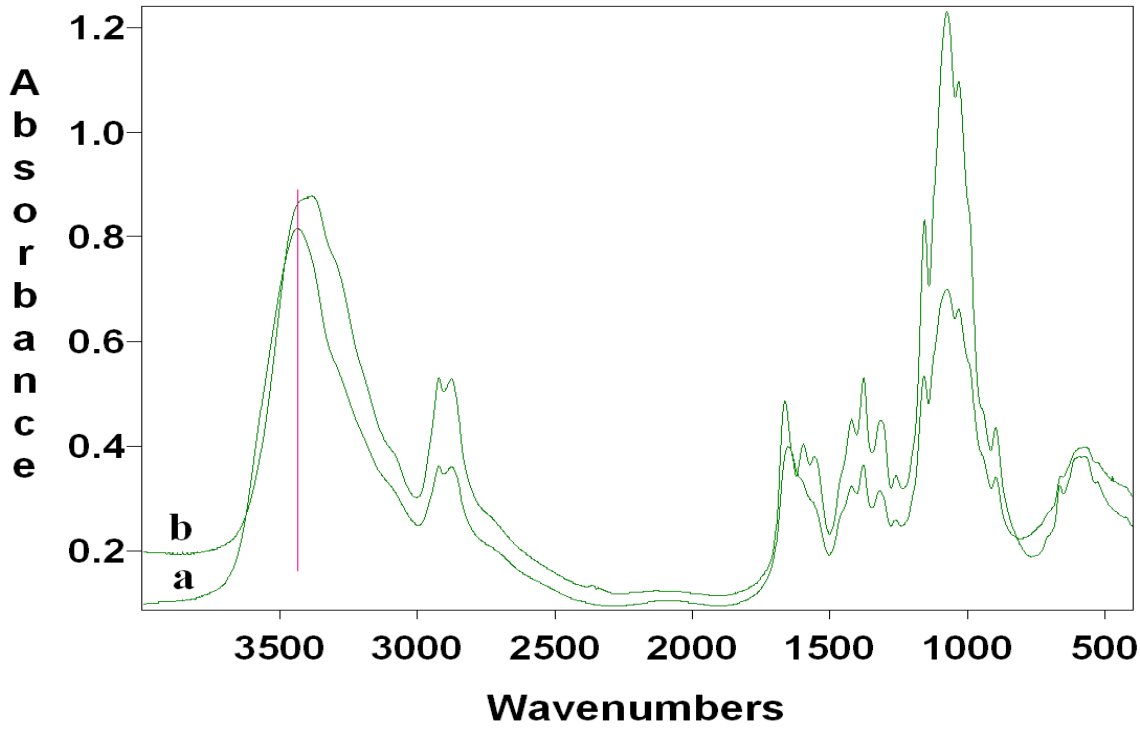



Figure 91a. FTIR spectra of original chitosan P4 (a) and MCChB powder (b)

These wavenumbers are usually described as crystallization-sensitive[258].
Increase in optical density at 1380 $cm^{-1}$ as compared with original chitosan is explained by the rise in crystallinity[259].

The degree of deacetylation of chitosan MCChB powder, calculated on basis of the method as described in Chapter 1., section 4.4. from the ratio of $A_{1650}/A_{3450}$ was 71.7% as compared with that of initial chitosan of 64.8%.

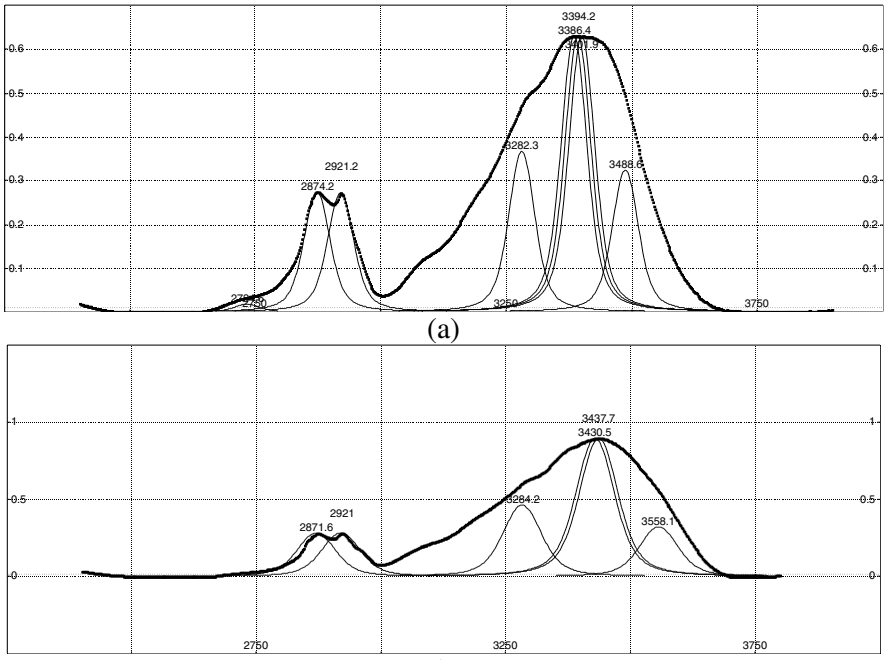



Figure 91b. Separation of FTIR spectra of chitosan (a) and MCChB powder (b) range from 3800 $cm^{-1}$ to 2400 $cm^{-1}$

4.9.2.2. MCChB powder containing casein

FTIR spectra of MCChB powders containing casein (Figure 65a) differed from those of the chitosan by:

⇒ the presence of a peak at the wavenumber of 3290 $cm^{-1}$ responsible for N-H stretch (hydrogen-bonded. symmetric),

⇒ a strong intensity of band at the wavenumber of 1590 $cm^{-1}$ responsible for amide II and at 1650 $cm^{-1}$ resulting from the amide I band,

⇒ several peaks near the amide I band at ca. 1650 $cm^{-1}$ corresponding to the hydrogen bonded amide groups were separated using the PeakFit programme,

⇒ an absence of peak at the wavenumber 1150 $cm^{-1}$ responsible for C-O-C (ether bond),

The separation of IR spectrum of casein range from 1800 – 1200 $cm^{-1}$ is shown in Figure 92.

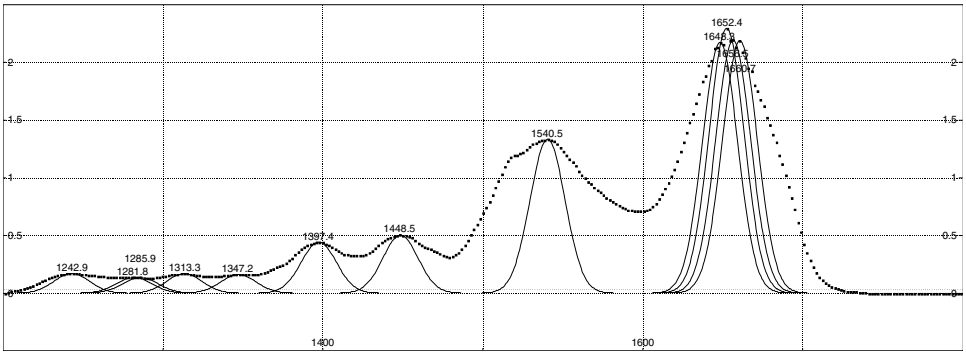



Figure 92. Separation of the casein FTIR spectra range of wavenumber from 1800 $cm^{-1}$ to 1200 $cm^{-1}$

The IR spectra of obtained MCChB powders containing casein are presented in Figure 93.

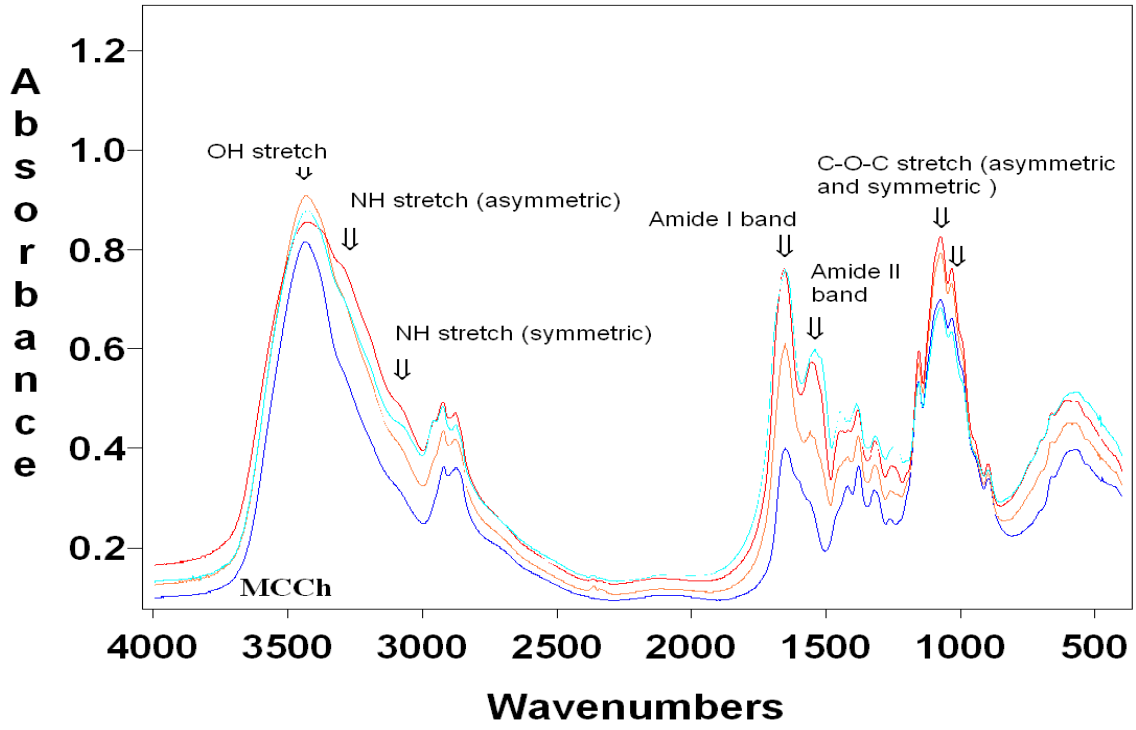



Figure 93. FTIR spectra of MCChB powder (blue line) and MCChB powders containing casein: orange – 33 wt% casein; red – 50 wt% casein; navy – 60 wt% casein; ⇓ indicates the changes in morphology of MCChB powders with the increase in casein amount

The intensity of the bands close to 1150 $cm^{-1}$ and 1050 $cm^{-1}$ as well as at 1650 $cm^{-1}$ decreased with the increase in the casein content in MCChB powders. The absorption of amide II was shifted to the lower wavenumber with the increase in casein content (resolved at 1540 $cm^{-1}$ for MCChB powder without casein and at 1590 $cm^{-1}$ for MCChB powder containing casein with concentration in the coagulation bath higher than 50 wt%). The shift of N-H stretches (symmetric and asymmetric) to a higher wavenumber was observed with an increase in content of casein in MCChB powders.

Table 54. Ratio of amide I to OH stretch and C-H stretch to C-O-C stretching band as well as CH stretch to C-O-C stretch

| Symbol of sample | Amide I/O-H stretch (%) | Amide I/C-O-C stretch | CH stretch/C-O-C stretch |
|---|---|---|---|
| Ca0.12 | 40.3 | 0.68 | --- |
| Ca0.25 | 41.3 | 0.74 | --- |
| Ca0.5 | 45.0 | 1.37 | --- |
| Ca1.0 | 46.7 | 1.58 | --- |
| Ca1.5 | 48.4 | 2.19 | --- |
| Kr0.12 | 22.9 | 0.28 | 0.29 |
| Kr0.25 | 25.4 | 0.28 | 0.30 |
| Kr0.5 | 32.3 | 0.33 | 0.33 |
| Kr1.0 | 34.9 | 0.37 | 0.38 |
| Kr1.5 | 35.7 | 0.36 | 0.39 |

The ratio of amide I band to O-H stretch successively increased with the increase in casein content (Table 54.). This fact confirms that during coagulation of MCChB gel-like dispersion the sorption of casein takes place.

4.9.2.2. MCChB powder containing keratin

The IR spectrum of keratin showed:

⇒ the presence of the peak at 1233 $cm^{-1}$ probably corresponding to the aliphatic amines (primary or secondary) (Figure 65b).

⇒ the strong intensities in the range of 1450 $cm^{-1}$– 1300 $cm^{-1}$ result from the CH band: at 1390 $cm^{-1}$ corresponding to the symmetrical vibration of $CH_3$ band, at 1452 $cm^{-1}$- asymmetrical vibration of $CH_3$ band.

⇒ the strong peaks at 1640 $cm^{-1}$ resulted from amide I (overlap: C=O stretch - amide I band at 1640 $cm^{-1}$and N-H band at 1640 $cm^{-1}$). Several peaks near the amide I band (at wavenumbers of 1610 $cm^{-1}$; 1641 $cm^{-1}$; 1645 $cm^{-1}$; 1652 $cm^{-1}$ as well as at 1684 $cm^{-1}$) were separated using the PeakFit programme. In addition, the N-H bending of secondary amines absorbs near 1620 $cm^{-1}$ - 1560 $cm^{-1}$.

⇒ the shift of carbonyl groups absorption to a higher wavenumber possessed the frequency of free carboxyl group (usually absorbed at 1760 $cm^{-1}$) describing the esters of amino acids (usually absorbed in a range of 1750 $cm^{-1}$-1735 $cm^{-1}$).

⇒ the weak group of peaks at the wavenumber of 2337 $cm^{-1}$ - 2360 $cm^{-1}$ corresponded to the frequencies of amino acids containing sulphur (S-H stretching). The decrease to the lower wavenumbers can be explained by the presence of interactions between C=S as well as C-N stretching or some hydrogen interactions.

⇒ the presence of methyl groups resulted in strong absorption in a range of 2960 $cm^{-1}$ - 2850 $cm^{-1}$, much stronger than in the case of the MCChB IR spectrum. Absorption at 2853 $cm^{-1}$ corresponded to the symmetric stretching of $CH_2$, at 2874 $cm^{-1}$ to the symmetric stretching of $CH_3$, and at 2925 $cm^{-1}$ and 2960 $cm^{-1}$ to the asymmetric stretching of $CH_2$ or $CH_3$.

⇒ strong intensity at 3071 $cm^{-1}$ and at 3218 $cm^{-1}$ resulting from the N-H stretch of primary hydrogen bonded amide (asymmetric and symmetric) superimposed on an OH stretching band. The absence of C-O-C stretch in comparison to the MCChB IR spectrum was observed.

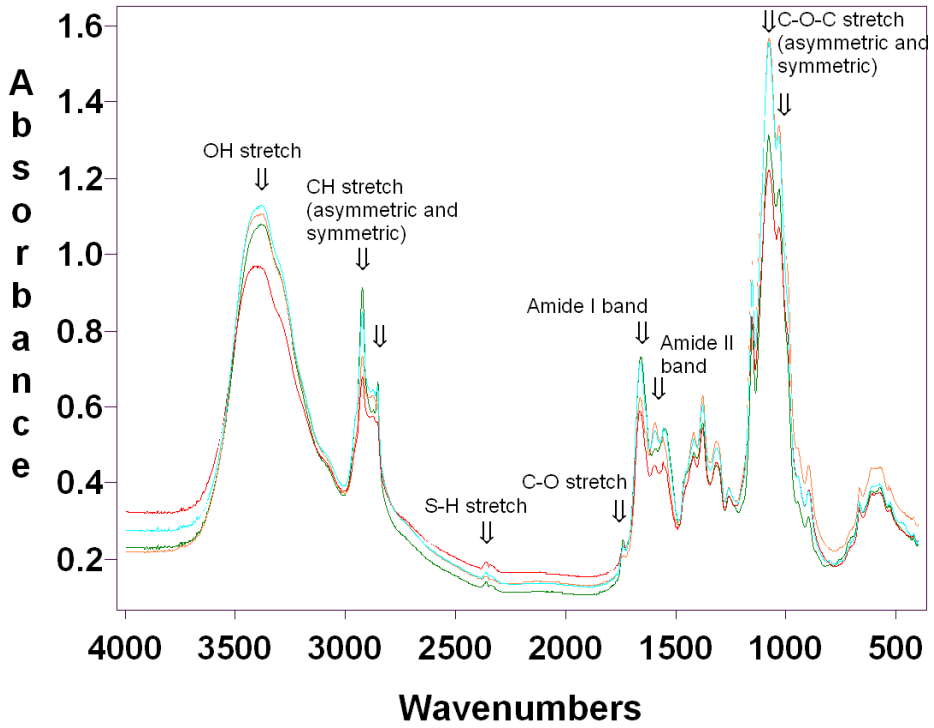



Figure 94. FTIR spectra of MCChB powders containing keratin: red – 20 wt% keratin, orange – 33 wt%, navy – 50 wt%, green – 60 wt%; ⇓ indicates the changes in morphology of MCChB powders with the increased in keratin content

Increase in keratin content in MCChB powders caused the increase in aliphatic compounds (increase in intensity of CH stretches). The absorption corresponding to symmetric vibration of $CH_3$ band becomes much stronger with the increase in keratin content. At the same time, the intensities of asymmetric vibration of $CH_3$ and $CH_2$ bands lowered. The absorption at 1380 $cm^{-1}$ increased as was compared to FTIR spectrum of MCChB powder. The band at 1590 $cm^{-1}$, resulting from the amide II, is also resolved. The intensities of peaks at 1735 $cm^{-1}$ as well as at 2350 $cm^{-1}$ increased with increasing

of keratin content. N-H stretch was shifted to a lower wavenumber because of being superimposed by an O-H band (Figure 94).
The IR spectra of MCChB-keratin powder in the range from 700 $cm^{-1}$ to 1200 $cm^{-1}$, did not present significant differences. An increase in absorption was observed at the frequency of 1378 $cm^{-1}$ corresponding to the crystalline regions. The background separation using PeakFit resolved the intensity increases of peaks at 1550 $cm^{-1}$ (this band decreased to lower wavenumber with the addition of keratin), at 1745 $cm^{-1}$ and at 2350 $cm^{-1}$ with increase in keratin content. The increase in absorption at this frequency was proportional to the increase in keratin concentration. At the same time, the peak at 1590 $cm^{-1}$, resulting from $NH_2$ bending of amide II, was decreased. An increase in keratin content resulted in the appearance of new peaks at 1610 $cm^{-1}$, 1630 $cm^{-1}$ as well as 1680 $cm^{-1}$ - 1690 $cm^{-1}$. The increase in intensity was found at the frequency of 1740 $cm^{-1}$ - 1730 $cm^{-1}$. These observations show the potentiality of several hydrogen intra- and interactions among chitosan chains and protein molecules. The decrease in the band at 1590 $cm^{-1}$ as well as the decrease to lower frequencies with the increase in protein content was caused by the creation of several interactions of amine groups of chitosan and protein. The increase in the absorption was also observed for aliphatic C-H stretching (especially for $CH_2$ stretches) close to 2900 $cm^{-1}$ (Figure 95.). The region resulting from superimposed O-H and NH stretching bands at 3100- 3300 $cm^{-1}$ was shifted to higher wavenumber.

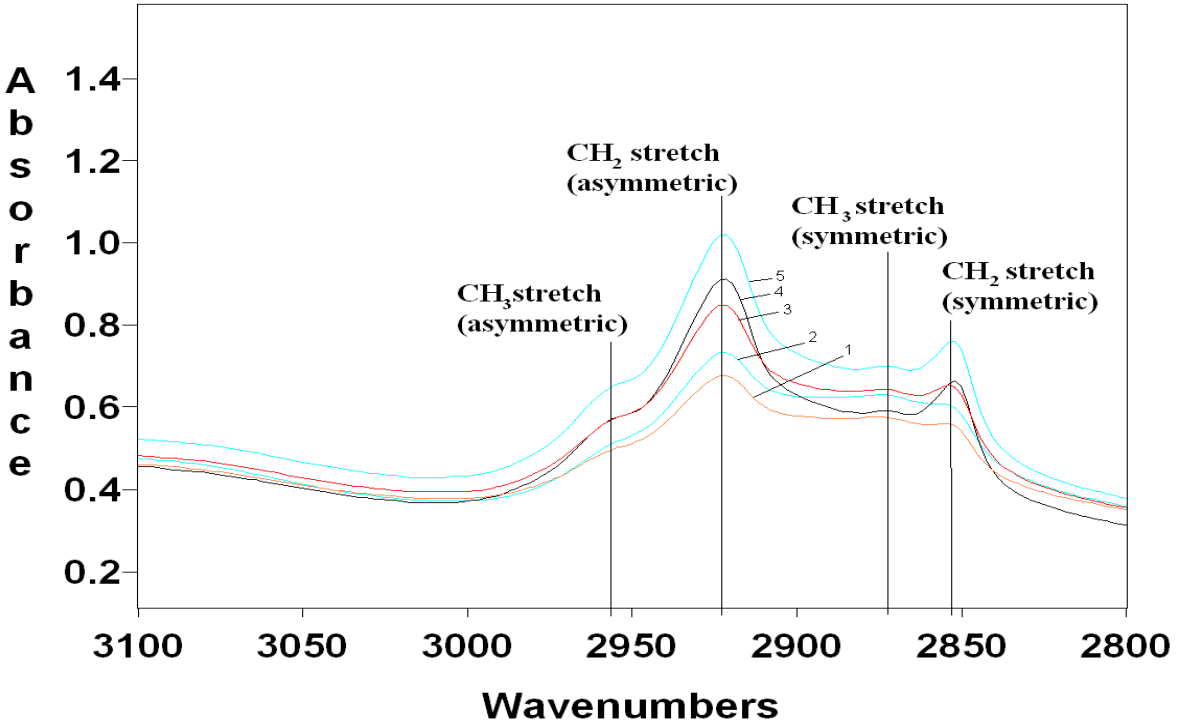



Figure 95. Spectra of MCCh-keratin powder (range 2700 $cm^{-1}$ - 3000 $cm^{-1}$): 1 – MCChB; 2 – MCChB in bath containing 20 wt% keratin; 3 – MCChB in bath containing 33 wt% keratin; 4 - MCChB in bath containing 50 wt% keratin; 5 - MCChB in bath containing 60 wt% keratin;

4.9.3. Assessment of crystallinity index

The films and powder of MCChB were prepared according to the procedures as described in section 3.2. and 3.2.1. using chitosan P4 transformed to a MCChB gel-like dispersion and dried directly from an aqueous solution without (MCChB powder) or with (MCChB films) the presence of glycerol.
The highest value of crystallinity index (CrI) was obtained for MCChB powder containing the lowest amount of casein. For powdered MCChB prepared in the presence of 20 wt% casein the crystallinity index was drastically reduced. Excellent film-forming behaviour was observed for MCChB in the presence of glycerol on the basis of a product coagulated in a bath containing casein with a concentration lower than 33 wt%. It was detected that film-forming was correlated with the decrease in crystallinity, when the concentration of casein higher than 33 wt%. It can be concluded that the concentrations of casein lower than 33 wt% limit the film-forming behaviour as well the increase in CrI.
This observation can be explained by the lower crystallinity regions with increases in casein content.

The high content of casein may influence the orientation of the polymer chains. The globular structure of casein can influence the formation of barriers between the chains of chitosan responsible for formation of the film (Table 55, Figures 96-97.).

Table 55. Crystallinity of MCCh powders dried from aqueous gel-like dispersion and MCCh films prepared in the presence of glycerol

| Protein content[a)] (wt%) | Crystallinity index (%) of MCChB forms | | | |
|---|---|---|---|---|
| | Powder[1] containing casein | Film[2] containing casein | Powder[1] containing keratin | Film[2] containing keratin |
| 0 | 28.9 | 34.7 | 28.9 | 34.7 |
| 11 | 38.1 | 39.4 | 34.5 | 42.1 |
| 20 | 34.4 | 24.9 | 35.0 | 45.3 |
| 33 | 14.6 | 14.0 | 34.3 | 42.6 |
| 40 | 12.1 | (*) | 30.4 | 34.0 |
| 60 | 11.9 | (*) | 25.2 | 21.7 |

[1] – prepared from aqueous gel-like dispersion,
[2] – prepared from aqueous gel-like dispersion in the presence of glycerol,
[a)] – concentration of casein in the coagulation bath, * - film was not formed.

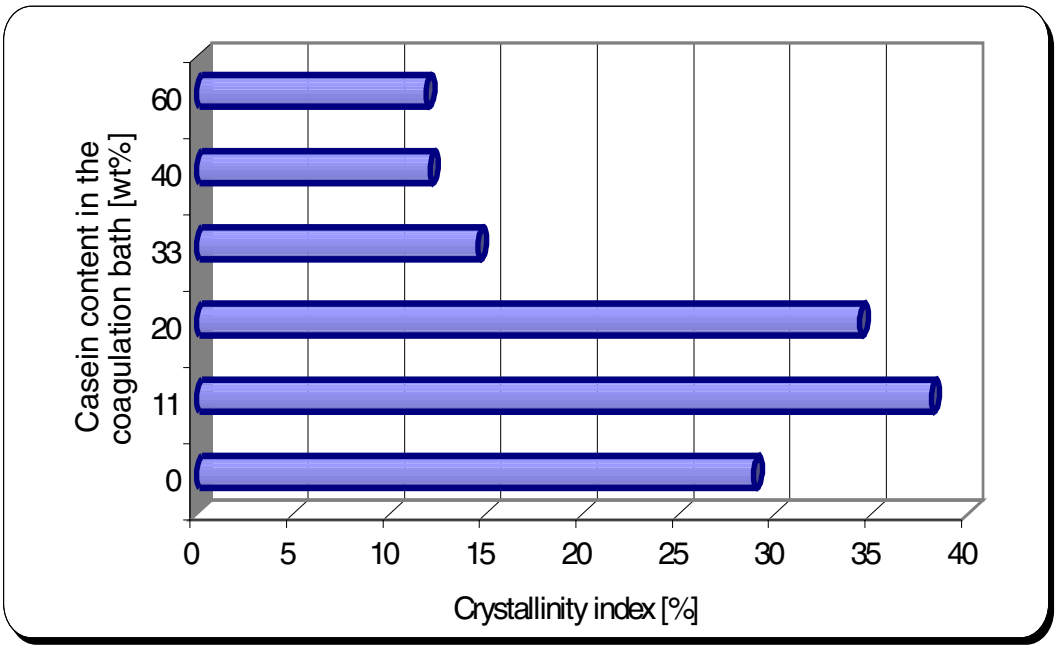



Figure 96. The effect of casein content in the coagulation on the crystallinity index of MCChB powder

For better visualization of the effect of CrI on the mechanical properties of MCChB films, the change in the crystallinity is discussed with its mechanical properties.
The tensile strength was dependent on the value of the crystallinity index (Figure 97). The highest values of mentioned parameter was obtained for casein content in the coagulation bath lower than 20 wt%. A similar fact was noted in the relation of the crystallinity index of MCChB powder as shown on Figure 96. Further increase of the casein content in the coagulation bath caused a reduction in the above parameter, except for elongation at break.
Keratin introduction during coagulation did not strongly influence the crystallinity index, as was in the case with the presence of casein. However, the CrI were considerately higher, compared with the unmodified MCChB films.
The decrease in CrI was stronger for MCChB films with glycerol than in the case of the MCCh-Kr powders. A maximum of crystallinity was observed for films and powders prepared in coagulation baths containing keratin with a concentration of 20 wt% (Figures 98-98).

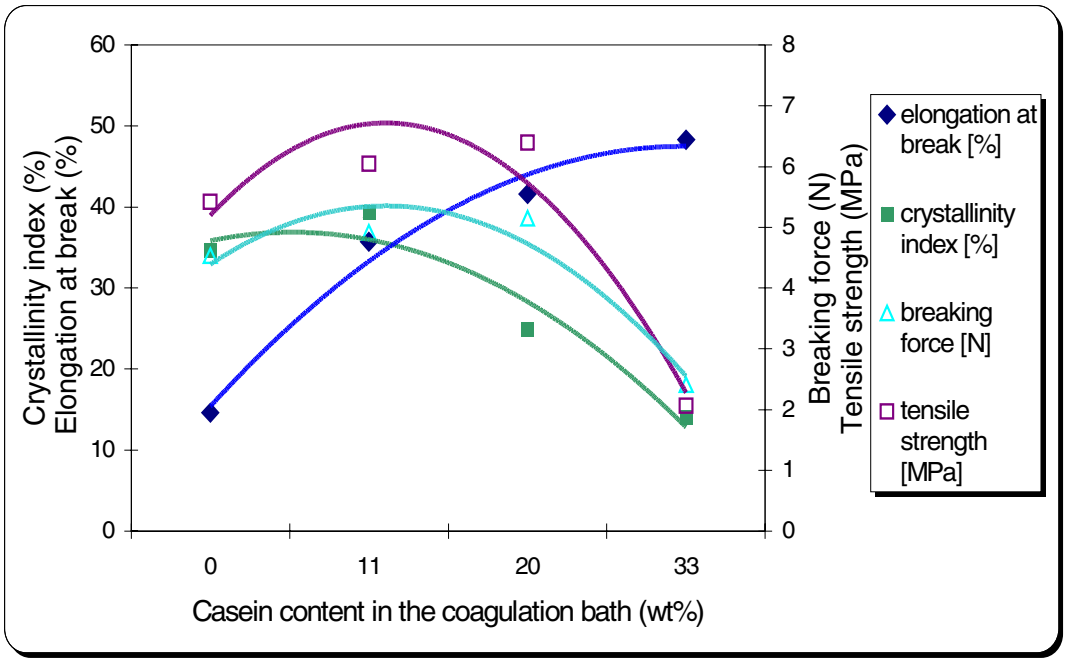



Figure 97. The effect of the amount of casein in the coagulation bath on the index of crystallinity and mechanical properties of MCChB film

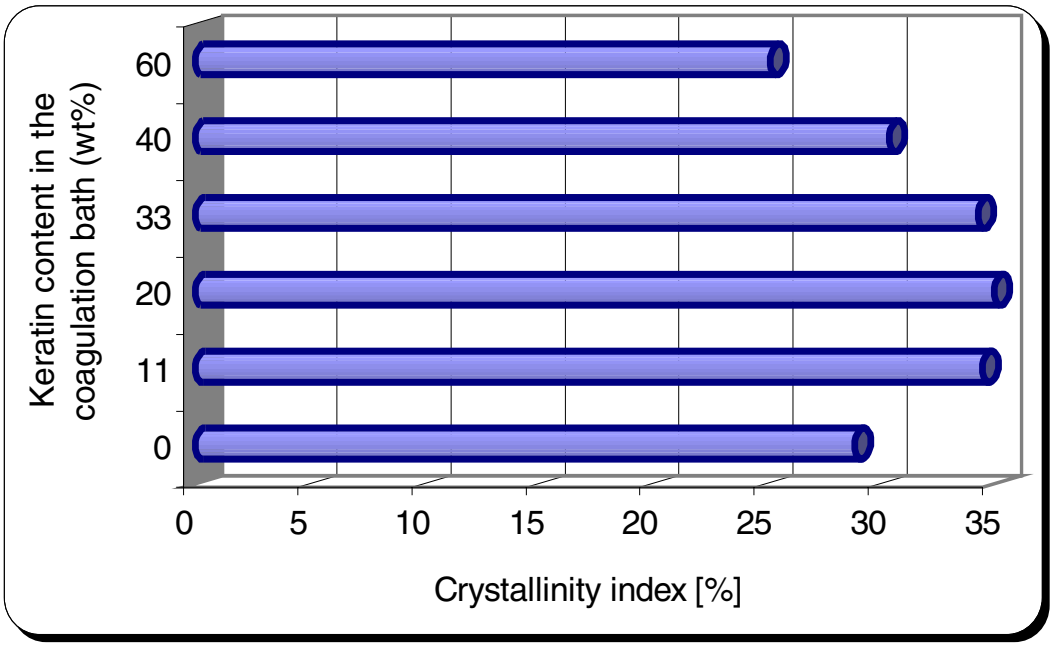



Figure 98. The effect of keratin content in the coagulation bath on the crystallinity index of MCChB powder

The presence of glycerol during film preparation significantly increased the crystallinity index for MCChB-Kr films, in contrast with MCChB powder containing keratin. In the case of the alteration of mechanical properties of the MCCh-keratin films, the same relationship was observed as for MCChB film containing casein (Table 55.).

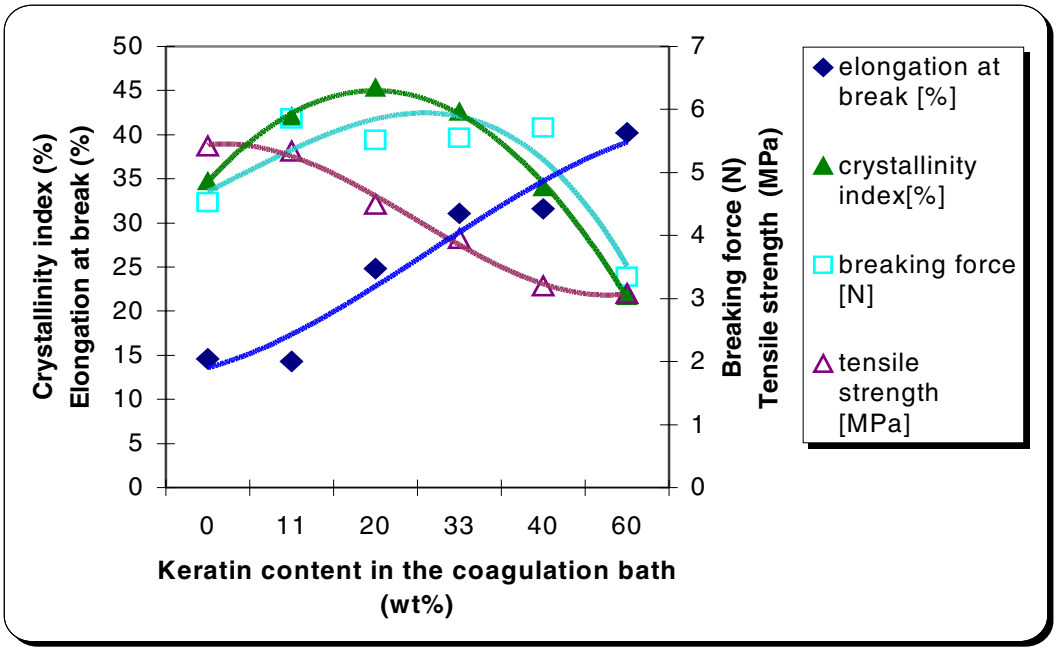



Figure 99. The effect of presence of keratin in the coagulation bath on the crystallinity index and mechanical properties of MCChB films

4.9.4. Optical microscope investigations of water-swollen microcrystalline chitosan aggregates

Microcrystalline chitosan prepared by MCChA or MCChB method was examined by using the optical microscopy. The microphotographs show the aggregates in the aqueous dispersion of gel-like dispersions (Figures 100-104).

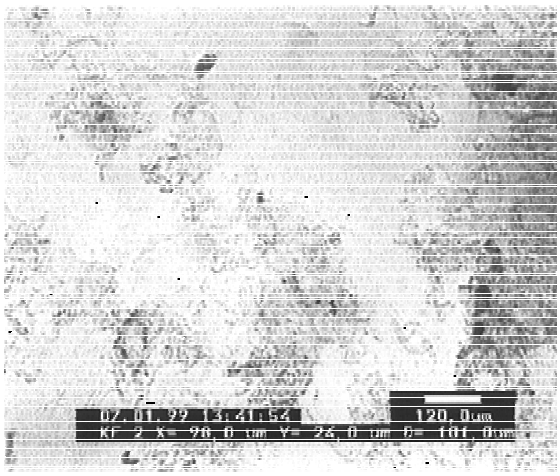


Figure 100. Optical microscope microphotographs of MCChA gel-like dispersion prepared on from chitosan solution containing 23 wt% sodium chloride (x100)

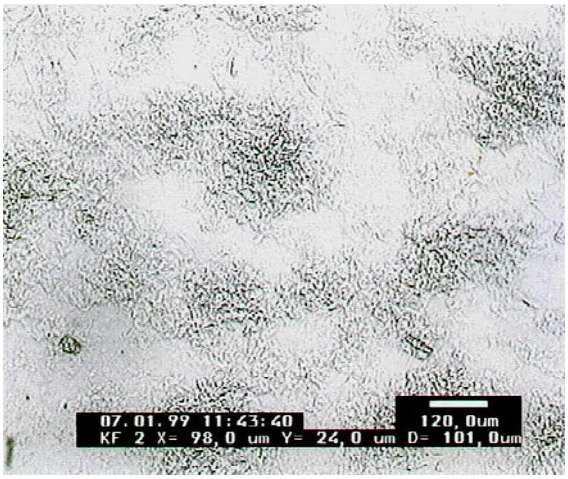



Figure 101. Optical microscope microphotographs of MCChB gel-like dispersion prepared from chitosan solution containing 23 wt% sodium chloride (x100)

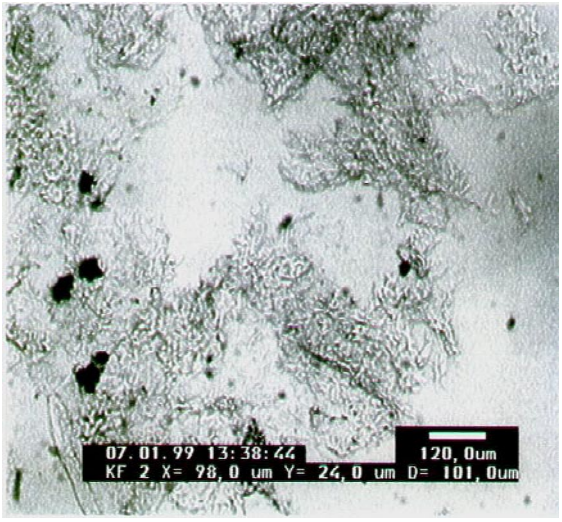



Figure 102. Optical microscope microphotographs of MCChB gel-like dispersion prepared from chitosan solution containing 38 wt% g sodium chloride (x100)

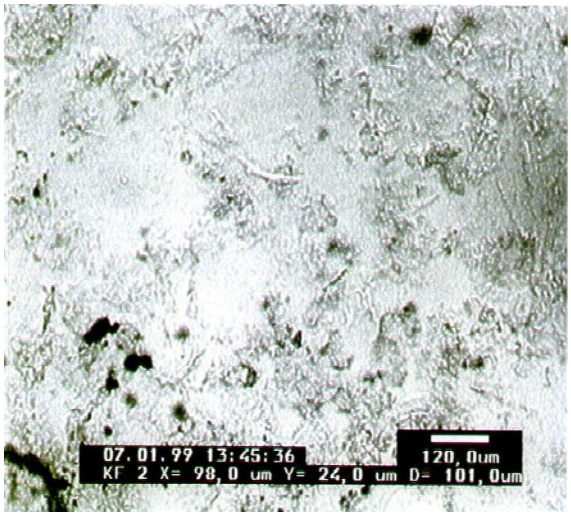



Figure 103. Optical microscope microphotographs of MCChB gel-like dispersion prepared in coagulation bath containing 20 wt% casein (x100)

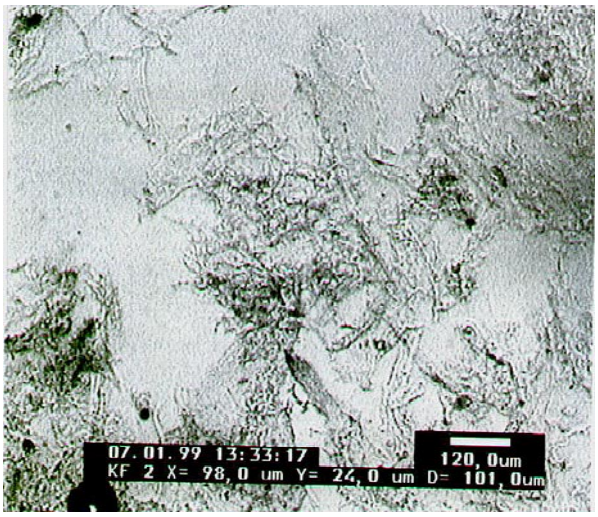



Figure 104. Optical microscope microphotographs of MCChB gel-like dispersion prepared in coagulation bath containing 20 wt% keratin (x100)

The aggregation of microcrystalline dispersion increased with the increasing in NaCl concentration up to 23 wt% in the coagulation bath. This also depends on the method of preparation of the microcrystalline gel-like dispersion (Figures 100-102.). The presence of sodium chloride with a concentration of 23 wt% in the coagulation bath caused the formation of fine aggregates of MCChB gel-like dispersion (Figure 101). At the same time, the MCChA procedure formed much higher aggregates (Figure 100). The presence of protein in the coagulation bath during the preparation of MCChB gel-like dispersion affected the structure of aggregates, increasing the accumulation of microcrystalline particles (Figures 103-104.)

4.10. Preparation of MCChB films containing proteins

Optimal conditions of MCChB film preparation were found as described below:

⇒ concentration of sodium chloride of 23 wt% in coagulation bath,
⇒ concentration of proteins in the coagulation bath between 20 wt% and 33 wt%,
⇒ polymer content of 1.4 wt%, glycerol content of 1wt%.

The aim of this research was to find the relationship among the mechanical properties, swelling parameters, degree of deacetylation, $\overline{M}_v$, as well as origin of chitosan.

The determination of WRV, Wc, and Cs were carried out according to the method as described in section 2.1., 2.2. and 2.3. WRV and Wc describe the susceptibility of product (MCChB gel-like dispersion or a formed film) to swelling of water, reflecting at the same time an increase in the products internal surface. Swelling coefficient (Cs) characterizes the hydrophility behaviour of formed films. The determination of DD as well as $\overline{M}_v$ of MCChB powders was described in section 2.11. and 2.12.

4.10.1. Determination of the changes in $\overline{M}_v$ and DD of chitosan in the MCChB powder

A reduction in $\overline{M}_v$ was noted for powdered MCChB compared to the initial chitosan. Moreover, the most extensive degradation effect was observed for chitosan showing the highest molecular weight (G3mA- reduction of 27%; PB08 - 23%; PB10 - 22%) (Table 56, Figure 105).

Table 56. Some properties of chitosan and MCChB powder

| Symbol of sample | Original chitosans $M_V$ [a] | WRV [b] (%) | DD (%)[c] by titration | Ash Content [%] | MCChB powder $M_V$ [a] ($x10^{-5}$) | WRV [b] (%) | DD (%)[c] by titration |
|---|---|---|---|---|---|---|---|
| G3mA | 264,000 | 153.9 | 66.3 | 1.2 | 194,000 | 512.3 | 73.9 |
| M3A | 187,000 | 171.1 | 73.5 | 4.5 | 168,000 | 439.7 | 79.2 |
| G3mB | 135,000 | 99.7 | 81.8 | 1.1 | 125,000 | 405.6 | 82.6 |
| M3mB | 143,000 | 99.8 | 90.5 | 1.1 | 131,000 | 407.0 | 90.5 |
| M1mC | 111,000 | 135.9 | 85.6 | 1.1 | 97,000 | 413.6 | 86.7 |
| M6mC | 64,000 | 88.7 | 92.6 | 1.1 | 60,000 | 391.6 | 93.9 |
| PB8 | 248,000 | 65.2 | 89.9 | 2.6 | 191,000 | 445.6 | 88.6 |
| PB10 | 227,000 | 56.8 | 98.4 | 2.5 | 178,000 | 428.2 | 98.0 |
| MCCh I-3/1/A | 237,000 | 74.5 | 97.6 | 0 | 186,000 | 460.0 | 97.0 |
| P4 | 130,000 | 79.5 | 69.6 | 0.6 | 123,000 | 449.3 | 71.7 |

[a] determined by viscometry. [b] determined gravimetrically, [c] DD determined according to the method as described in section 2.11. [227]

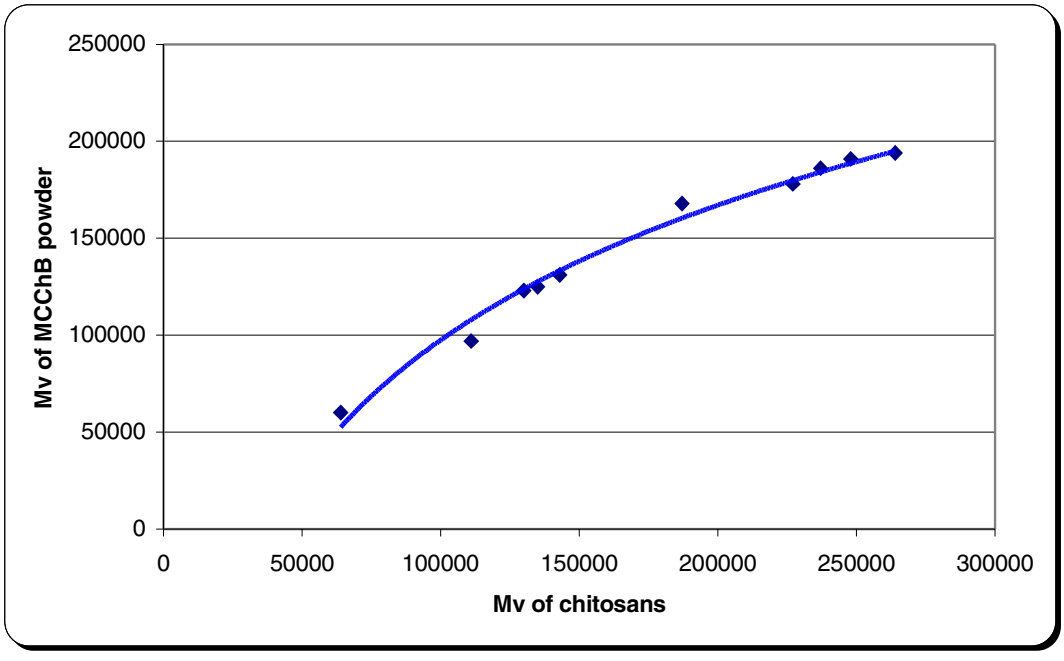



Figure 105. Correlation of $\overline{M}_v$ of chitosan and MCChB powder

An increase in DD was found for the samples possessed the relatively lowest DD (Figure 106). The above observation can be explained by the removal of the insoluble particles after dissolution of chitosan in aqueous acetic acid. During filtration of the solution, the insoluble particles, indicated significantly lower DD, were removed. The increase in DD was found for the MCChB showed the strong reduction in $\bar{M}_v$ (G3mA – $\bar{M}_v$ reduction of 27%, increase in DD of 10.3%; M3A - reduction in $\bar{M}_v$ of 6.5%, increase in DD of 7.2%). This observation was not noted for chitosans with the highest DD.

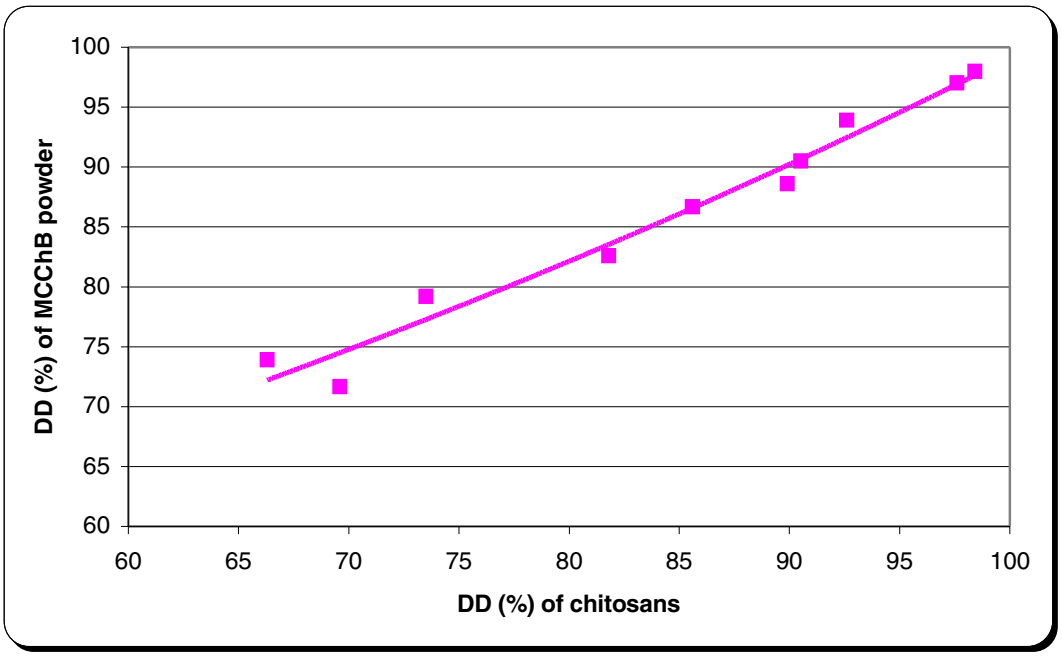



Figure 106. Correlation of the degree of deacetylation of chitosan and MCChB

The degradation of the MCChB gel-like dispersion while it was coagulated in pH ~ 10.0 as well as removal of the insoluble chitosan fractions yielded an increase in DD and decrease in $\bar{M}_v$.
The MCChB powders possessed 3-4 times higher WRV in contrast with the initial polymer. WRV was increased with the increase in $\bar{M}_v$ and decrease in DD. The formation the fibrous-like forms of MCChB was obtained for chitosan G3mA and M3A, which possessed relatively low DD and high $\bar{M}_v$. Processing of MCChB gel-like dispersion significantly affected the molecular structure of polymer. During the aggregation of chains as well as coagulation of polymer, a product showed better swelling behaviour (retention of water) and hydrophility character was obtained.

4.10.2. Determination of swelling parameters of MCChB gel-like dispersion and MCChB films

The dependence of swelling properties of MCChB gel-like dispersion and formed films on $\bar{M}_v$ and DD of prepared MCChB was studied below.

Table 57. The swelling parameters of MCChB gel-like dispersion and MCChB film

| Sample | MCChB gel-like dispersion | | MCChB films | | |
|---|---|---|---|---|---|
| | WRV (%) | Wc (%) | WRV (%) | Cs (%) | Wc (%) |
| G3mA | 439.7 | 81.2 | 217.8 | 238.5 | 68.5 |
| M3A | 479.0 | 83.7 | 139.4 | 147.9 | 58.2 |
| G3mB | 406.6 | 80.3 | 130.2 | 168.6 | 56.6 |
| M3mB | 407.0 | 80.3 | 67.7 | 160.1 | 39.2 |
| M1mC | 413.6 | 80.5 | 101.4 | 115.4 | 50.3 |
| M6mC | 391.6 | 79.6 | 97.7 | 126.1 | 49.4 |
| PB8 | 445.6 | 81.7 | 168.4 | 196.7 | 67.6 |
| PB10 | 428.2 | 81.1 | 165.9 | 241.6 | 62.4 |
| MCCh I-3/1/A | 460.0 | 82.4 | 166.7 | 267.6 | 62.7 |

A decrease in WRV and Wc of MCChB gel-like dispersion when decrease in $\overline{M}_v$ and DD was found (Table 57, Figures 107-108). The highest values of the above parameters were obtained for MCChB gel-like dispersion derived from a high-molecular chitosan with relatively low DD.
The WRV of MCChB films significantly depended on $\overline{M}_v$. It was lowered when reductions in $\overline{M}_v$ and increases in DD was observed (Figure 109). The reduction in $\overline{M}_v$ of MCChB indicated a decrease in Wc. At the same time, Wc of MCChB films decreased with the increase in DD (Figure 110).
The swelling coefficient (Cs) decreased with the reduction in $\overline{M}_v$ as well as DD. The highest values of Cs were found for MCChB films having relatively high $\overline{M}_v$ (ranging from 150,000 Da to 190,000 Da) and high DD (Figure 111).
The above observation leads to the conclusion that the change in swelling properties of MCChB gel-like dispersion and films depended in a significant extent on $\overline{M}_v$ of MCChB. At the same time, the dependence of it on DD was not noted.

### 4.10.3. Determination of swelling parameters of MCChB gel-like dispersion and MCChB films containing casein

MCChB gel-like dispersion prepared in a coagulation bath containing 20 wt% or 33 wt% casein as well as formed films were used for determination of the influence of proteins addition on swelling properties.

Table 58. The swelling coefficients of MCChB gel-like dispersion and MCChB films prepared in presence of 20 wt% of casein

| Symbol of sample | MCChB gel-like dispersion | | MCChB films | | |
|---|---|---|---|---|---|
| | WRV (%) | Wc (%) | WRV (%) | Cs (%) | Wc (%) |
| G3mA | 472.0 | 83.6 | 218.8 | 238.0 | 68.6 |
| M3A | 512.1 | 83.7 | 180.0 | 189.1 | 64.5 |
| G3mB | 508.6 | 83.6 | 197.7 | 196.1 | 66.4 |
| M3mB | 480.0 | 82.7 | 178.8 | 175.0 | 64.1 |
| M1mC | 515.9 | 83.9 | 137.8 | 149.5 | 57.9 |
| M6mC | 604.7 | 85.8 | 137.8 | 179.2 | 57.9 |
| PB8 | 471.6 | 82.5 | 194.2 | 255.1 | 66.0 |
| PB10 | 434.0 | 81.3 | 174.3 | 188.2 | 63.5 |
| MCCh I-3/1/A | 552.0 | 84.7 | 185.4 | 189.8 | 64.7 |

The addition of 20 wt% of casein during the preparation of MCChB gel-like dispersion resulted in the effect of $\overline{M}_v$ and DD on the swelling behaviour of the product. WRV and Wc for MCChB gel-like dispersion indicated an increase in WRV and Wc in comparison to gel-like dispersions without protein (Figures 112-113.).
WRV and water content coefficients were lowered with a decrease in $\overline{M}_v$ of both MCChB gel-like dispersion and films. The increase in Wc and WRV values for MCChB gel-like dispersion aggregated from low $\overline{M}_v$ of MCChB with high DD were noted. WRV of formed films indicated the highest level for high $\overline{M}_v$ of the original MCChB. The decrease in WRV and Wc with the increase in DD was noted (Table 58, Figure 114).
The highest value of Wc was found for MCChB films prepared with relatively high $\overline{M}_v$ of MCChB with low DD. The change in Wc was identical to that obtained for WRV describing this type of MCChB film (Figure 115).
The high $\overline{M}_v$ of MCChB at low and medium DD resulted in the highest swelling coefficient of MCChB-casein films. For highly-deacetylated MCChB, the swelling coefficient was reduced (Figure 116).
A strong increase in WRV and Wc was observed for MCChB gel-like dispersions microcrystalline chitosan showing fibre-like consistency, which was produced from insect. Nevertheless, similar values of swelling parameters of MCChB were noted in comparison to samples with similar properties.

The increase in casein content changed remarkable the properties of MCChB gels. The addition of 33 wt% of casein in a coagulation bath notably increased WRV and Wc of MCChB gel-like dispersion (Table 59).

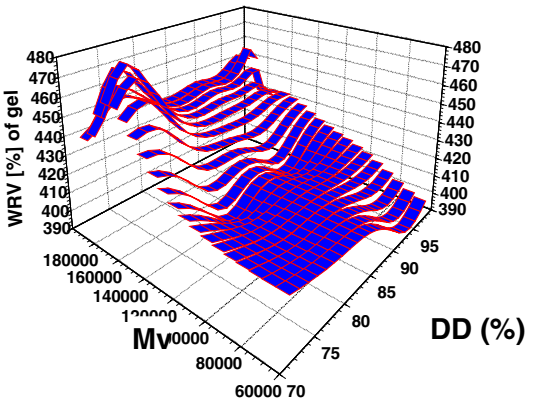



Figure 107. The effect of DD and $\bar{M}_v$ weight on WRV of MCChB gel-like dispersion

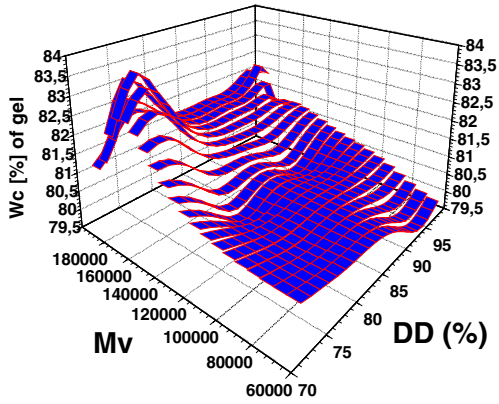



Figure 108. The effect of DD and $\bar{M}_v$ on Wc of MCChB gel-like dispersion

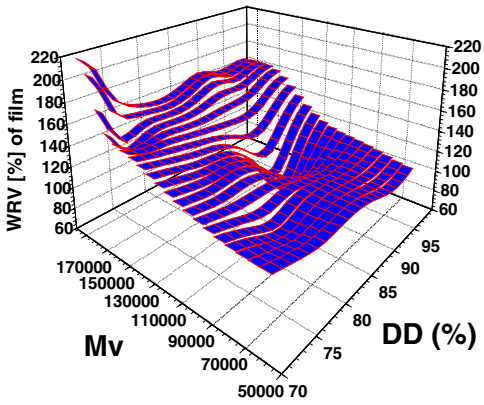



Figure 109. The effect of DD and $\bar{M}_v$ on WRV of MCChB films

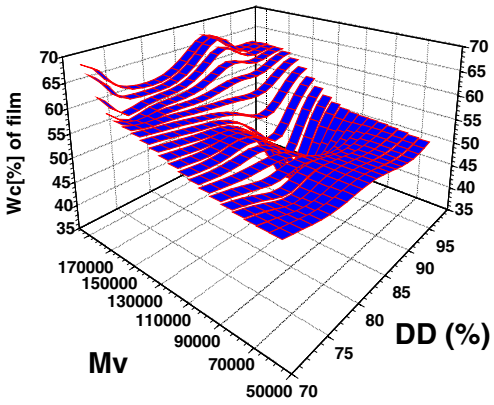



Figure 110. The effect of DD and $\bar{M}_v$ on Wc of MCChB films

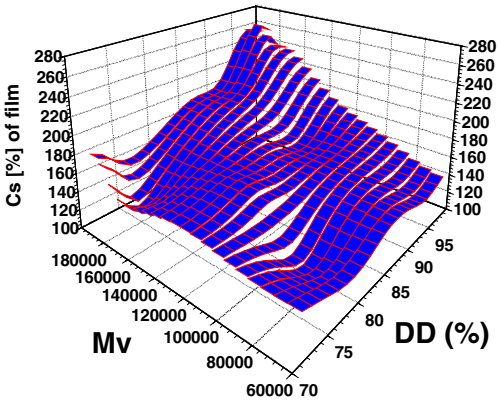



Figure 111. The effect of DD and $\bar{M}_v$ on Cs of MCChB films

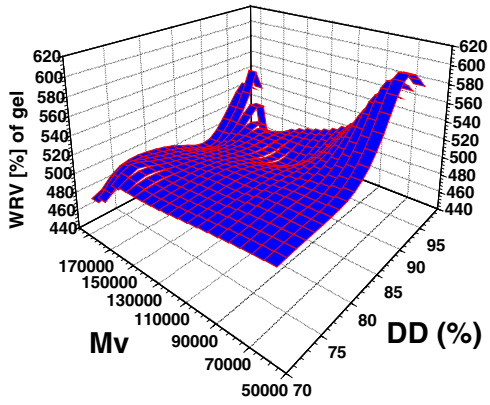



Figure 112. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB gel-like dispersion prepared in presence of 20 wt% casein

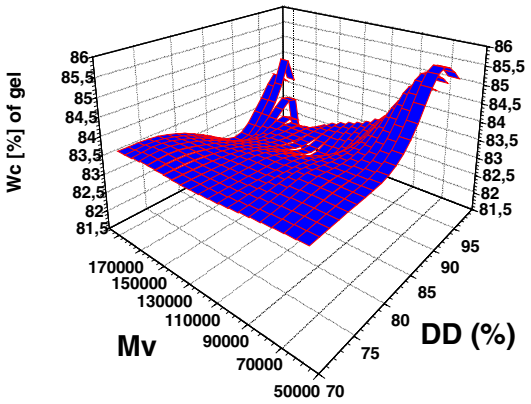



Figure 113. The effect of DD and $\bar{M}_v$ of chitosan on Wc of MCChB gel-like dispersion prepared in presence of 20 wt% casein

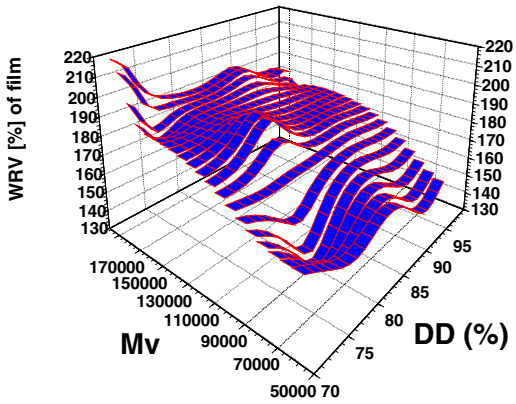



Figure 114. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB film prepared in presence of 20 wt% casein

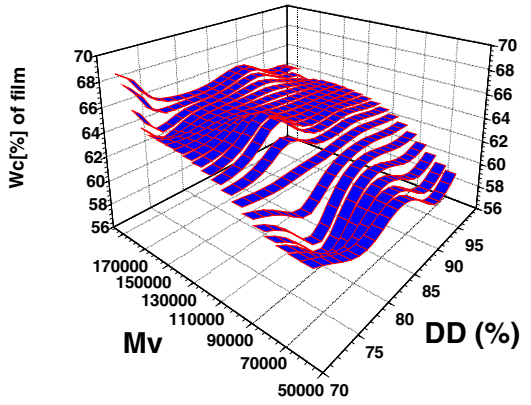



Figure 115. The effect of DD and $\bar{M}_v$ of chitosan on Wc of MCChB film prepared in presence of 20 wt% casein

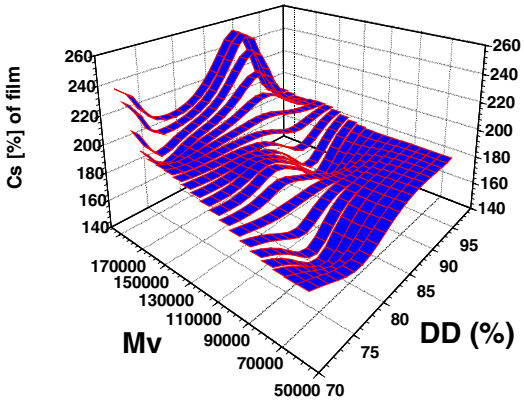



Figure 116. The effect of DD and $\bar{M}_v$ of chitosan on Cs of MCChB film prepared in presence of 20 wt% casein

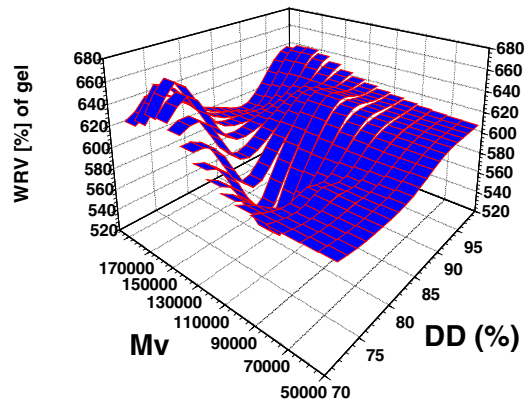



Figure 117. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB gel-like dispersion prepared in presence of 33 wt% casein

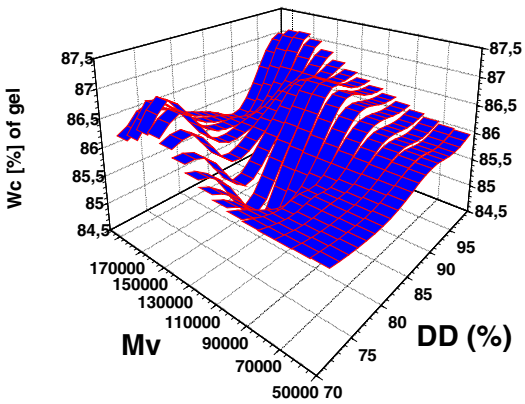



Figure 118. The effect of DD and $\bar{M}_v$ chitosan on Wc of MCChB gel-like dispersion prepared in presence of 33 wt% casein

Table 59. The parameters of MCChB gel-like dispersion and MCChB films prepared in presence of 33 wt% casein

| Symbol of sample | MCChB gel-like dispersion | | MCChB films | | |
|---|---|---|---|---|---|
| | WRV (%) | Wc (%) | WRV (%) | Cs (%) | Wc(%) |
| G3mA | 624.0 | 86.2 | 235.4 | 254.3 | 69.7 |
| M3A | 663.9 | 86.9 | 243.7 | 241.1 | 70.0 |
| G3mB | 574.2 | 84.9 | 232.5 | 257.0 | 69.9 |
| M3mB | 630.2 | 86.8 | 155.8 | 204.0 | 60.9 |
| M1mC | 597.1 | 85.8 | 154.2 | 183.8 | 60.7 |
| M6mC | 613.4 | 86.0 | 223.3 | 244.5 | 69.1 |
| PB8 | 492.5 | 83.1 | 208.4 | 267.4 | 67.6 |
| PB10 | 645.8 | 87.2 | 178.6 | 199.3 | 64.1 |

The highest values of WRV and Wc were obtained for samples showing high $\overline{M}_v$. The decrease in $M_v$ of MCChB caused lower swelling parameters (Figures 117-118). WRV and Wc of MCChB-Ca films similarly depended on $\overline{M}_v$ and DD of MCChB. An increase in DD resulted in decrease in WRV and WC in the case of samples possess high $\overline{M}_v$ (Figures 119-120). High values of WRV were also observed for low $\overline{M}_v$ of MCChB with high DD. The same observation was found for the swelling coefficient (Figure 121). An increase in Cs was observed for films prepared from MCChB with high $M_v$, which had low or medium DD. At the same time, a high Cs was obtained for a low $\overline{M}_v$ of MCChB showing a high degree of deacetylation (93 %). The ability of MCCh-casein films to retain water was related to the value of $\overline{M}_v$ of MCChB. Two peaks were observed: for high $\overline{M}_v$ of MCChB with low DD as well as for low $\overline{M}_v$ of MCChB having a high DD. This fact suggests that casein modification resulted in increase in the ability of MCChB gel-like dispersion and film to high swelling.

4.10.4. Determination of swelling parameters of MCChB gel like dispersions and MCChB films containing keratin

MCChB gel-like dispersion prepared in presence of 20 wt% or 33 wt% keratin and films were used in this stage of research. High WRV and Wc for MCChB-keratin gel-like dispersion prepared in a presence of 20 wt% keratin with low $\overline{M}_v$ and high DD was found (Table 60). The WRV and Wc of gel-like dispersions decreased with the reduction in $\overline{M}_v$ (Figures 122-123.).

Table 60. The swelling coefficients of MCChB gel-like dispersion and MCChB films prepared in presence of 20 wt% keratin

| Symbol of sample | MCChB gel-like dispersion | | MCChB films | | |
|---|---|---|---|---|---|
| | WRV (%) | Wc (%) | WRV (%) | Cs (%) | Wc (%) |
| G3mA | 372.2 | 78.9 | 130.5 | 129.5 | 56.6 |
| M3A | 377.9 | 79.1 | 112.8 | 140.7 | 53.0 |
| G3mB | 343.5 | 81.5 | 137.2 | 137.2 | 57.8 |
| M3mB | 370.3 | 78.7 | 101.4 | 130.2 | 50.3 |
| M1mC | 344.0 | 77.5 | 83.7 | 121.3 | 45.6 |
| M6mC | 437.4 | 81.1 | 186.5 | 132.4 | 65.1 |
| PB8 | 360.5 | 77.8 | 147.2 | 151.2 | 59.5 |
| PB10 | 408.4 | 80.4 | 115.2 | 145.7 | 53.5 |
| MCCh I-3/1/A | 370.1 | 78.1 | 148.1 | 153.8 | 59.7 |

The presence of 20 wt% keratin in the coagulation bath resulted in decrease in both the WRV and Wc coefficients of films in comparison to MCChB or MCChB-casein forms. The highest WRV and Wc was obtained for low $M_v$ of MCChB possessing a high DD. WRV and Wc decreased with an increase in $\overline{M}_v$ and reduction in DD (Figures 124-125.). The increase in $\overline{M}_v$ of MCChB with high DD caused

the increase in swelling coefficient. By lowering DD and $\bar{M}_v$, the above-mentioned coefficient decreased (Figure 126). The increase in keratin content to 33 wt% caused an increase in WRV and Wc of MCChB gel-like dispersions in contrast to the MCChB-casein gel-like dispersion prepared under the same conditions (Table 61). The highest values of these coefficients were obtained for high $\bar{M}_v$ of MCChB (Figures 127-128.). Both WRV and Wc of MCChB-keratin films showed a decrease with increases in DD. The high $\bar{M}_v$ of MCChB attained the highest levels of WRV and Wc (Figures 129-130.).

Table 61. The parameters of MCChB gel-like dispersion and MCChB films prepared in presence of 33 wt% keratin

| Symbol of sample | MCChB gel like dispersion | | MCChB films | | |
|---|---|---|---|---|---|
| | WRV (%) | Wc (%) | WRV (%) | Cs (%) | Wc (%) |
| G3mA | 470.6 | 82.5 | 155.9 | 214.7 | 60.9 |
| M3A | 480.4 | 82.8 | 128.4 | 194.1 | 56.2 |
| G3mB | 378.2 | 78.9 | 177.7 | 203.6 | 64.0 |
| M3mB | 417.0 | 80.6 | 168.9 | 159.7 | 62.0 |
| M1mC | 387.9 | 79.3 | 160.1 | 162.7 | 61.6 |
| M6mC | 403.4 | 80.1 | 101.3 | 196.4 | 50.3 |
| PB8 | 423.4 | 81.0 | 178.8 | 230.8 | 64.1 |
| PB10 | 505.3 | 82.7 | 137.6 | 150.2 | 57.9 |

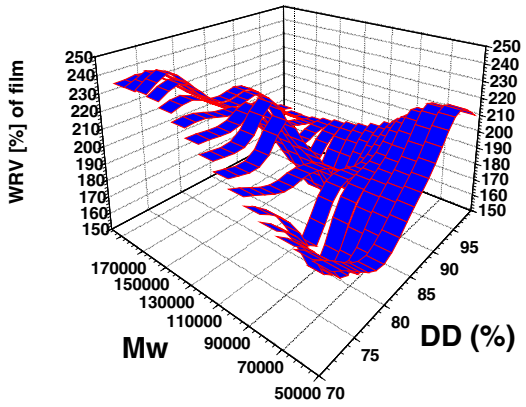



Figure 119. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB film prepared in presence of 33 wt% casein

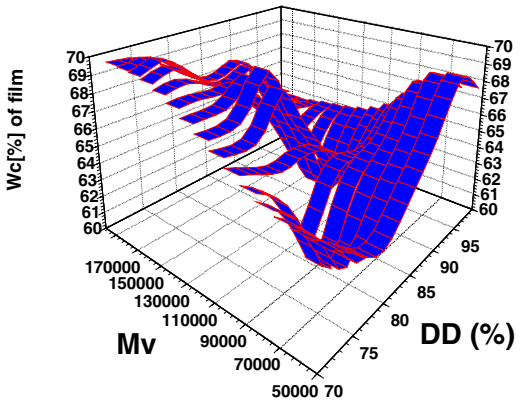



Figure 120. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB film prepared in presence of 33 wt% casein

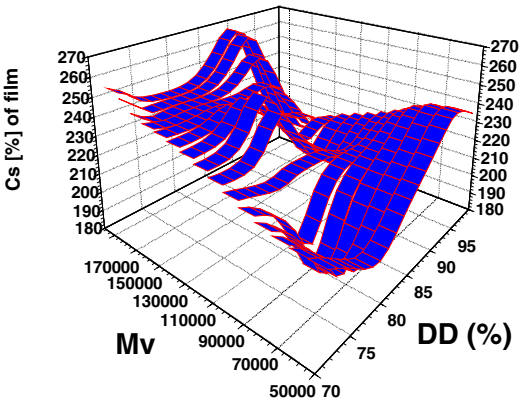



Figure 121. The effect of DD and $\bar{M}_v$ of chitosan on Cs of MCChB film prepared in presence of 33 wt% casein

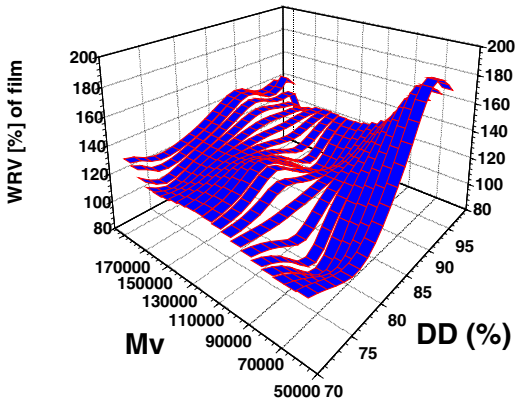



Figure 122. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB gel-like dispersion prepared in presence of 20 wt% keratin

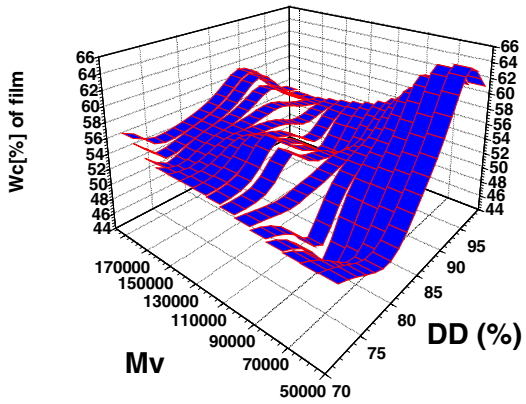



Figure 123. The effect of DD and $\bar{M}_v$ of chitosan on Wc of MCChB gel-like dispersion prepared in presence of 20 wt% keratin

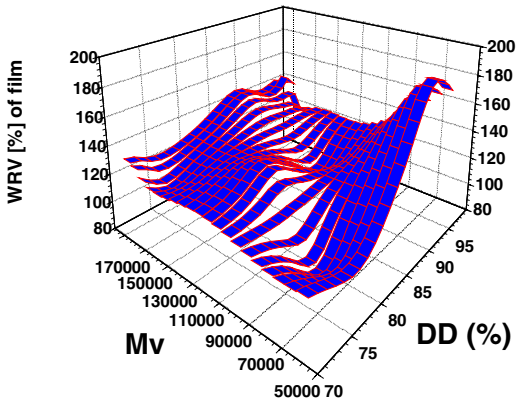



Figure 124. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB film prepared in presence of 20 wt% keratin

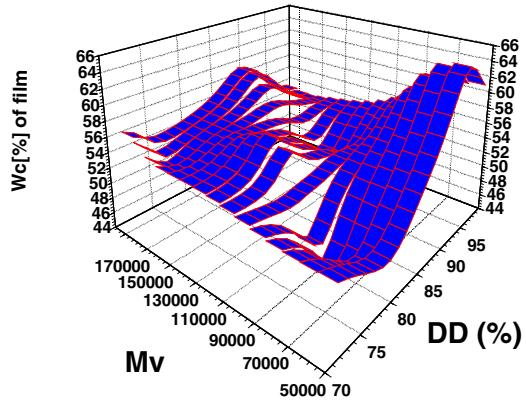



Figure 125. The effect of DD and $\bar{M}_v$ of chitosan on Wc of MCChB film prepared in presence of 20 wt% keratin

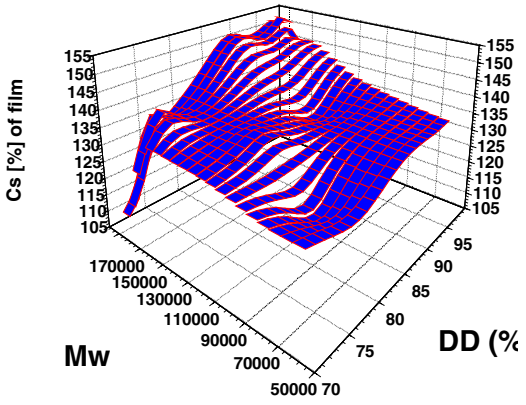



Figure 126. The effect of DD and $\bar{M}_v$ of chitosan on Cs of MCChB film prepared in presence of 20 wt% keratin

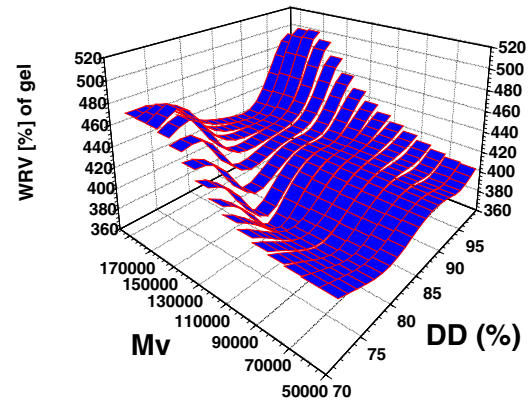



Figure 127. The effect of DD and $\bar{M}_v$ of chitosan on WRV of MCChB gel-like dispersion prepared in presence of 33 wt% keratin

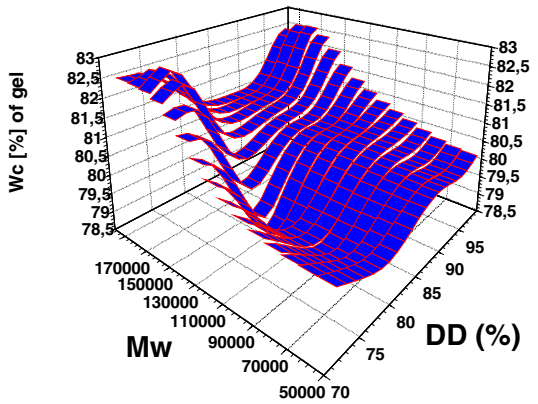



Figure 128. The effect of DD and $\bar{M}_v$ of chitosan on Wc of MCChB gel-like dispersion prepared in presence of 33 wt% keratin

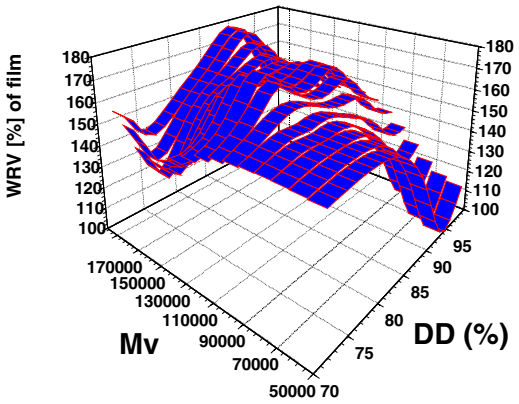



Figure 129. The effect of DD and $\overline{M}_v$ of chitosan on WRV of MCChB film prepared in presence of 33 wt% keratin

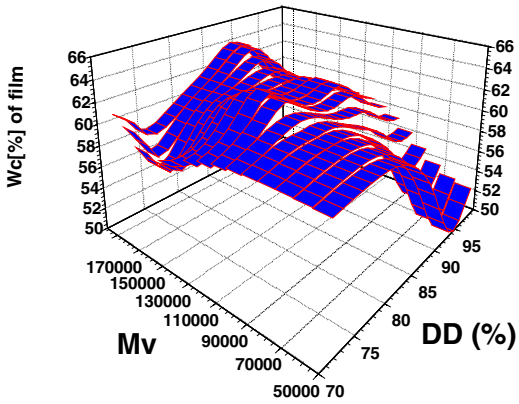



Figure 130. The effect of DD and $\overline{M}_v$ of chitosan on Wc of MCChB film prepared in presence of 33 wt% keratin

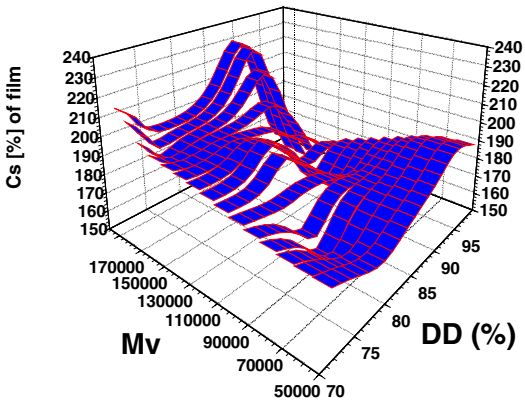



Figure 131. The effect of DD and $\overline{M}_v$ of chitosan on Cs of MCChB film prepared in presence of 33 wt% keratin

The increases in $\overline{M}_v$ of MCChB yielded an increase in the swelling coefficient of MCChB-keratin films. Increase in DD and the reduction in $\overline{M}_v$ of MCChB produced a decrease in Cs except for MCChB-keratin films formed from highly-deacetylated MCChB with low $\overline{M}_v$ (Figure 131).

4.10.5. Determination of mechanical properties of chitosan films with or without proteins

The mechanical properties of MCChB films were determined according to the method as described in section 2.13. During this research stage, MCChB films prepared by the modified method in coagulation bath both with or without 20 wt% of proteins (casein or keratin) were used.

The high $\overline{M}_v$ of MCChB caused the highest regress in breaking force of films (Table 62, Figure 132). In addition, the samples showed a high value of DD yielded a high breaking force. The same observation was detected for the tensile strength of films. MCChB with low $\overline{M}_v$ and low DD produced films, which possessed the lowest values of tensile strength (Figure 133).

This fact leads to the conclusion that increases in $\overline{M}_v$ plays the most important role in preparation of MCChB films with high mechanical strength. At the same time, a high DD of MCChB supports an increase in the mechanical strength of MCChB films. The elongation at break had a maximum for the high $\overline{M}_v$ of MCChB.

These parameters did not show significant dependence on DD of MCChB (Figure 134). Films made from low $\overline{M}_v$ MCChB did not show high elongation at break.

The addition of 20 wt% of casein during MCChB coagulation resulted in a significant change in the mechanical properties of MCChB films (Table 63).

Table 62. The mechanical properties of MCChB films

| Symbol of sample | Tensile strength (MPa) | Coefficient of the alteration of the charge (%) | Applied breaking force (N) | Elongation at break (%) | Coefficient of the alteration of the elongation (%) |
|---|---|---|---|---|---|
| G3mA | 3.7 | 26.3 | 2.38±1.02 | 16.6±3.3 | 24.2 |
| M3A | 9.1 | 10.1 | 9.59±1.02 | 25.3±3.2 | 20.9 |
| G3mB | 4.0 | 23.5 | 4.34±1.21 | 18.9±3.8 | 51.5 |
| M3mB | 11.7 | 25.5 | 17.40±4.70 | 26.5±12.1 | 43.5 |
| M1mC | 8.6 | 31.1 | 6.44±1.54 | 9.9±1.6 | 21.7 |
| M6mC | 7.8 | 19.7 | 7.60±1.57 | 11.0±3.4 | 29.3 |
| PB8 | 12.0 | 16.6 | 14.70±3.1 | 20.3±2.9 | 31.0 |
| PB10 | 6.5 | 33.0 | 4.69±1.19 | 15.0±3.2 | 28.1 |
| MCCh I-3/1/A | 5.9 | 27.0 | 4.70±1.20 | 14.9±4.2 | 23.4 |

Table 63. The mechanical properties of MCChB films prepared in presence of 20 wt% casein

| Symbol of sample | Tensile strength (MPa) | Coefficient of the alteration of the charge (%) | Applied breaking force (N) | Elongation at break (%) | Coefficient of the alteration of the elongation (%) |
|---|---|---|---|---|---|
| G3mA | 6.84 | 23.1 | 7.60±2.18 | 35,8±12,9 | 29,2 |
| M3A | 11,70 | 23,3 | 10,60±2,3 | 18,0±4,8 | 29,0 |
| G3mB | 5,06 | 33,2 | 5,58±1,41 | 10,8±3,6 | 37,2 |
| M3mB | 6,81 | 31,6 | 5,73±1,39 | 11,6±4,0 | 45,0 |
| M1mC | 5,13 | 31,4 | 7,76±1,13 | 8,9±1,9 | 24,8 |
| M6mC | 7,01 | 21,0 | 5,49±0,88 | 7,8±1,5 | 24,6 |
| PB8 | 4,26 | 40,4 | 3,71±1,26 | 28,9±11,6 | 47,9 |
| PB10 | 4,11 | 19,0 | 3,76±0,51 | 13,7±2,0 | 20,4 |
| MCCh I-3/1/A | 5,59 | 30,3 | 4,26±1,57 | 9,7±1,52 | 15,7 |

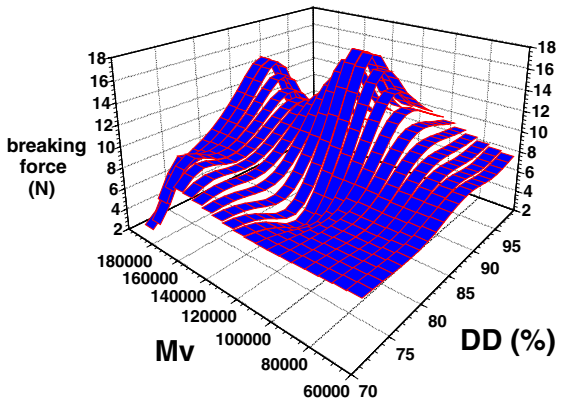



Figure 132. The effect of DD and $\bar{M}_v$ of MCChB on breaking force of films

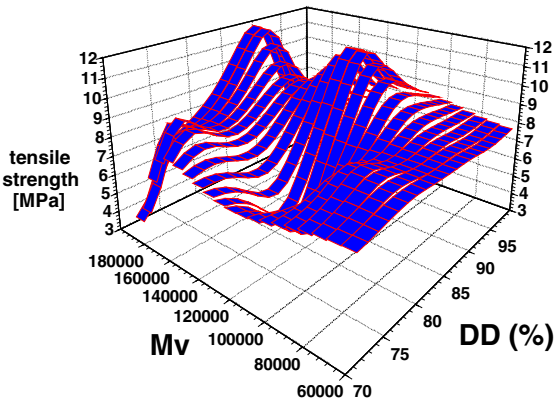



Figure 133. The effect of DD and $\bar{M}_v$ of MCChB on tensile strength of films

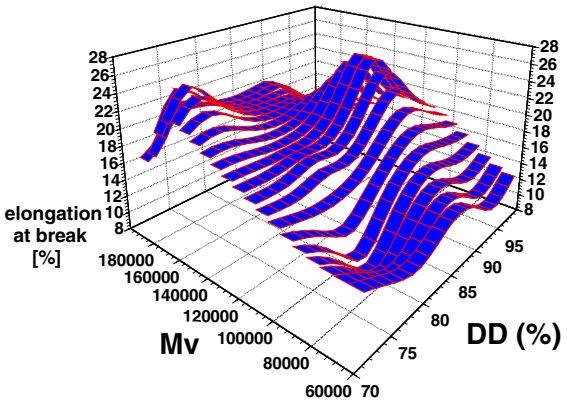



Figure 134. The effect of DD and $\overline{M}_v$ of MCChB on elongation at break of films

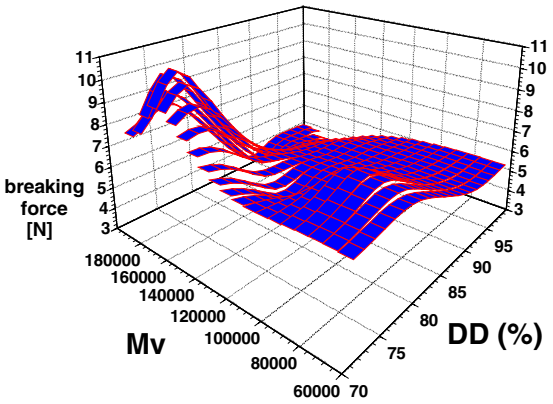



Figure 135. The effect of DD and $\overline{M}_v$ of MCChB on applied breaking force of films prepared in presence of 20 wt% casein

The highest value of applied breaking force was found for MCChB films prepared form MCChB having high $\overline{M}_v$ (175,000 Da) and the lowest DD. The applied breaking force of MCChB films modified by casein decreased with a reduction in $\overline{M}_v$ and with the increase in DD of MCChB (Figure 135). The same observation was discovered for the change in tensile strength. As indicated in the case of applied breaking force, the highest value of tensile strength of films was noted for high $\overline{M}_v$ of MCChB as well as for the lowest DD. MCChB with a high DD showed a smaller tensile strength of films (Figure 136). The elongation at break decreased with the reduction in $\overline{M}_v$ as well as with the increase in DD of MCChB (Figure 137). The highest parameter of elongation at break of films was obtained for MCChB showed a high $\overline{M}_v$ and low or medium DD.

MCChB showed the highest DD and high $\overline{M}_v$ led to the lowest applied breaking force of MCChB-keratin films (Table 64). The highest breaking force of films was found, when MCChB with high $\overline{M}_v$ was used, however a significant increase was noted in the case of MCChB with high DD (Figure 138). The same relationship was observed for tensile strength, but its dependence on DD was insignificant (Figure 139). The elongation at break decreased with the reduction in DD of MCChB. The highest elongation parameter was noted for a high $\overline{M}_v$ of MCChB with high DD (Figure 140). The above results suggest that addition of protein during the preparation of MCChB gel-like dispersion and the sources of chitosan strongly affect the mechanical properties of films. The type and the concentration of proteins change to the effect of $\overline{M}_v$ and DD of MCChB powder on the mechanical properties.

Table 64. The mechanical properties of MCChB films prepared in presence of 20 wt% of keratin

| Symbol of sample | Tensile strength (MPa) | Coefficient of the alteration of the charge (%) | Applied breaking force (N) | Elongation at break (%) | Coefficient of the alteration of the elongation (%) |
|---|---|---|---|---|---|
| G3mA | 5,25 | 29,0 | 6,30±2,91 | 13,2±6,1 | 29,2 |
| M3A | 8,47 | 56,7 | 7,51±3,94 | 10,4±3,9 | 40,4 |
| G3mB | 6,0 | 35,1 | 6,10±0,79 | 11,5±5,9 | 31,6 |
| M3mB | 10,0 | 21,1 | 12,20±2,7 | 21,3±4,8 | 21,3 |
| M1mC | 3,49 | 24,9 | 2,81±0,59 | 14,9±3,2 | 26,0 |
| M6mC | 5,75 | 26,8 | 4,21±1,20 | 14,8±5,3 | 28,9 |
| PB8 | 4,31 | 24,7 | 2,90±0,89 | 7,2±3,2 | 36,0 |
| PB10 | 8,8 | 15,5 | 9,90±1,54 | 19,8±3,6 | 21,1 |
| MCCh I-3/1/A | 7,03 | 16,3 | 5,21±1,15 | 15,4±2,44 | 15,8 |

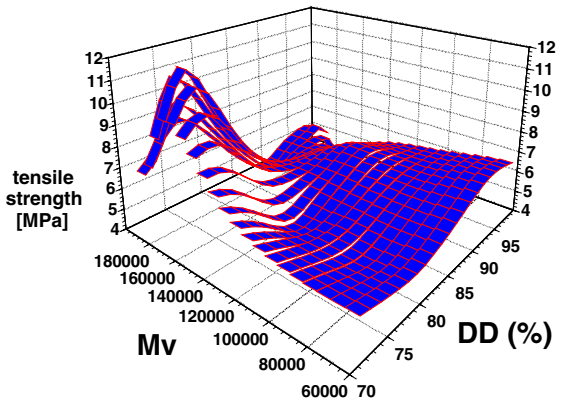



Figure 136. The effect of DD and $\bar{M}_v$ of MCChB on tensile strength of films prepared in presence of 20 wt% casein

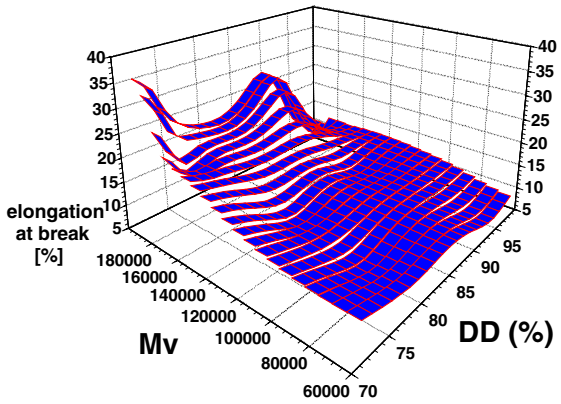



Figure 137. The effect of DD and $\bar{M}_v$ of MCChB on elongation at break of films prepared in presence of 20 wt% casein

The strongest MCChB films and highest swelling behaviour (with or without protein) were prepared from chitosan having $\bar{M}_v$ from 200 kDa to 150 kDa and a relatively low DD. The origin of chitosan seems to be rather unimportant.

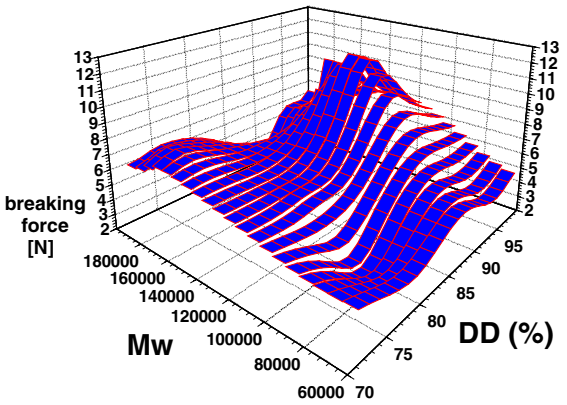



Figure 138. The effect of DD and $\bar{M}_v$ of MCChB on applied breaking force of films prepared in presence of 20 wt% keratin

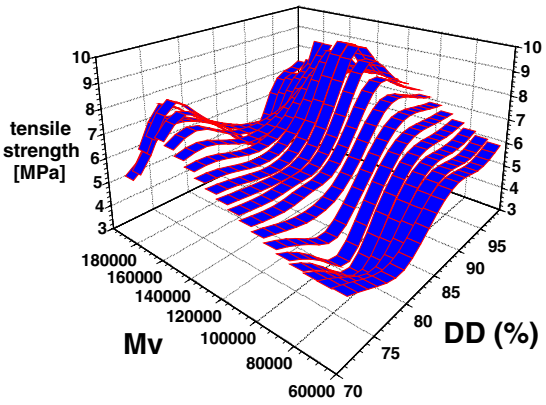



Figure 139. The effect of DD and $\bar{M}_v$ of MCChB on tensile strength of films prepared in presence of 20 wt% keratin

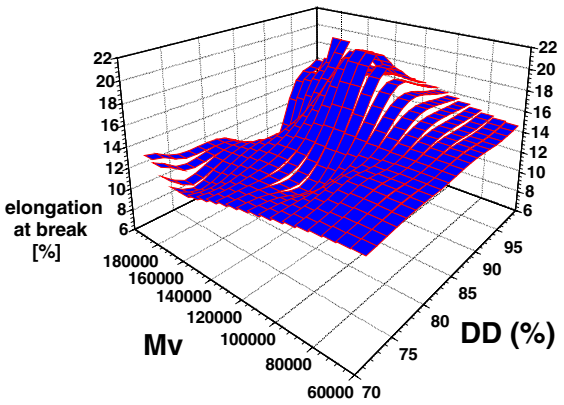



Figure 140. The effect of DD and $\bar{M}_v$ of MCChB on elongation at break of films prepared in presence of 20 wt% keratin

4.10.6. Scanning electron microscopy investigation of MCChB films

The morphological properties of MCChB films with or without proteins were determined by scanning electron microscopy (SEM). The appearance of MCChB films is presented in Figures 141-150. The SEM microphotographs of surfaces indicate that the addition of proteins changes the roughness of films. Casein addition produced a significant increase in coarseness of film surface, especially for MCChB films prepared in presence of the highest amount of protein. On the other hand, the addition of keratin resulted in more smooth surface.

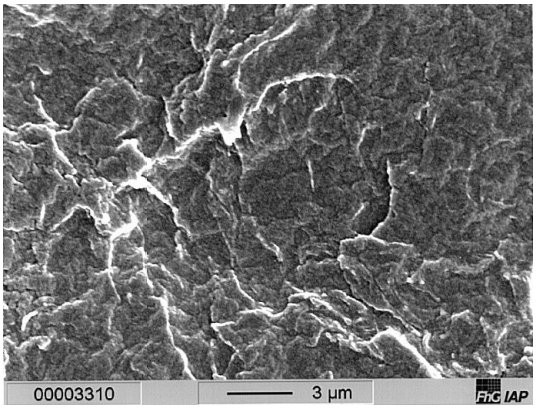



Figure 141. SEM micrograph of the surface of MCChB film

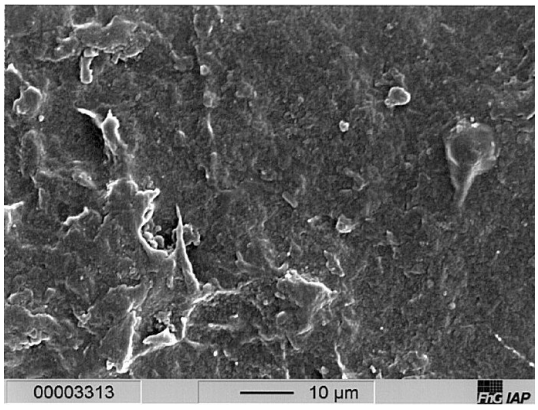



Figure 142. SEM micrograph of the surface of MCChB films prepared in presence of 11 wt% casein

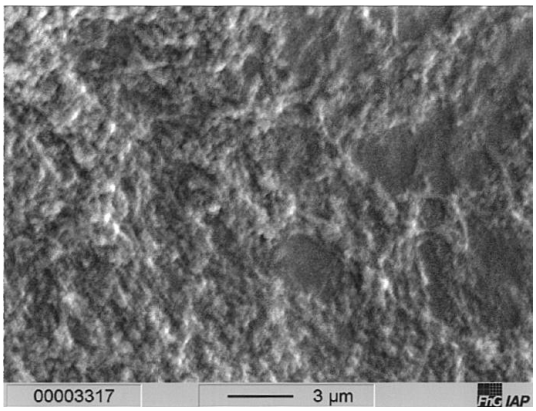



Figure 143. SEM micrograph of the surface of MCCh films prepared in presence of 33 wt% casein

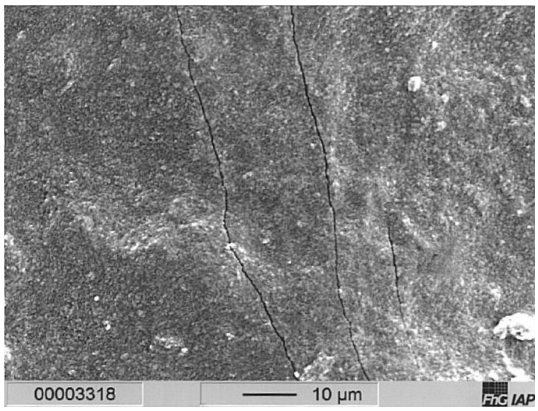



Figure 144. SEM micrograph of the surface of MCCh films prepared in presence of 11 wt% keratin

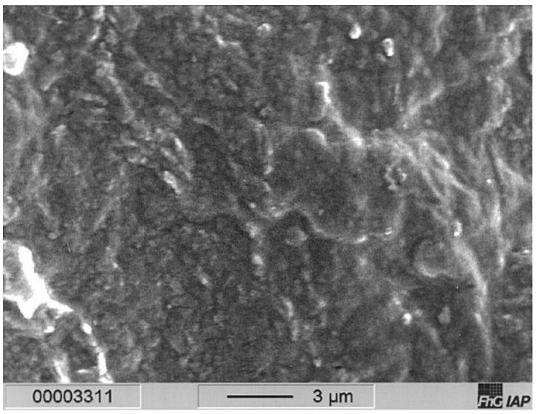



Figure 145. SEM micrograph of the surface of MCCh films prepared in presence of 60 wt% keratin

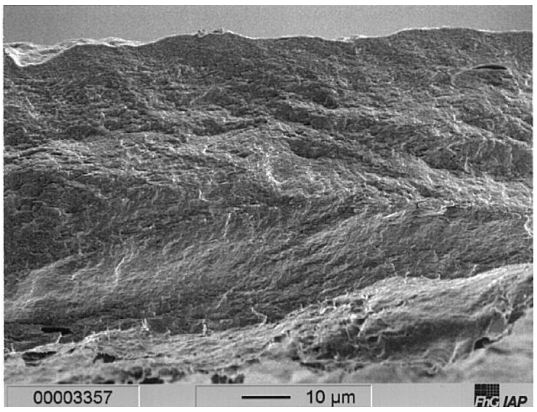



Figure 146. Cross-section of MCChB film

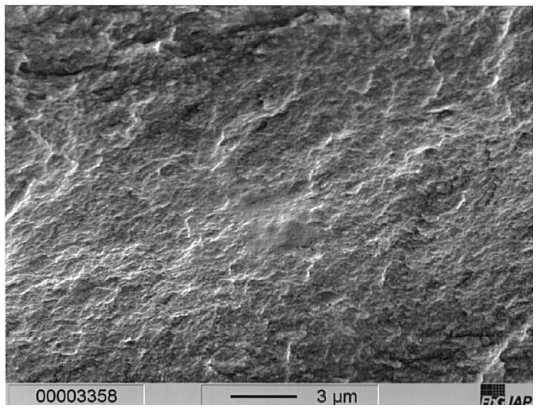



Figure 147. Cross-section of MCChB film

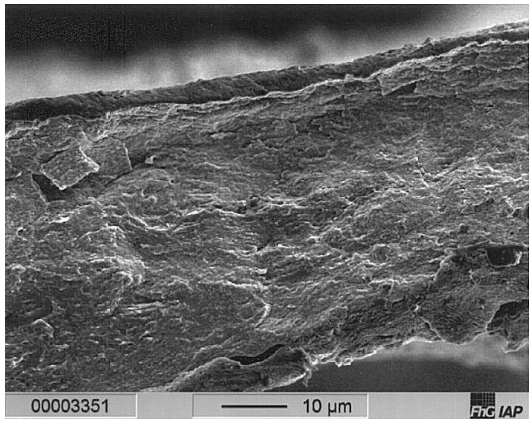



Figure 148. The cross-section of MCChB film prepared in presence of 11 wt% keratin

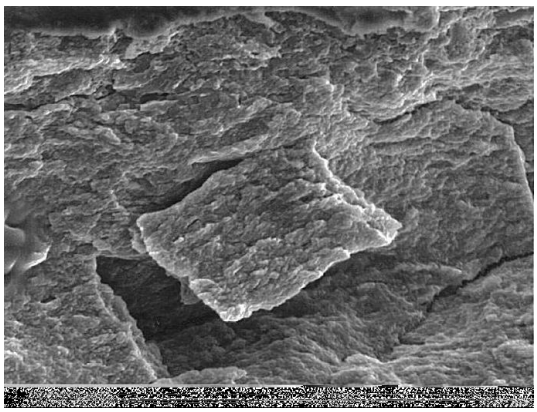


Figure 149. Cross-section of MCChB film prepared in presence of 11 wt% keratin

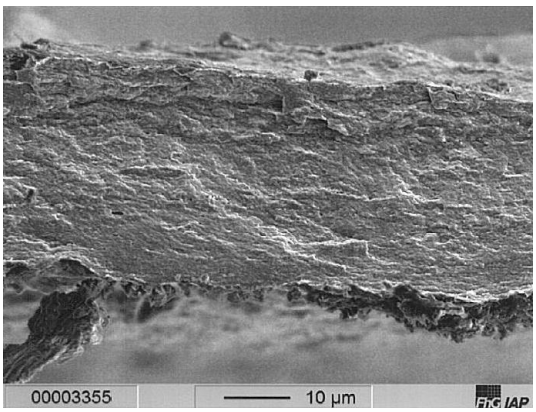



Figure 150. Cross-section of MCChB film prepared in presence of 11 wt% casein

4.11. Determination of the swelling properties of MCChB films cross-linked by glutaraldehyde (GA)

The effects of crosslink of MCChB films prepared from chitosan M3A or M3mB by GA are shown in Table 65 and Figure 151.

Table 65. Swelling parameters of cross-linked MCChB films

| Sample | Molar ratio GA:$NH_2$ | pH | Time of cross-linking (h) | WRV (%) | Wc (%) | Cs (%) | WRV in 1 wt% acetic acid (aq.) (%) | Wc in 1 wt% acetic acid (aq.) (%) |
|---|---|---|---|---|---|---|---|---|
| M3A | 3.0 | 7.0 | 24 | 53.5 | 34.8 | 54.7 | 75.5 | 43.0 |
| | 1.0 | 7.0 | 24 | 53.2 | 34.7 | 54.6 | 87.0 | 46.5 |
| | 0.5 | 7.0 | 24 | 57.7 | 36.6 | 58.8 | 97.4 | 49.3 |
| | 0.25 | 7.0 | 24 | 76.2 | 43.3 | 80.7 | 163.9 | 62.1 |
| | 0.11 | 7.0 | 24 | 136.2 | 57.4 | 139.8 | 270.1 | 72.3 |
| M3 mB | 3.0 | 7.0 | 24 | 45.8 | 31.4 | 50.5 | 67.7 | 40.4 |
| | 0.11 | 7.0 | 4 | 117.4 | 54.0 | 128.2 | 610.6 | 85.9 |
| M3A | 3.0 | 7.0 | 4 | 61.3 | 38.0 | 65.2 | 79.3 | 44.3 |
| | 0.11 | 7.0 | 4 | 136.8 | 57.8 | 140.2 | 778.2 | 88.6 |
| | 0.11 | 7.0 | 0.5 | 143.4 | 58.9 | 144.5 | 790.6 | 88.8 |
| | 0.11 | 7.0 | 0.25 | 147.3 | 59.6 | 145.9 | 1065.2 | 91.4 |
| | 6.0 | 7.0 | 0.5 | 88.0 | 46.8 | 93.8 | 97.4 | 49.3 |
| | 6.0 | 7.0 | 0.25 | 119.1 | 54.4 | 121.6 | 174.0 | 63.5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 6.0 | 7.0 | 0.12 | 122.7 | 54.6 | 138.4 | 319.1 | 76.1 |

A decrease in WRV with the increase in concentration of GA and with prolongation of cross-link was observed (Figure 151.).
A twofold increase in GA:DD ratio caused a decrease in WRV of films, especially in 1 wt% aqueous acetic acid by ca. 50%.
A decrease in DD of chitosan also affected the solubility of samples. In the case of films prepared from the highly-deacetylated MCChB, WRV and Cs were significantly lower compared to films from M3A chitosan.
However, the insoluble films possessing the lowest WRV and Wc shrunk during air-drying after soaking in 1 wt% aqueous acid solution.
The application of low concentrated solution of GA and shorter reaction times yielded MCChB films having considerable friability.

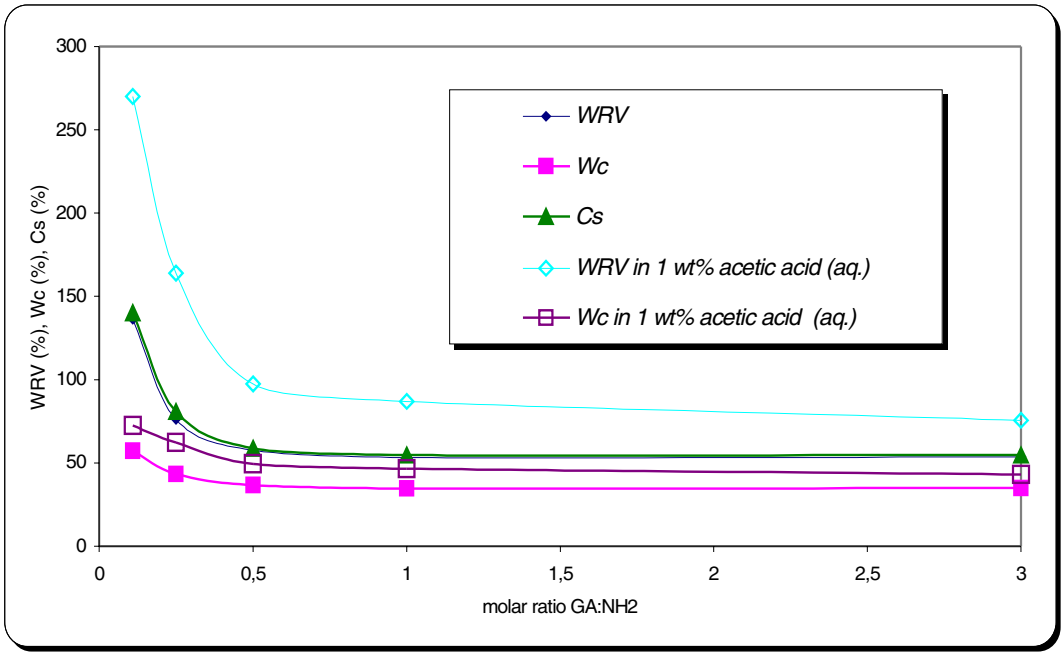



Figure 151. The effect of concentration of GA on WRV, Wc, and Cs of cross-linked MCChB films (cross-linking carried out for 24 h)

4.12. Determination of swelling properties of MCChB films prepared from cross-linked gel-like dispersions

Swelling parameters of MCChB films prepared from M3A chitosan cross-linked in an aqueous solution of GA are shown in Table 67.
The decrease in WRV with the increase in glutaraldehyde concentration and the prolongation of cross-linking time were also observed for the cross-linked MCChB films (Figure 152).
A decrease in pH of the reaction mixture caused increase in swelling parameters.
This observation was noted during investigations of WRV and Wc in 1 wt% aqueous acetic acid.
The highest swelling parameters were found for the films prepared using gel-like dispersion cross-linked at pH=6.0, except those sample cross-linked for a short time (Figure 153).
This fact may be explained with the solubility of chitosan at lower pH. The prolongation of reaction with GA at pH lower than 6.0 caused acid resistance. However, it is necessary to remember that the Schiff base formed during cross-linking of chitosan is susceptible to acid hydrolysis[260].
MCChB films produced from cross-linked gel-like dispersions showed a change of colour from yellow to dark-red.
The prolongation of the reaction time and the application of highly concentrated solutions of GA resulted in shrinked film.

Table 66. Swelling parameters of MCChB films prepared from cross-linked MCChB gel-like dispersion

| Sample | Molar ratio GA:$NH_2$ | pH | Time of cross-linking (h) | WRV (%) | Wc (%) | Cs (%) | WRV in 1 wt% acetic acid (aq.) (%) | Wc in 1 wt% acetic acid (aq.) (%) |
|---|---|---|---|---|---|---|---|---|
| M3A | 4.00 | 7.0 | 4 | 70.3 | 42.3 | 71.2 | 100.0 | 51.9 |
| | 2.00 | 7.0 | 4 | 70.5 | 42.3 | 74.6 | 105.6 | 52.3 |
| | 1.00 | 7.0 | 4 | 76.5 | 43.4 | 77.5 | 128.9 | 56.3 |
| | 0.11 | 7.0 | 4 | 103.2 | 50.8 | 108.0 | 387.4 | 79.5 |
| | 0.11 | 6.0 | 4 | 125.3 | 55.6 | 158.8 | 553.5 | 84.3 |
| | 0.11 | 5.0 | 4 | 128.8 | 56.3 | 199.3 | 375.4 | 79.0 |
| | 0.11 | 6.0 | 2 | 151.9 | 60.3 | 184.9 | 511.4 | 83.7 |
| | 0.11 | 5.0 | 2 | 151.0 | 60.2 | 204.0 | 378.4 | 79.1 |
| | 0.11 | 7.0 | 0.5 | 162.7 | 62.0 | 191.8 | 622.2 | 86.2 |
| | 0.11 | 6.0 | 0.5 | 162.8 | 62.0 | 158.1 | 399.0 | 80.0 |
| | 0.11 | 5.0 | 0.5 | 135.7 | 57.6 | 144.8 | 373.9 | 78.9 |
| | 0.11 | 7.0 | 24 | 96.1 | 49.0 | 107.8 | 466.6 | 82.4 |
| | 0.11 | 6.0 | 24 | 135.6 | 57.6 | 151.9 | 575.5 | 85.1 |
| | 0.11 | 5.0 | 24 | 122.4 | 55.0 | 141.6 | 239.5 | 70.5 |

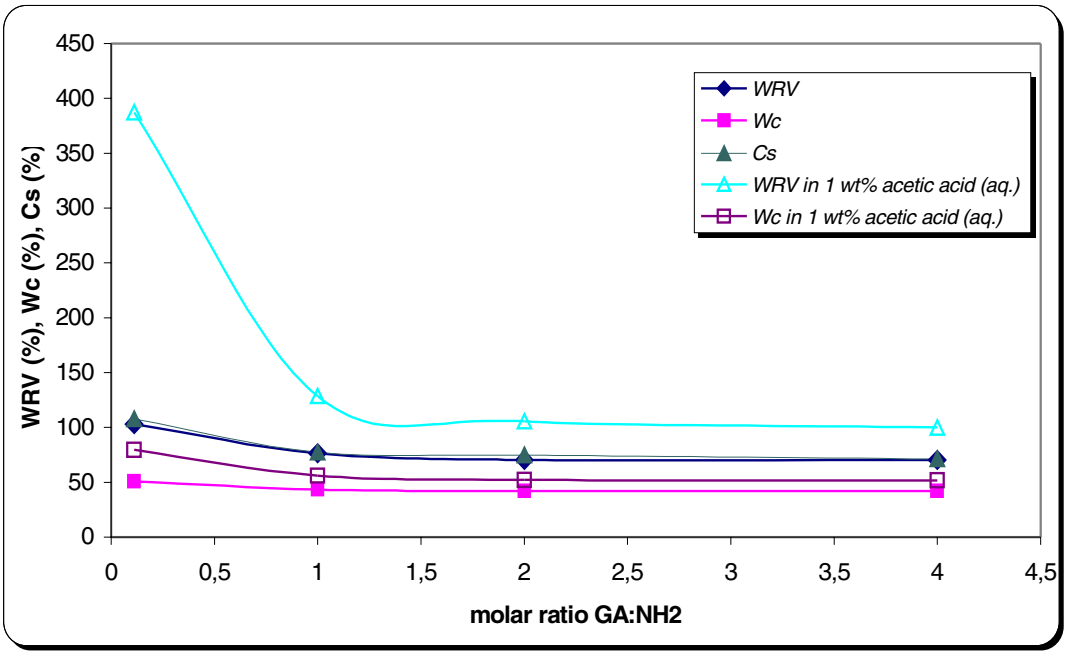



Figure 152. The effect of the concentration of GA on swelling parameters of MCChB films prepared using cross-linked MCChB gel-like dispersion (cross-link carried out for 4 h)

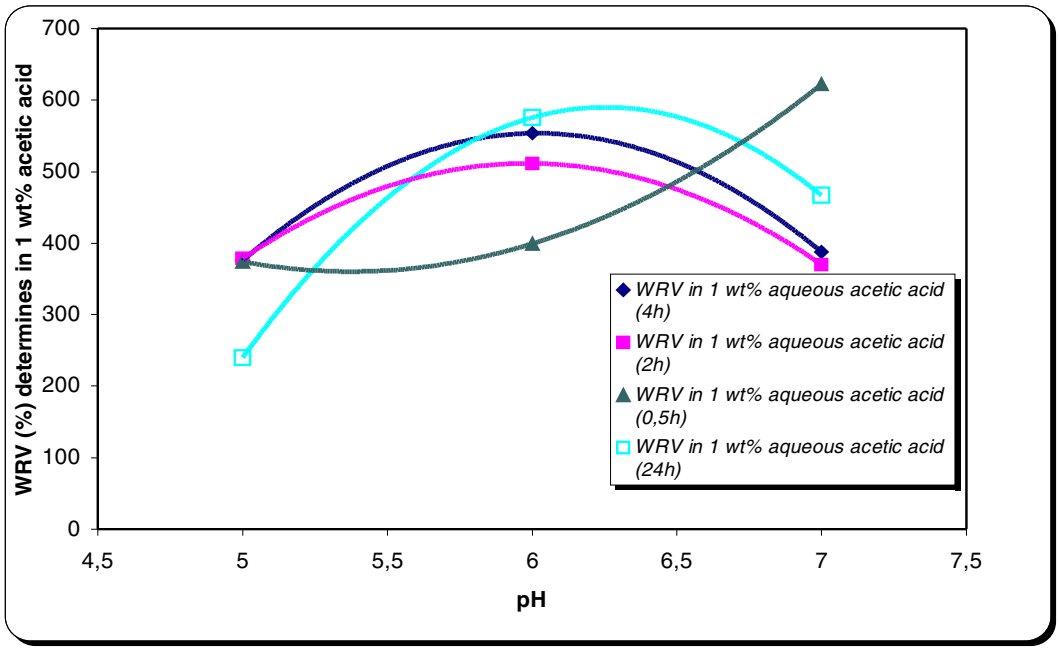



Figure 153. Changes in WRV of MCChB films cross-linked at various pH

4.13. Preparation of MCChB-cellulose paper containing proteins

The aim of this stage of the research was to prepare paper sheets containing microcrystalline chitosan with or without proteins. The procedure of MCChB-cellulose paper preparation was carried out according to the introduction of MCChB gel-like dispersion to cellulose pulp before paper formation or MCChB precipitation onto the cellulose fibre at pH=10 during sheet formation as described in sections 3.7. and 3.8.

4.13.1. Determination of swelling parameters for paper formed by the introduction of MCChB gel-like dispersion

The swelling parameters of paper formed by the introduction of MCChB gel-like dispersion are shown in Table 67.

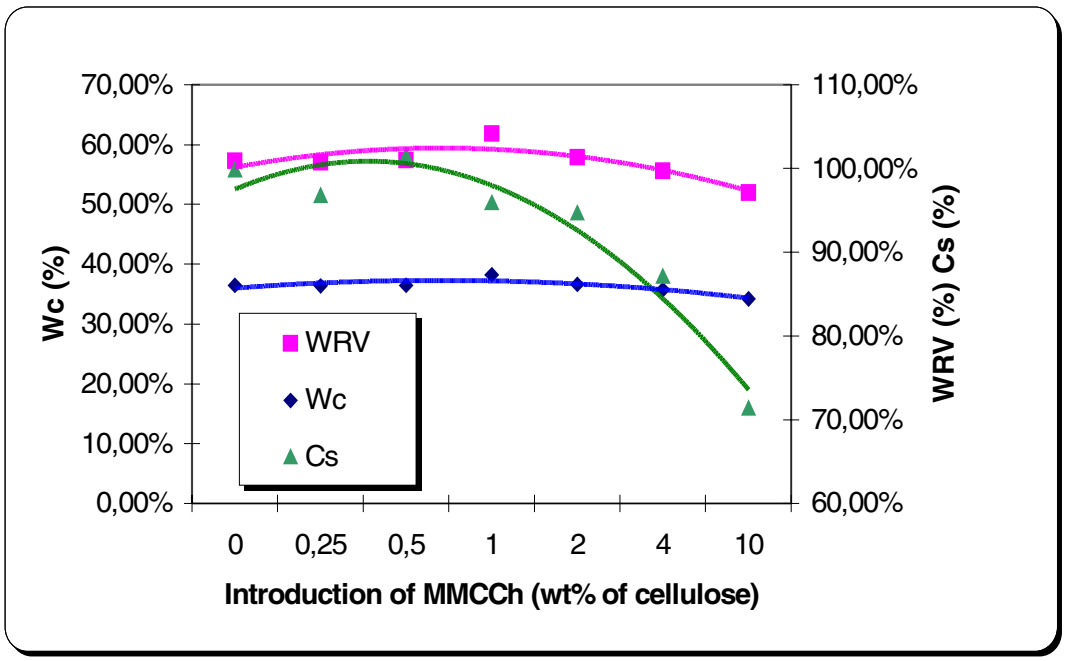



Figure 154. The effect of the concentration of introduced MCChB gel-like dispersion on paper swelling parameters

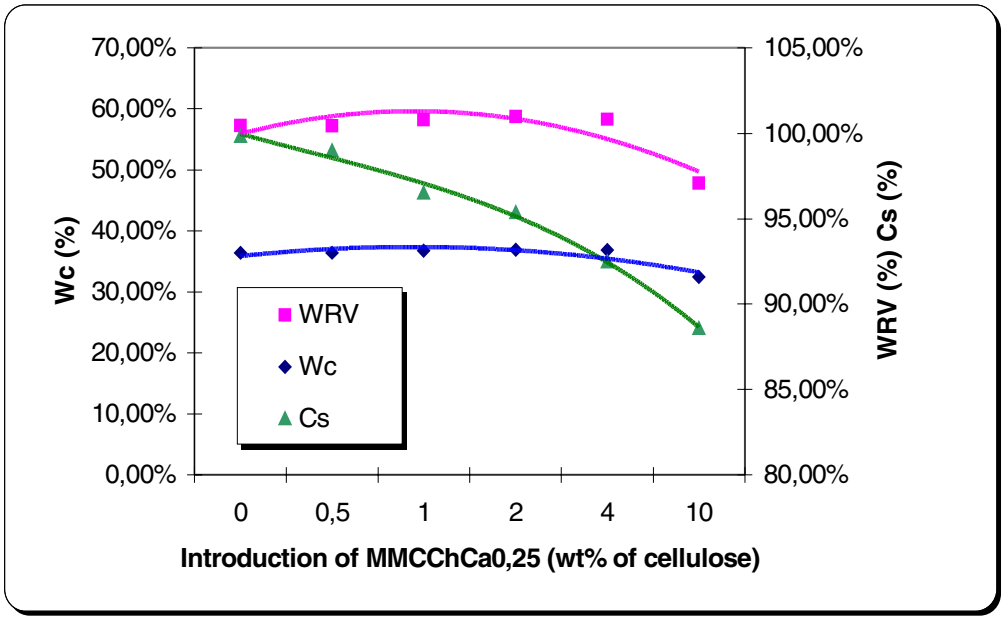



Figure 155. The effect of the concentration of introduced MCChB gel-like dispersion prepared in presence of 20 wt% casein on paper swelling parameters

Table 67. Swelling parameters of paper formed by the introduction of MCChB gel-like dispersion with or without protein

| Concentration of MCChB gel-like dispersion (wt%) | WRV(%) | Wc (%) | Cs (%) |
|---|---|---|---|
| Control (paper sheets without MCChB) | 57.3 | 36.4 | 99.8 |
| 0.25 | 57.1 | 36.4 | 96.8 |
| 0.5 | 57.4 | 36.5 | 101.5 |
| 1.0 | 61.9 | 38.2 | 95.9 |
| 2.0 | 57.9 | 36.6 | 94.7 |
| 4.0 | 55.7 | 35.8 | 87.1 |
| 10.0 | 52.0 | 34.2 | 71.4 |
| *Paper with addition of MCChB gel-like dispersion prepared in presence of 20 wt% casein* | | | |
| Control (paper sheets without MCChB) | 57.3 | 36.4 | 99.8 |
| 0.5 | 62.3 | 38.4 | 103.2 |
| 1.0 | 58.2 | 36.8 | 96.5 |
| 2.0 | 58.7 | 36.9 | 95.4 |
| 4.0 | 58.3 | 36.8 | 92.5 |
| 10.0 | 47.8 | 32.4 | 88.6 |
| *Paper with addition of MCChB gel-like dispersion prepared in presence of 20 wt% keratin* | | | |
| Control (paper sheets without MCChB) | 57.3 | 36.4 | 99.8 |
| 0.5 | 59.3 | 37.2 | 105.5 |
| 1.0 | 48.6 | 32.7 | 80.1 |
| 2.0 | 51.5 | 34.0 | 93.2 |
| 4.0 | 49.5 | 32.8 | 90.2 |
| 10.0 | 47.0 | 32.0 | 86.5 |

An increase in MCChB gel-like dispersion content caused a decrease in WRV, Wc, and Cs of paper sheets prepared by the introduction method (see section 3.7.) (Figure 154). This fact is especially visible in the reduction of the swelling coefficient. A similar observation was noted for cellulose papers containing MCChB-protein. However, the increase in MCChB-protein yielded in lower WRV and Wc as compared with paper sheets containing MCChB without protein. The increase in the

retention of MCChB-protein up to 0.5 wt% resulted in the increase in swelling coefficient (Figure 155. and 152). Further increase in MCChB content caused decrease in swelling parameters.

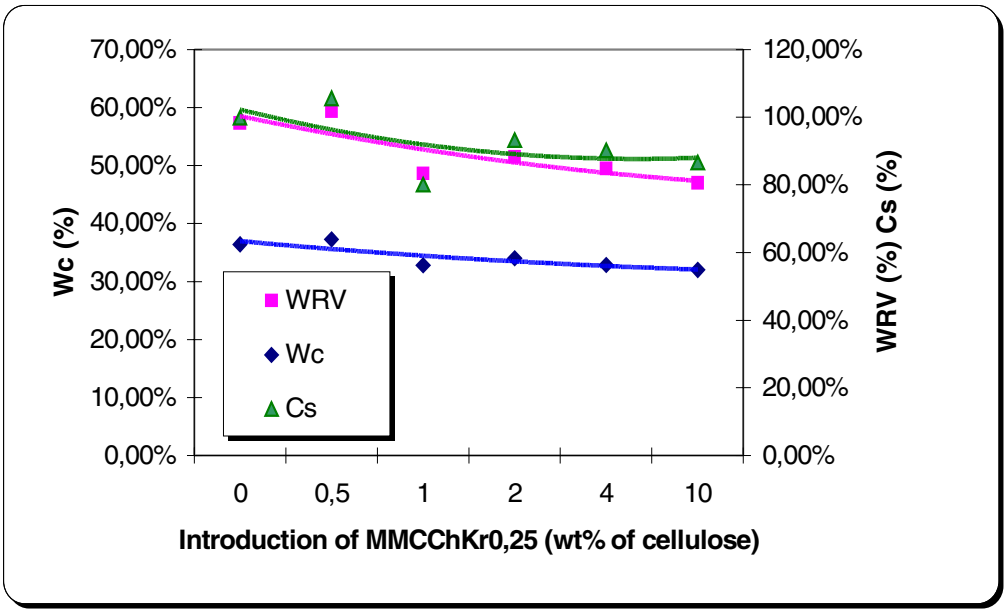



Figure 156. The effect of the concentration of introduced MCChB gel-like dispersion prepared in presence of 20 wt% keratin on paper swelling parameters

4.13.2. Determination of swelling parameters for paper formed by precipitation of MCChB gel-like dispersion with or without proteins

The paper sheets produced by precipitation of MCChB gel-like dispersion (see section 3.8.) showed a notable decrease in swelling parameters with the increase in MCChB retention (Table 68, Figure 157.).

Table 68. Swelling coefficients of paper formed with precipitated MCChB gel-like dispersion with or without protein

| Concentration of MCChB gel-like dispersion (wt%) | WRV(%) | Wc (%) | Cs (%) |
|---|---|---|---|
| Control (paper sheets without MCChB) | 57.3 | 36.4 | 99.8 |
| 0.5 | 42.9 | 30.0 | 101.1 |
| 1.0 | 42.1 | 29.6 | 95.3 |
| 2.0 | 41.4 | 29.3 | 86.9 |
| 4.0 | 40.3 | 29.0 | 87.3 |
| 10.0 | 38.2 | 27.6 | 89.1 |
| *Paper with precipitated MCChB gel-like dispersion prepared in presence of 20 wt% casein* | | | |
| Control (paper sheets without MCChB) | 57.3 | 36.4 | 99.8 |
| 0.50 | 50.6 | 33.6 | 117.6 |
| 1.00 | 50.0 | 33.4 | 119.6 |
| 2.00 | 48.2 | 32.0 | 97.8 |
| 4.00 | 47.2 | 31.9 | 96.6 |
| 10.00 | 38.5 | 27.8 | 88.4 |
| *Paper with precipitated MCChB gel-like dispersion prepared in presence of 20 wt% keratin* | | | |
| Control (paper sheets without MCChB) | 57.3 | 36.4 | 99.8 |
| 0.5 | 52.0 | 39.1 | 108.6 |
| 1.0 | 58.5 | 36.9 | 116.6 |
| 2.0 | 45.4 | 31.2 | 93.3 |
| 4.0 | 46.2 | 31.4 | 93.2 |
| 10.0 | 44.5 | 31.0 | 91.2 |

Their swelling parameters indicated lower value as compared for suitable paper prepared by MCChA introduction.
The application of MCChB gel-like dispersion with proteins affected a decrease in the WRV, Wc and Cs of paper, resulting in lower swelling parameters than for the direct introduction of MCChB (Figures 158-159.). However, low content of precipitated MCChB containing proteins caused increase in swelling parameters of paper sheets.

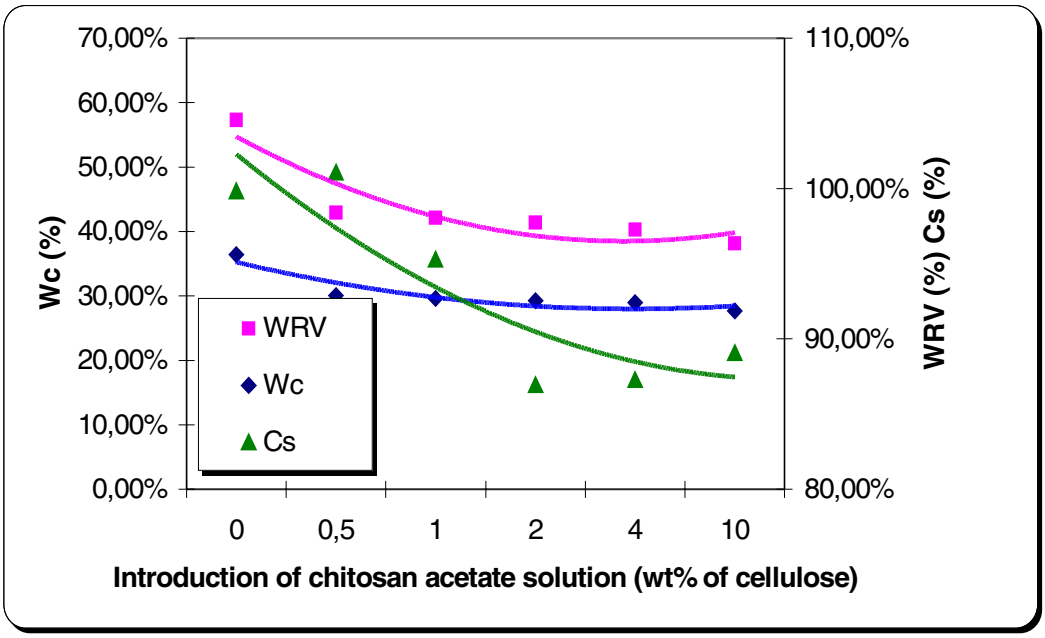



Figure 157. The effect of the concentration of chitosan acetate on paper swelling parameters

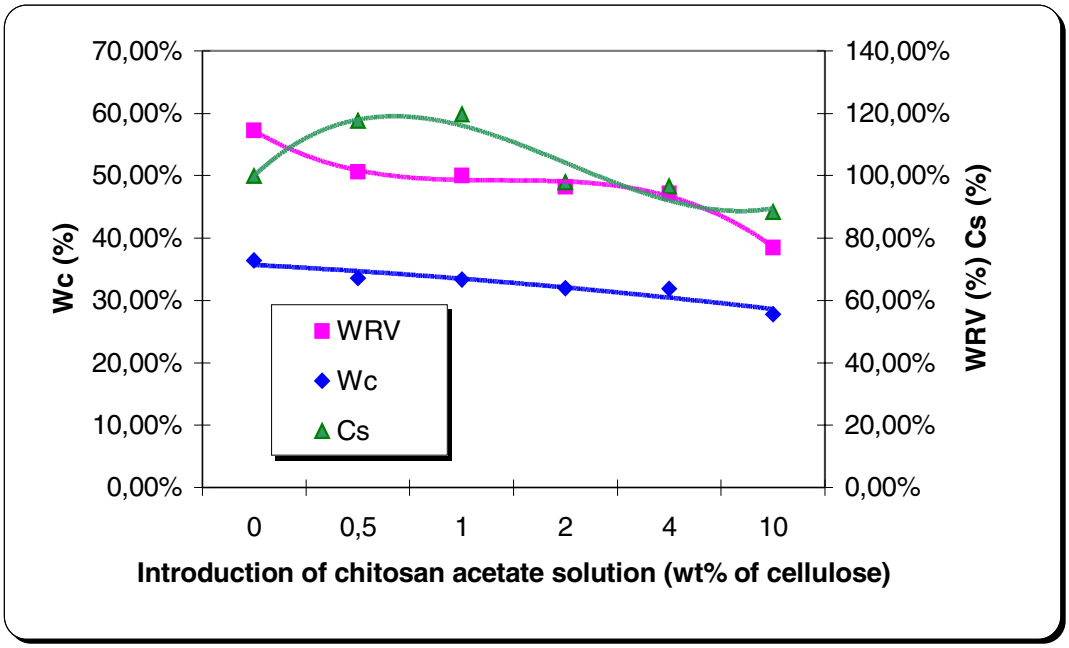



Figure 158. The effect of the concentration of chitosan acetate precipitated in presence of 20 wt% casein on paper swelling parameters

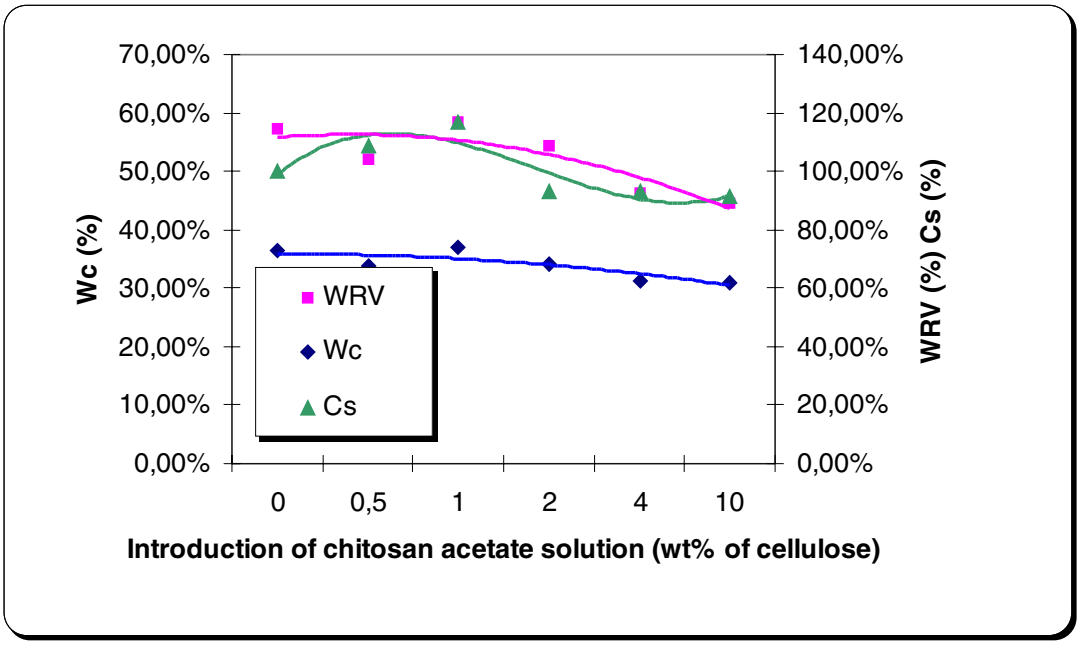



Figure 159. The effect of the concentration of chitosan acetate precipitated in presence of 20 wt% keratin on paper swelling parameters

Further increase in the concentration of MCChB-protein gel-like dispersion resulted in a considerable decrease in these parameters.
This observation suggests that while MCChB with protein is precipitated, its adsorption onto the cellulose fibers is significantly better as compared with paper with directly introduced MCChB. The distance between fibres decreased and the space between neighboring chains is filled by the precipitated MCChB particles. In the case of the direct MCChB introduction method, the relatively smaller numbers of hydrogen bonds between chitosan-cellulose chains results in a lower reduction in swelling parameters. The increase in swelling parameters of paper containing MCChB-protein with amount from 0.5 wt%, to 1.0 wt% is apparently due to water retention by protein.

4.13.3. Estimation of the MCChB retention in the paper sheets

The lower amount of introduced initial chitosan acetate caused considerably higher retention of MCChB in paper sheets, both prepared by precipitation or direct introduction of MCChB. Further increase in chitosan concentration up to 4 wt% resulted in higher retention for the direct introduction of MCChB. The application of MCChB gel-like dispersion with protein affected a decrease in the MCChB retention, especially for casein (Table 69, Figure 160).

Table 69. Retention of MCChB in paper sheets

| Chitosan introduction (wt%) | Retention (wt%) | | | | | |
|---|---|---|---|---|---|---|
| | MCChB | | MCChB containing casein | | MCChB containing keratin | |
| | Direct introduction method | Precipitation Method | Direct introduction method | Precipitation method | Direct introduction method | Precipitation method |
| 1 | 0.93 ± 0.03 | 0.90 ± 0.07 | 0.95 ± 0.08 | 0.90 ± 0.12 | 0.88 ± 0.12 | 0.62 ± 0.18 |
| 2 | 1.78 ± 0.08 | 2.06 ± 0.11 | 1.52 ± 0.05 | 1.54 ± 0.15 | 1.84 ± 0.16 | 2.24 ± 0.15 |
| 4 | 3.74 ± 0.05 | 3.60 ± 0.08 | 3.14 ± 0.13 | 2.98 ± 0.16 | 3.34 ± 0.20 | 3.21 ± 0.13 |
| 10 | 9.73 ± 0.21 | 8.40 ± 0.28 | 6.56 ± 0.20 | 5.60 ± 0.33 | 7.40 ± 0.30 | 6.82 ± 0.13 |

The functional groups of proteins may change the chemical as well as supermolecular behaviour of MCChB such as decrease of the amount of non-bonded amine groups with decrease in polycationic behaviour, the negative affect of the anionic groups of protein on the formation of the interactions among cellulose and chitosan chains, modification of supermolecular structure of biopolymers.

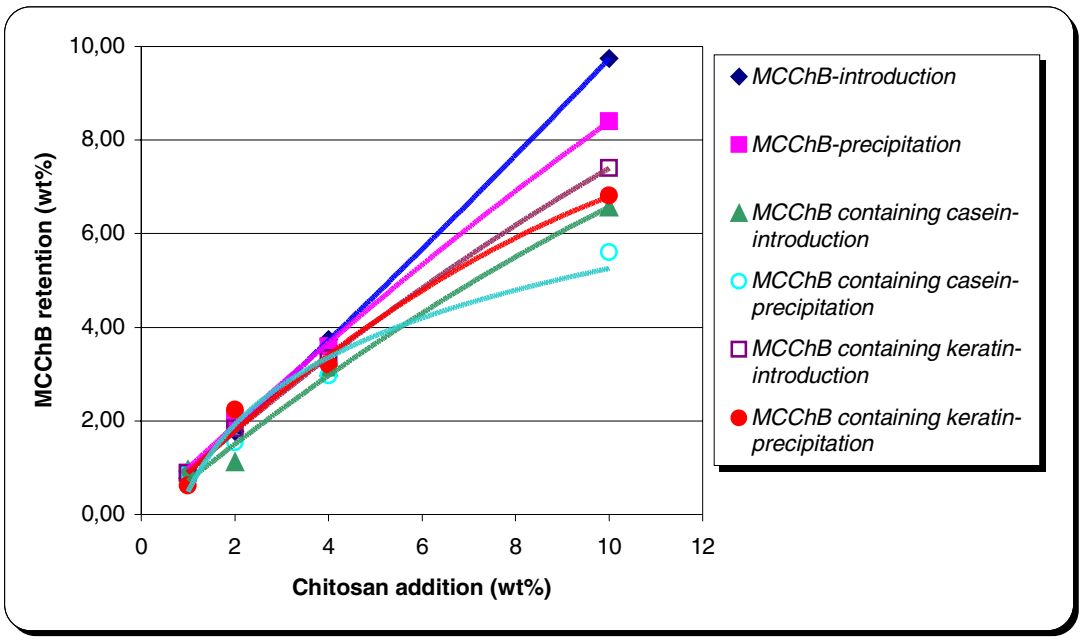



Figure 160. Introduction of MCChB vs. retention of MCChB in paper sheets

The functional groups of proteins may change the chemical as well as supermolecular behaviour of MCChB such as decrease of the amount of non-bonded amine groups with decrease in polycationic behaviour, the negative affect of the anionic groups of protein on the formation of the interactions among cellulose and chitosan chains, modification of supermolecular structure of biopolymers.

4.13.4. Mechanical properties of paper containing MCChB gel-like dispersion at low relative humidity

The mechanical properties of paper sheets prepared by the direct introduction of MCChB increased with the increase in chitosan retention (Table 70, Figure 161).

Table 70. Mechanical properties of paper sheets produced with different amount of MCChB possibly containing protein at rel. humidity of 60%

| Concentration of MCChB | Elongation at break (%) | Tensile strength (MPa) | Young's modulus (GPa) |
|---|---|---|---|
| | *Direct introduction method* | | |
| Control (paper sheets without MCChB) | 1.79 ± 0.15 | 7.20 ± 0.28 | 1.19 ± 0.10 |
| 0.25 | 1.85 ± 0.19 | 7.23 ± 0.26 | 1.11 ± 0.09 |
| 1.0 | 1.85 ± 0.18 | 7.37 ± 0.20 | 1.08 ± 0.1 |
| 2.0 | 1.73 ± 0.20 | 8.20 ± 0.47 | 1.10 ± 0.15 |
| 2.0 containing casein | 1.85 ± 0.29 | 7.70 ± 0.40 | 1.33 ± 0.12 |
| 2.0 containing keratin | 1.00 ± 0.20 | 5.50 ± 0.70 | 1.17 ± 0.08 |
| 4.0 | 2.36 ± 0.31 | 10.56 ± 0.39 | 1.31 ± 0.10 |
| 4.0 containing casein | 1.74 ± 0.37 | 7.90 ± 0.70 | 1.33 ± 0.05 |
| 4.0 containing keratin | 2.00 ± 0.10 | 8.80 ± 0.30 | 1.34 ± 0.06 |
| 10.0 | 2.57 ± 0.19 | 13.32 ± 0.56 | 1.70 ± 0.13 |
| | *Precipitation method* | | |
| Control (paper sheets without MCChB) | 1.79 ± 0.15 | 7.20 ± 0.28 | 1.19 ± 0.10 |
| 1.0 | 2.27 ± 0.24 | 7.70 ± 0.49 | 0.96 ± 0.12 |
| 2.0 | 2.43 ± 0.14 | 9.54 ± 0.22 | 1.31 ± 0.10 |
| 2.0 containing casein | 2.30 ± 0.20 | 8.20 ± 0.30 | 1.01 ± 0.18 |
| 2.0 containing keratin | 1.90 ± 0.30 | 7.70 ± 0.30 | 1.13 ± 0.12 |
| 4.0 | 2.36 ± 0.24 | 10.88 ± 0.41 | 1.34 ± 0.18 |
| 4.0 containing casein | 2.20 ± 0.20 | 9.80 ± 0.50 | 1.30 ± 0.20 |
| 4.0 containing keratin | 2.00 ± 0.20 | 8.70 ± 0.30 | 1.23 ± 0.15 |
| 10.0 | 2.20 ± 0.38 | 10.69 ± 0.42 | 1.48 ± 0.15 |

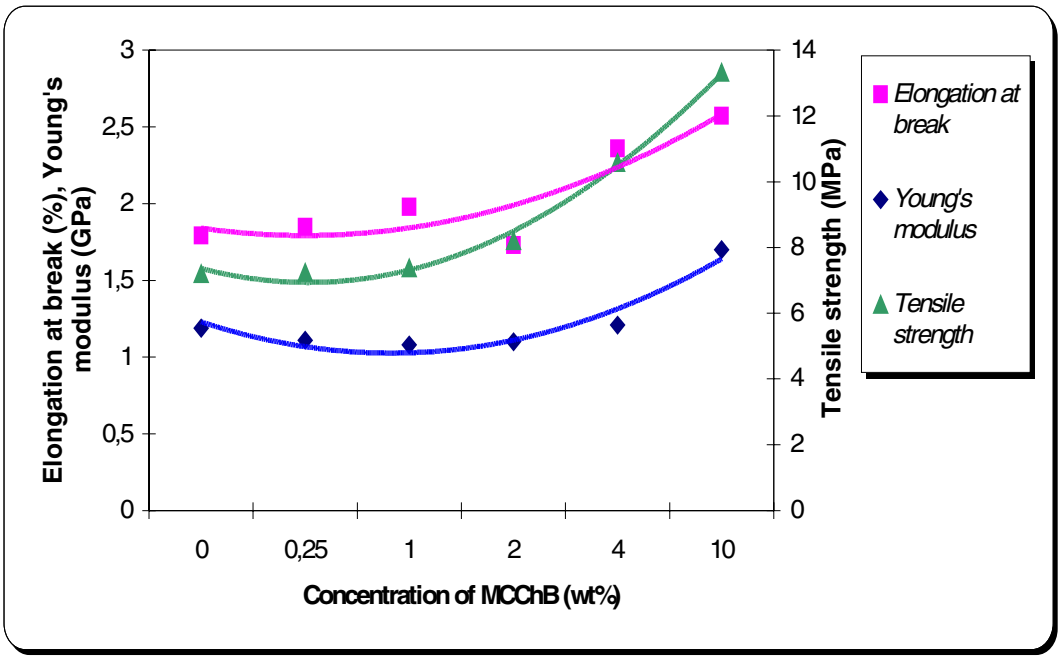



Figure 161. The effect of the concentration of MCChB on the mechanical properties of paper sheets prepared by direct introduction method

The highest tensile strength was found for the highest concentration of introduced MCChB. The same observation for elongation at break and Young's modulus was noted. However, the direct introduction of MCChB up to 2 wt% resulted in the decrease in Young's modulus of paper sheets. Further increase in MCChB content caused an increase in Young's modulus.
In the case of cellulose paper with precipitated MCChB, the increase in elongation at break as well as tensile strength was noted up to chitosan concentrations of 4 wt%. When a higher amount of chitosan was added, a relatively smaller increase in tensile strength with a decrease in elongation at break was found. The Young's modulus values increased with the increase of precipitated amount of chitosan (Figure 162).

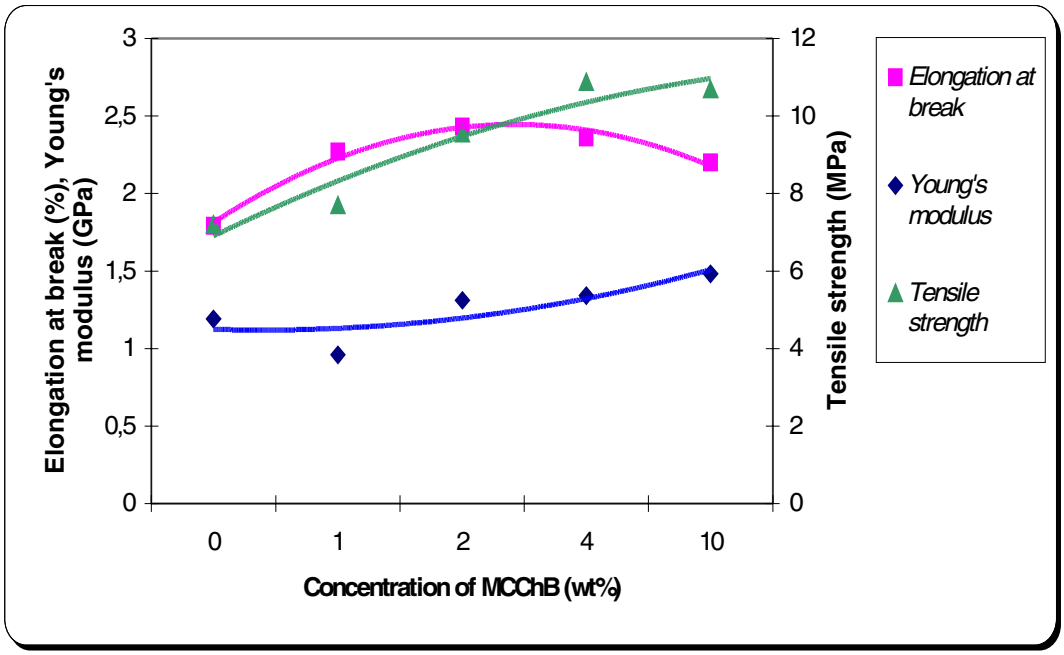



Figure 162. The effect of the concentration of MCChB on the mechanical properties of paper sheets prepared from suspension of fibres with precipitated chitosan

The above observation are explained by the formation of hydrogen bonds with energy suitable to improve the mechanical properties of paper sheets.

The addition of protein during preparation of MCChB gel-like dispersion caused a decrease in the tensile strength of paper sheets as compared to suitable paper sheets containing MCChB without protein. However, its value was higher than determined for control (Figure 163).

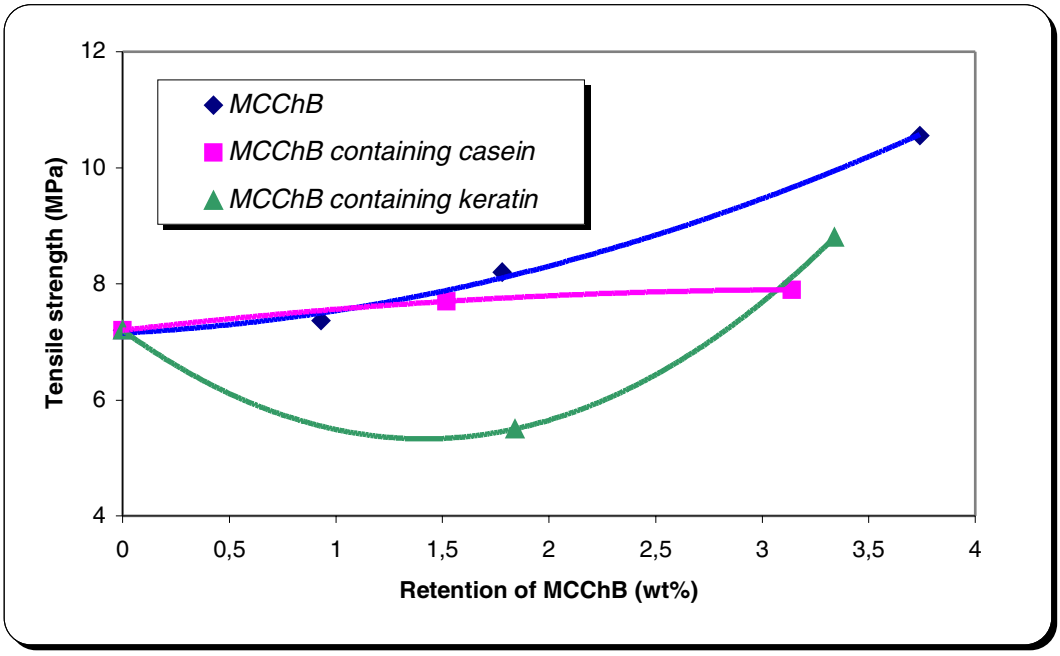



Figure 163. The effect of MCChB retention with or without proteins on the tensile strength of paper sheets prepared by the direct introduction method

The increase in the quantity of MCChB containing proteins caused the increases in tensile strength. Higher values of Young's modulus were noted for paper sheets containing directly introduced MCChB with casein. Nevertheless, the increase in MCChB containing keratin influenced a reduction in these parameters in comparison to paper sheets containing MCChB without protein (Figure 164).

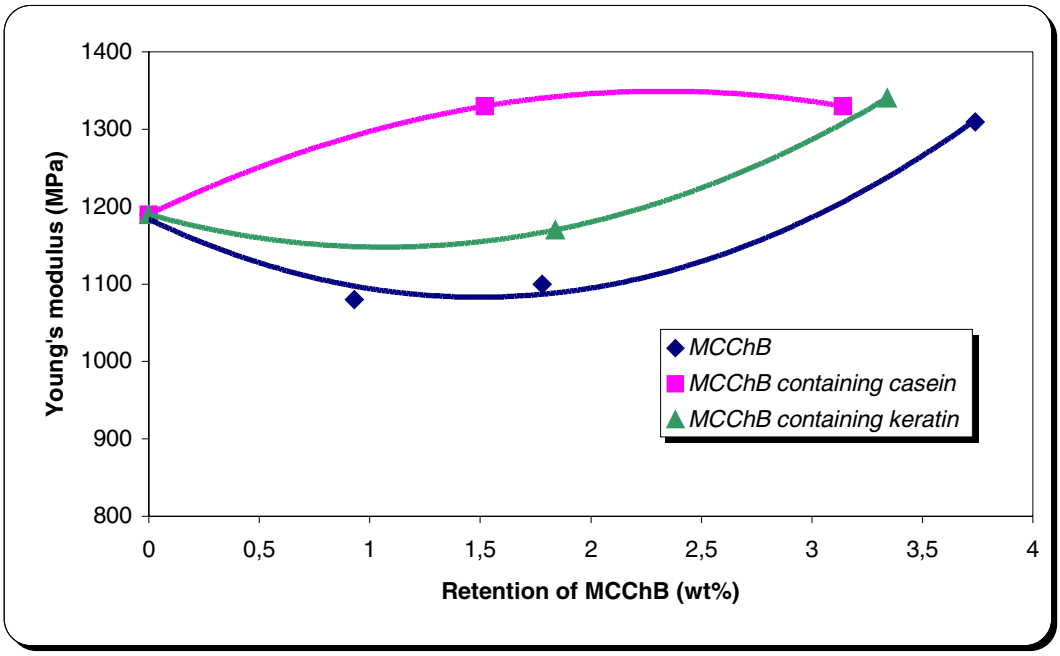



Figure 164. The effect of MCChB retention with or without proteins on Young's modulus of paper sheets prepared by the direct introduction method

The precipitation of MCChB containing proteins yielded a higher value of tensile strength of paper sheets, in contrast to the sheets obtained by the direct introduction method (Figure 165).

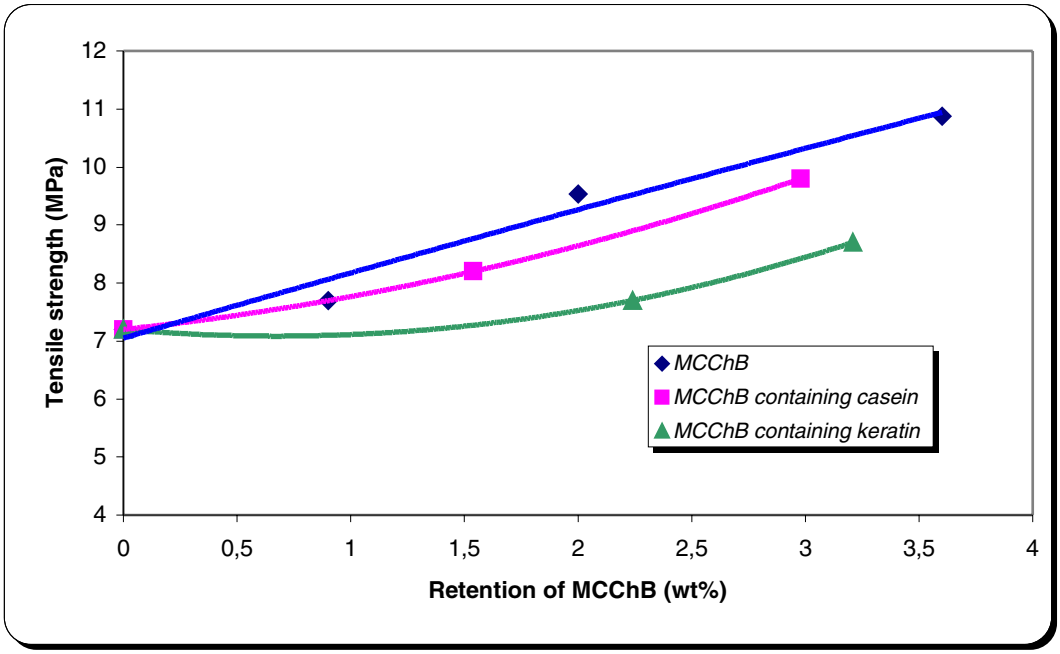



Figure 165. The effect of MCChB retention with or without proteins on tensile strength of paper sheets prepared by the precipitation method

Nevertheless, their parameters showed lower values than paper sheets formed by MCChB precipitation. The difference between tensile strength of paper sheets containing MCChB without protein and with introduced protein was lower in the case of presence of casein.

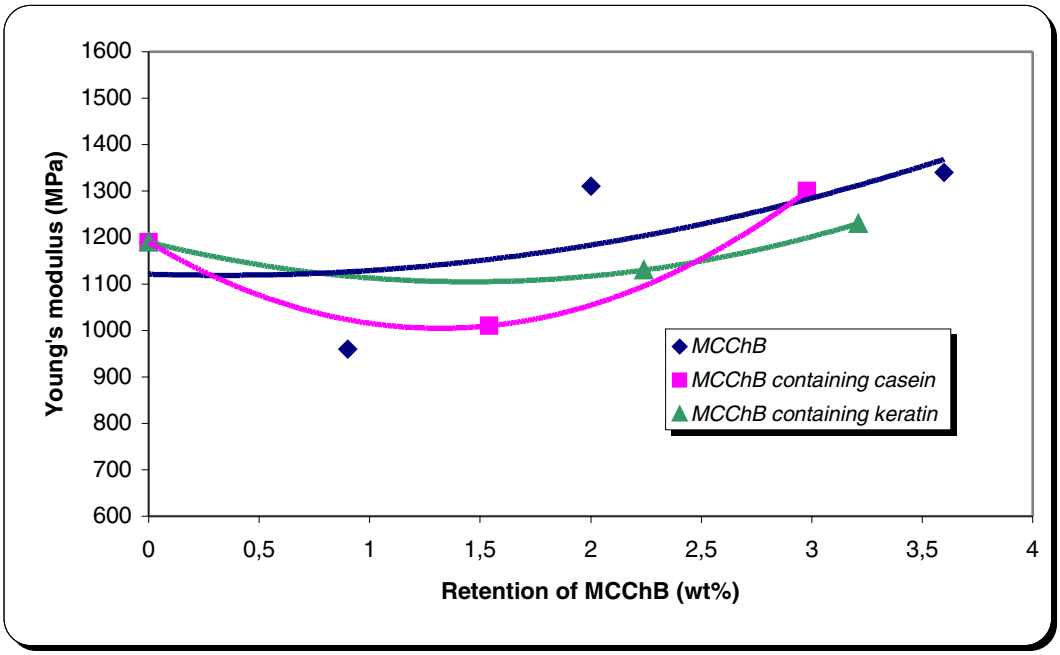



Figure 166. The effect of MCChB retention with or without proteins on Young's modulus of paper sheets prepared by the precipitation method

Lower Young's modulus have found with paper sheets containing MCChB with protein. However, the increase in MCChB retention resulted in an increase in this parameter. For the highest retention value of MCChB, the Young's modulus of paper sheets containing MCChB with casein corresponded to the parameter obtained for paper-MCChB sheets without incorporated proteins (Figure 166).

The protein molecules interacting with chitosan chains blocked the free amine groups of chitosan as well as breaking down the ionic and hydrogen bonds between chitosan and cellulose. This phenomenon resulted in lower values of tensile strength. However, paper sheets with MCChB-protein possessed significantly better mechanical properties than the control ones.

Paper sheets containing ca. 4 wt% of MCChB with proteins showed similar mechanical properties to paper sheets containing MCChB without protein.

4.13.5. Mechanical properties of paper containing MCChB gel-like dispersion at rel. humidity of 90%

Because of water effects the mechanical properties of paper, it was important to investigate its mechanical properties in high rel. humidity.
Increase in rel. humidity caused a decrease in tensile strength and Young's modulus of paper sheets and a weak increase in elongation at break. However, an increase in the mechanical properties compared to the reference was noted.
The decrease in tensile strength and Young's modulus has been observed for all paper sheets containing MCChB with proteins. The more aliphatic keratin affected considerable an increase in tensile strength compared to paper sheets with directly introduced MCChB (Table 71). The addition of protein resulted in smaller differences between the mechanical properties at low and high rel. humidity (Table 71, Figure 167).

Table 71. Mechanical properties of wet paper sheets produced with different amount of MCChB with or without protein at rel. humidity of 90%

| Concentration of MCChB | Elongation at break (%) | Tensile strength (MPa) | Young's modulus (GPa) |
|---|---|---|---|
| | *Direct introduction method* | | |
| Control (paper sheets without MCChB) | 3.20 ± 0.18 (178.8%) | 5.82 ± 0.17 (80.8%) | 0.75 ± 0.04 (63.1%) |
| 4.0 | 3.17± 0.35 (134.3%) | 7.54 ± 0.62 (71.4%) | 0.88 ± 0.05 (72.5%) |
| 4.0 containing casein | 3.11± 0.36 (178.7%) | 6.47 ± 0.35 (81.9%) | 0.69 ± 0.10 (52.0%) |
| 4.0 containing keratin | 2.84 ± 0.45 (142.0%) | 6.98 ± 0.37 (79.3%) | 0.79 ± 0.06 (59.3%) |
| | *Precipitation method* | | |
| Control (paper sheets without MCChB) | 3.20 ± 0.18 (178.8%) | 5.82 ± 0.17 (80.8%) | 0.75 ± 0.04 (63.1%) |
| 4.0 | 3.15 ± 0.52 (133.5%) | 8.20 ± 0.74 (75.4%) | 0.76 ± 0.13 (56.6%) |
| 4.0 containing casein | 3.66 ± 0.31 (166.4%) | 7.57 ± 0.40 (77.2%) | 0.71 ± 0.08 (54.4%) |
| 4.0 containing keratin | 3.63 ± 0.30 (181.5%) | 7.06 ± 0.24 (81.1%) | 0.73 ± 0.06 (59.3%) |

**(...%)** - Percent of original value (ratio of measured parameter at high rel. humidity to its obtained at low rel. humidity)

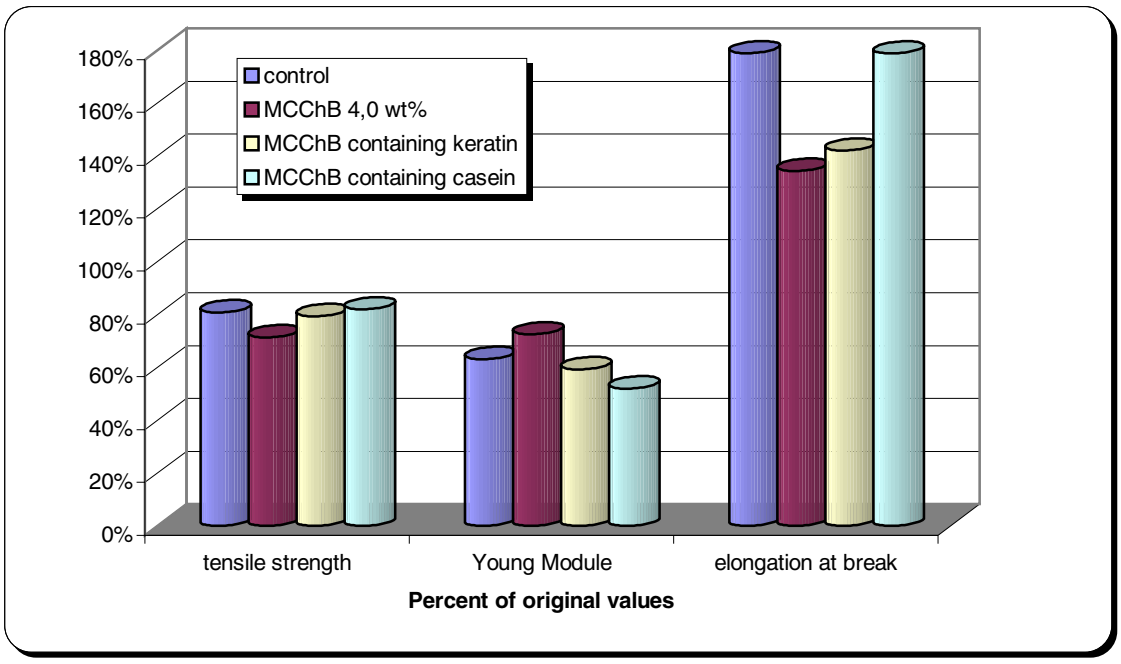



Figure 167. Percent of original value of mechanical properties of paper sheets measured at rel. humidity of 90% prepared by the MCChB directly introduction method

Similar observation was noted for paper sheets prepared by the precipitation methods. However, the lowest difference between the mechanical properties of dry and wet form was found for paper-MCChB containing keratin (Figure 168). The difference in Young's module among the paper-MCChB with and without protein is not notable.

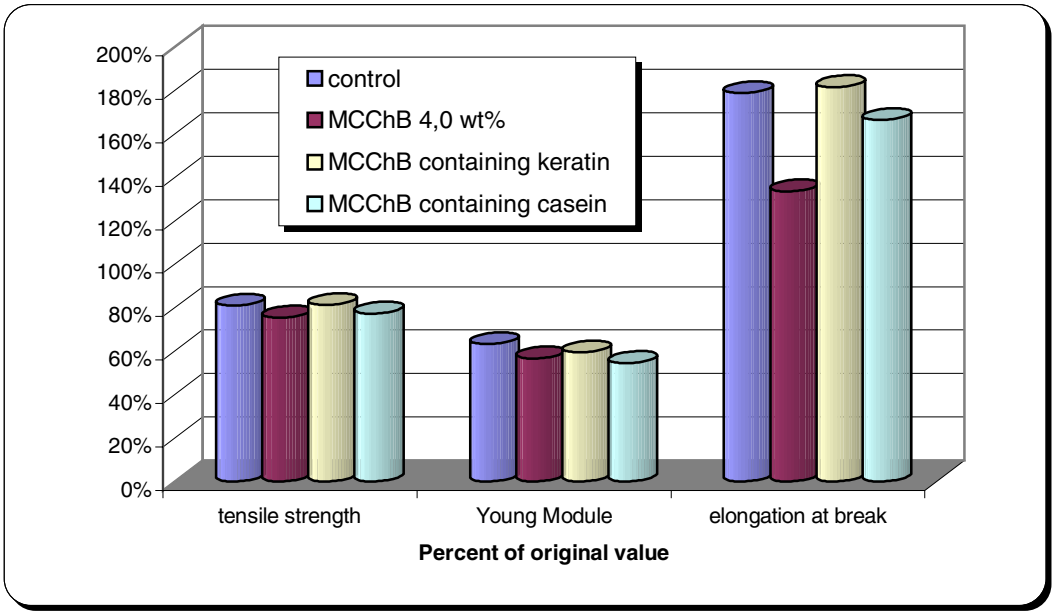



Figure 168. Percent of original value of mechanical properties of paper sheets at rel. humidity of 90% prepared by the MCChB precipitation method

4.13.6. Optical microscope investigation of water-swollen MCChB-cellulose paper containing protein

A crossection of the paper sheets allowed for their morphological analysis, as shown in Figures 169–174. The coat-like structure of MCChB was observed among the separated fibres of the paper sheets containing its high amount. At lower concentration of MCChB, 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 and the precipitation of MCChB gel-like dispersion preferred the formation of coating fibres. The coat-like fibres are often visible for the papers sheets prepared with introduction of protein.


Figure 169. Optical photograph (x100) of used cellulose fibres

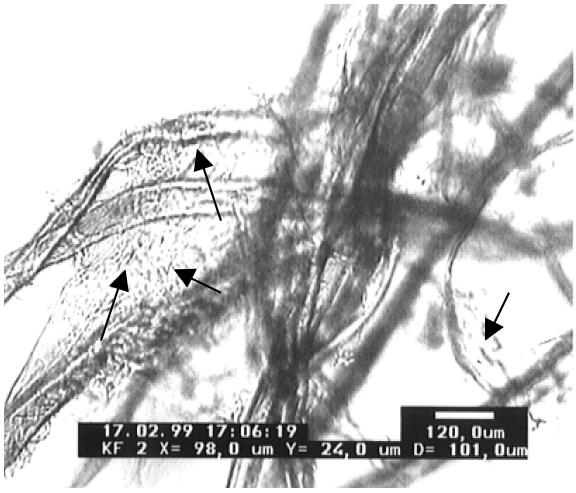



Figure 170. Optical photograph (x100) of used cellulose fibres with direct introduced MCChB gel-like dispersion prepared in a bath containing 20 wt% casein. ⇓ shows the web-like structure of MCChB

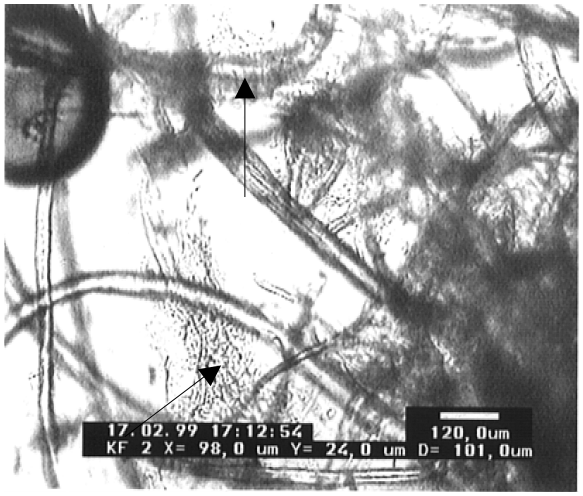



Figure 171. Optical photograph (x100) of used cellulose fibres with precipitated MCChB gel-like dispersion prepared in a bath containing 20 wt% casein. ⇓ shows the coated cellulose fibres by MCChB


Figure 172. Optical photograph (x100) of used cellulose fibre with precipitated MCChB gel-like dispersion prepared in a bath containing 20 wt% casein. ⇓ shows the coated cellulose fibres by MCChB

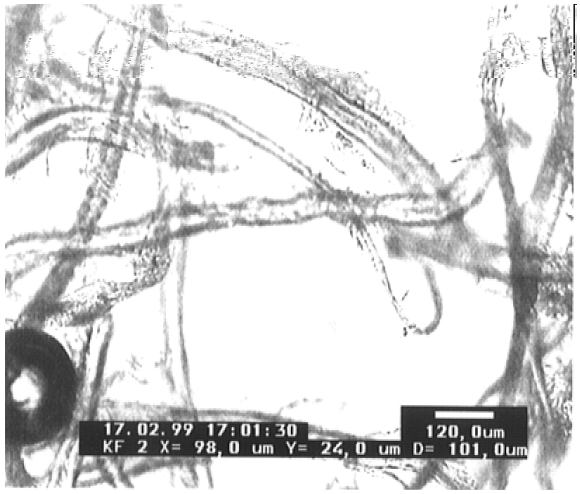



Figure 173. Optical photograph (x100) of used cellulose fibres with precipitated MCChB gel-like dispersion prepared in a bath containing 20 wt% keratin. ⇓ shows the coated cellulose fibres by MCChB


Figure 174. Optical photograph (x100) of used cellulose fibres with precipitated MCChB gel-like dispersion prepared in a bath containing 20 wt% keratin. ⇓ shows the coated cellulose fibres by MCChB

4.14. Biodegradation of chitosan films in the presence of the *Aspergillus fumigatus*

4.14.1. Incubation of chitosan films with *Aspergillus fumigatus*

Chitosan films prepared from acidic solution by drying and subsequent regeneration with aqueous NaOH with or without casein were used during this investigation to the biodegradation using fungus *Asp. fumigatus*.

During biodegradation, chitosan films became wrinkled shrunk, and incomplete at the edges or in the middle of the sample. They were decoloured and strongly deformed. A good susceptibility for biodegradation was observed to films showed low DD (ranging from 60% to 73%). Moreover, the susceptibility of chitosan films for degradation increased when initial chitosan with low DD was applied. The results of the biodegradation are presented in Tables 72-79. The films regenerated in an aqueous sodium hydroxide containing casein possessed the lowest degradation velocity as found in the reduction in viscosity and loss of weight. The films with the lowest WRV showed a decrease in their ability to degradation.

It suggests that the most important factor for film decomposition is the widely expanded internal surface of films. Low WRV, responsible for lower porosity and compact structure of films caused difficulty in the fungal enzymes penetration into the structure of films, which yields a slow decomposition. The longer time necessary for swelling of films prolonged the biodegradation. The increase in viscosity during the biodegradation of films with the lowest WRV leads to the conclusion that low $\bar{M}_v$ chitosan was released from the films into the fermentation bath. At the same time, the increase in the amount of high $\bar{M}_v$ polymer was found with the increase in viscosity as a function of $\bar{M}_v$.

Table 72. Biodegradation of chitosan films G3mA in the presence of *A. fumigatus*

| Sample | Time (week) | Intrinsic viscosity (dL/g) | WRV (%) | Wc (%) | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film (%) | Change in viscosity (%) |
|---|---|---|---|---|---|---|---|
| G3mA - 1% ethanol | 0 | 9.58 | 177.0 | 64.0 | -- | -- | -- |
| | 1 | 7.93 | 141.4 | 58.6 | 50.52 | 9.0 | 17.2 |
| | 2 | 6.30 | 130.7 | 56.7 | 70.38 | 15.1 | 34.2 |
| | 3 | 3.02 | 125.9 | 54.5 | 91.07 | 30.9 | 68.5 |
| | 4 | 2.55 | 88.2 | 46.9 | 186.57 | 33.4 | 73.4 |
| G3mA - 1% protein | 0 | 12.02 | 40.2 | 29.3 | -- | -- | -- |
| | 1 | 13.2 | 59.4 | 37.3 | 43.58 | 2.2 | -9.8 |
| | 2 | 14.50 | 54.2 | 35.2 | 64.77 | 4.7 | -20.6 |
| | 3 | 15.52 | 53.9 | 35.0 | 148.36 | 5.6 | -29.1 |
| | 4 | 14.52 | 35.0 | 25.9 | 198.58 | 6.3 | -20.8 |
| G3mA - 1% water | 0 | 14.26 | 136.1 | 58.0 | -- | -- | -- |
| | 1 | 8.43 | 114.7 | 53.4 | 28.68 | 4.8 | 40.9 |
| | 2 | 6.78 | 164.2 | 62.2 | 46.41 | 7.2 | 52.5 |
| | 3 | 3.75 | 136.4 | 57.7 | 68.99 | 15.1 | 73.7 |
| | 4 | 3.53 | 135.5 | 57.6 | 126.60 | 19.6 | 75.2 |
| G3mA - 2% ethanol | 0 | 12.61 | 185.9 | 65.8 | -- | -- | -- |
| | 1 | 10.87 | 186.5 | 65.1 | 20.51 | 5.5 | 13.8 |
| | 2 | 7.23 | 167.7 | 63.2 | 27.89 | 6.0 | 42.7 |
| | 3 | 7.27 | 152.9 | 60.5 | 64.34 | 7.1 | 42.3 |
| | 4 | 7.20 | 135.0 | 57.5 | 136.61 | 8.7 | 42.9 |
| G3mA - 2% protein | 0 | 11.68 | 103.8 | 50.8 | -- | -- | -- |
| | 1 | 11.89 | 82.8 | 45.3 | 26.35 | 2.3 | -1.8 |
| | 2 | 11.67 | 79.7 | 44.3 | 48.66 | 3.5 | 0.1 |
| | 3 | 10.93 | 76.2 | 44.0 | 64.41 | 3.9 | 6.4 |
| | 4 | 10.68 | 75.5 | 43.0 | 100.79 | 6.0 | 8.6 |
| G3mA - 2% water | 0 | 16.96 | 192.8 | 66.8 | -- | -- | -- |
| | 1 | 14.43 | 101.5 | 50.4 | 28.25 | 3.5 | 14.9 |
| | 2 | 12.74 | 102.8 | 50.7 | 41.09 | 4.1 | 24.9 |
| | 3 | 12.45 | 101.1 | 50.3 | 55.84 | 5.2 | 26.6 |
| | 4 | 12.42 | 92.9 | 48.2 | 87.67 | 6.0 | 26.8 |

Table 73. Biodegradation of chitosan films M3A in the presence of *Asp. fumigatus*

| Sample | Time | Intrinsic viscosity | WRV | Wc | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film | Change in viscosity |
|---|---|---|---|---|---|---|---|
| | (week) | (dL/g) | (%) | (%) | | (%) | (%) |
| M3A - 1% ethanol | 0 | 10.86 | 128.9 | 55.4 | -- | -- | -- |
| | 1 | 10.01 | 123.3 | 54.9 | 34.53 | 7.6 | 7.8 |
| | 2 | 9.28 | 119.9 | 54.5 | 41.86 | 23.0 | 14.5 |
| | 3 | 7.97 | 101.5 | 51.3 | 63.09 | 28.5 | 26.6 |
| | 4 | 7.37 | 74.4 | 42.7 | 263.39 | 29.8 | 32.1 |
| M3A - 1% protein | 0 | 11.79 | 102.9 | 51.3 | -- | -- | -- |
| | 1 | 13.00 | 168.8 | 62.8 | 69.15 | 6.8 | -10.3 |
| | 2 | 10.79 | 160.3 | 61.6 | 78.65 | 11.5 | 8.5 |
| | 3 | 8.03 | 145.7 | 59.3 | 101.90 | 59.3 | 31.9 |
| M3A - 1% water | 0 | 16.18 | 145.3 | 67.3 | -- | -- | -- |
| | 1 | 13.75 | 135.3 | 57.9 | 28.74 | 4.0 | 15.0 |
| | 2 | 13.78 | 121.3 | 54.6 | 44.97 | 4.5 | 14.8 |
| | 3 | 12.76 | 115.2 | 53.2 | 72.80 | 4.8 | 21.1 |
| | 4 | 8.29 | 107.2 | 51.7 | 137.75 | 9.0 | 48.8 |
| M3A - 2% ethanol | 0 | 10.09 | 132.8 | 57.6 | -- | -- | -- |
| | 1 | 9.05 | 123.2 | 54.8 | 36.32 | 7.4 | 10.3 |
| | 2 | 8.90 | 119.7 | 54.5 | 54.29 | 5.6 | 11.8 |
| | 3 | 8.19 | 117.2 | 54.3 | 108.21 | 8.3 | 18.8 |
| | 4 | 8.68 | 112.3 | 50.0 | 230.12 | 10.0 | 14.0 |
| M3A - 2% protein | 0 | 11.44 | 163.1 | 65.7 | -- | -- | -- |
| | 1 | 11.57 | 141.1 | 58.5 | 25.26 | 4.7 | -1.1 |
| | 2 | 14.54 | 143.5 | 58.9 | 37.54 | 4.4 | -27.1 |
| | 3 | 14.03 | 119.3 | 54.4 | 54.92 | 6.2 | -22.6 |
| | 4 | 13.32 | 134.6 | 57.4 | 78.62 | 7.9 | -16.4 |
| M3A - 2% water | 0 | 13.22 | 176.1 | 68.9 | -- | -- | -- |
| | 1 | 13.00 | 112.7 | 53.0 | 31.23 | 5.8 | 1.7 |
| | 2 | 12.87 | 102.4 | 50.6 | 54.47 | 4.4 | 2.6 |
| | 3 | 11.29 | 102.3 | 50.6 | 107.12 | 6.2 | 14.6 |
| | 4 | 10.06 | 104.3 | 51.1 | 229.37 | 8.1 | 23.9 |

Table 74. Biodegradation of chitosan films G3mB in the presence of *A. fumigatus*

| Sample | Time | Intrinsic viscosity | WRV | Wc | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film | Change in viscosity |
|---|---|---|---|---|---|---|---|
| | (week) | (dL/g) | (%) | (%) | | (%) | (%) |
| G3mB - 1% protein | 0 | 10.26 | 84.2 | 46.5 | -- | -- | -- |
| | 1 | 12.24 | 73.8 | 42.5 | 78.92 | 3.2 | -19.3 |
| | 2 | 12.54 | 63.3 | 38.8 | 131.84 | 4.5 | -22.2 |
| | 3 | 11.89 | 68.3 | 39.2 | 141.73 | 5.4 | -15.9 |
| | 4 | 10.24 | 71.1 | 41.6 | 413.80 | 8.2 | 0.2 |
| G3mB - 1% water | 0 | 10.75 | 114.3 | 53.2 | -- | -- | -- |
| | 1 | 12.93 | 98.2 | 42.2 | 122.13 | 2.3 | -20.3 |
| | 2 | 13.84 | 62.9 | 38.6 | 152.40 | 3.0 | -28.7 |
| | 3 | 13.74 | 99.7 | 49.9 | 231.03 | 6.3 | -27.8 |
| G3mB - 2% protein | 0 | 9.53 | 112.4 | 53.1 | -- | -- | -- |
| | 1 | 10.84 | 73.3 | 42.3 | 43.05 | 5.0 | -13.7 |
| | 2 | 9.70 | 51.0 | 33.8 | 88.42 | 2.8 | -1.8 |
| | 3 | 9.40 | 65.9 | 22.7 | 107.89 | 5.5 | 1.4 |
| | 4 | 9.41 | 66.1 | 39.8 | 338.99 | 4.9 | 1.3 |
| G3mB - 2% water | 0 | 13.56 | 121.8 | 55.1 | -- | -- | -- |
| | 1 | 16.06 | 63.9 | 39.0 | 35.77 | 4.7 | -18.4 |
| | 2 | 16.78 | 94.2 | 48.5 | 46.41 | 5.0 | -23.7 |
| | 3 | 16.79 | 65.8 | 39.7 | 72.84 | 4.9 | -23.2 |
| | 4 | 14.59 | 71.6 | 41.7 | 197.80 | 8.5 | -6.9 |
| G3mB -1% ethanol | 0 | 13.25 | 104.7 | 50.6 | -- | -- | -- |
| | 1 | 12.39 | 80.1 | 44.5 | 96.12 | 2.9 | 6.5 |
| | 2 | 10.12 | 76.7 | 43.4 | 120.63 | 5.7 | 23.6 |
| | 3 | 9.88 | 75.8 | 43.0 | 220.61 | 6.2 | 25.4 |
| | 4 | 7.08 | 79.8 | 44.4 | 421.00 | 8.2 | 46.6 |

Table 75. Biodegradation of chitosan films M1mC in the presence of *A. fumigatus*

| Sample | Time | Intrinsic viscosity | WRV | Wc | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film | Change in viscosity |
|---|---|---|---|---|---|---|---|
| | (week) | (dL/g) | (%) | (%) | | (%) | (%) |
| M1mC - 2% ethanol | 0 | 11.44 | 109.2 | 52.1 | -- | -- | -- |
| | 1 | 13.68 | 120.6 | 54.7 | 26.55 | 2.5 | -19.6 |
| | 2 | 13.02 | 150.7 | 60.1 | 41.29 | 4.7 | -13.8 |
| | 3 | 12.36 | 142.2 | 58.7 | 70.04 | 5.5 | -8.0 |
| | 4 | 6.29 | 111.2 | 49.3 | 162.03 | 6.9 | 45.0 |
| M1mC - 2% protein | 0 | 8.98 | 114.7 | 53.5 | -- | -- | -- |
| | 1 | 8.81 | 110.2 | 53.2 | 44.42 | 3.8 | 1.9 |
| | 2 | 8.35 | 114.8 | 53.5 | 79.84 | 2.4 | 7.0 |
| | 3 | 8.07 | 79.0 | 44.1 | 149.14 | 3.9 | 10.1 |
| | 4 | 7.89 | 0.0 | 0.0 | 266.97 | 0.0 | 12.1 |
| M1mC - 1% water | 0 | 11.33 | 162.2 | 61.9 | -- | -- | -- |
| | 1 | 11.6 | 125.7 | 55.7 | 41.93 | 5.5 | -2.4 |
| | 2 | 13.62 | 131.2 | 57.1 | 52.70 | 7.4 | -20.2 |
| | 3 | 13.94 | 147.5 | 59.6 | 82.26 | 9.3 | -23.0 |
| | 4 | 11.2 | 123.2 | 56.0 | 136.08 | 0.0 | 1.1 |
| M1mC - 2% water | 0 | 12.08 | 117.2 | 54.2 | -- | -- | -- |
| | 1 | 10.91 | 86.1 | 46.3 | 65.42 | 3.6 | 9.7 |
| | 2 | 11.24 | 102.8 | 50.7 | 127.11 | 4.5 | 7.0 |
| | 3 | 10.79 | 92.9 | 48.2 | 221.47 | 5.2 | 10.7 |
| | 4 | 9.55 | 92.4 | 48.0 | 340.29 | 5.2 | 20.9 |

Table 76. Biodegradation of chitosan films M6mC in the presence of *A. fumigatus*

| Sample | Time | Intrinsic viscosity | WRV | Wc | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film | Change in viscosity |
|---|---|---|---|---|---|---|---|
| | (week) | (dL/g) | (%) | (%) | | (%) | (%) |
| M6mC - 1% protein | 0 | 9.56 | 106.2 | 51.2 | -- | -- | -- |
| | 1 | 9.45 | 138.6 | 58.1 | 51.81 | 1.87 | 1.2 |
| | 2 | 8.90 | 136.8 | 57.8 | 63.75 | 3.38 | 6.9 |
| | 3 | 8.15 | 102.3 | 50.6 | 114.62 | 4.47 | 14.7 |
| | 4 | 8.10 | 51.3 | 33.9 | 270.42 | 9.97 | 15.3 |
| M6mC - 2% ethanol | 0 | 7.47 | 101.2 | 49.6 | -- | -- | -- |
| | 1 | 9.19 | 67.5 | 40.3 | 18.83 | 5.9 | -23.0 |
| | 2 | 9.20 | 88.1 | 46.8 | 27.02 | 17.4 | -23.2 |
| | 3 | 8.23 | 89.1 | 47.1 | 37.66 | 22.3 | -10.2 |
| | 4 | 7.78 | 100.7 | 50.2 | 65.36 | 22.3 | -4.1 |
| M6mC - 2% protein | 0 | 9.16 | 114.7 | 54.3 | -- | -- | -- |
| | 1 | 10.11 | 117.2 | 54.0 | 44.42 | 5.0 | -10.4 |
| | 2 | 10.44 | 122.3 | 55.0 | 79.84 | 5.5 | -14.0 |
| | 3 | 10.85 | 145.3 | 59.2 | 149.14 | 7.0 | -18.4 |
| M6mC - 2% protein * | 0 | 8.58 | 106.5 | 54.0 | -- | -- | -- |
| | 1 | 8.24 | 102.1 | 50.5 | 30.80 | 4.4 | 4.0 |
| | 2 | 8.43 | 91.2 | 47.7 | 29.70 | 4.7 | 1.7 |
| | 3 | 8.56 | 83.1 | 45.4 | 61.89 | 4.9 | 0.2 |
| | 4 | 7.75 | 108.3 | 52.0 | 93.96 | 7.3 | 9.7 |
| M6mC - 2% protein ** | 0 | 8.03 | 79.5 | 45.2 | -- | -- | -- |
| | 1 | 8.01 | 54.3 | 35.2 | 24.39 | 4.1 | 0.2 |
| | 2 | 7.93 | 69.7 | 41.1 | 27.71 | 4.4 | 1.2 |
| | 3 | 7.50 | 88.4 | 46.9 | 52.39 | 4.8 | 6.6 |
| | 4 | 7.57 | 92.4 | 48.0 | 81.69 | 6.8 | 5.7 |
| M6mC - 2% water | 0 | 11.03 | 117.2 | 54.2 | -- | -- | -- |
| | 1 | 11.52 | 82.0 | 45.1 | 20.60 | 4.5 | -4.4 |
| | 2 | 9.97 | 80.5 | 44.6 | 25.90 | 4.5 | 9.6 |
| | 3 | 9.16 | 68.1 | 40.5 | 31.77 | 5.0 | 17.0 |
| | 4 | 7.04 | 71.4 | 41.7 | 51.10 | 5.7 | 36.2 |

* - films regenerated 48 h in 5 wt% NaOH aq. containing casein
** - films regenerated 72 h in 5 wt% NaOH aq. containing casein

Table 77. Biodegradation of chitosan films M3mB in the presence of *A. fumigatus*

| Sample | Time | Intrinsic viscosity | WRV | Wc | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film | Change in viscosity |
|---|---|---|---|---|---|---|---|
| | (week) | (dL/g) | (%) | (%) | | (%) | (%) |
| M3mB - 1% ethanol | 0 | 9.63 | 118.3 | 54.2 | -- | -- | -- |
| | 1 | 5.94 | 115.6 | 53.6 | 32.99 | 3.4 | 38.3 |
| | 2 | 4.58 | 112.6 | 53.0 | 43.68 | 9.3 | 52.4 |
| | 3 | 3.64 | 104.8 | 51.2 | 78.40 | 31.2 | 62.2 |
| | 4 | 3.34 | 109.2 | 52.2 | 100.65 | 32.6 | 65.3 |
| M3mB - 1% water | 0 | 8.83 | 162.1 | 62.7 | ---- | -- | -- |
| | 1 | 6.96 | 119.8 | 54.5 | 64.82 | 6.5 | 21.2 |
| | 2 | 4.94 | 105.2 | 51.3 | 124.58 | 4.3 | 44.1 |
| | 3 | 4.44 | 101.9 | 50.5 | 193.28 | 5.2 | 49.7 |
| | 4 | 4.34 | 100.0 | 50.3 | 501.08 | 28.8 | 50.8 |
| M3mB- 1% protein | 0 | 9.95 | 90.0 | 48.9 | -- | -- | -- |
| | 1 | 11.78 | 165.0 | 62.3 | 67.06 | 6.3 | -18.4 |
| | 2 | 11.42 | 124.0 | 55.4 | 91.08 | 6.5 | -14.8 |
| | 3 | 13.03 | 142.4 | 58.8 | 216.81 | 8.0 | -31.0 |
| | 4 | 12.64 | 133.3 | 57.1 | 446.69 | 9.0 | -27.0 |
| M3mB - 2% ethanol | 0 | 9.37 | 116.2 | 54.0 | -- | -- | -- |
| | 1 | 9.07 | 102.7 | 50.7 | 20.98 | 5.1 | 3.2 |
| | 2 | 8.19 | 112.8 | 53.0 | 25.23 | 5.0 | 12.6 |
| | 3 | 7.23 | 95.4 | 48.8 | 35.50 | 7.9 | 22.8 |
| | 4 | 7.03 | 97.5 | 49.4 | 78.22 | 8.4 | 25.0 |
| M3mB - 2% protein | 0 | 12.92 | 112.3 | 53.1 | -- | -- | -- |
| | 1 | 12.13 | 84.3 | 45.7 | 20.98 | 2.6 | 6.1 |
| | 2 | 11.05 | 95.1 | 48.7 | 27.91 | 2.8 | 14.5 |
| | 3 | 12.32 | 84.7 | 45.9 | 33.36 | 3.1 | 4.6 |
| | 4 | 12.27 | 87.2 | 46.6 | 95.92 | 3.2 | 5.0 |
| M3mB - 2% water | 0 | 11.85 | 198.2 | 70.9 | -- | -- | -- |
| | 1 | 11.19 | 152.7 | 60.4 | 38.92 | 3.9 | 5.6 |
| | 2 | 10.78 | 126.5 | 55.9 | 71.74 | 3.4 | 9.0 |
| | 3 | 10.73 | 115.8 | 53.7 | 94.77 | 5.0 | 9.5 |
| | 4 | 9.45 | 62.1 | 38.3 | 272.83 | 67.3 | 20.3 |

Table 78. Biodegradation of chitosan films PB08 in the presence of *A. fumigatus*

| Sample | Time (week) | Intrinsic viscosity (dL/g) | WRV (%) | Wc (%) | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film (%) | Change in viscosity (%) |
|---|---|---|---|---|---|---|---|
| PB 08 - 1% ethanol | 0 | 10.39 | 137.2 | 58.3 | -- | -- | -- |
| | 1 | 9.95 | 112.3 | 54.8 | 49.76 | 4.7 | 4.2 |
| | 2 | 9.10 | 78.5 | 44.0 | 46.80 | 5.0 | 12.4 |
| | 3 | 7.67 | 81.2 | 44.0 | 77.68 | 4.5 | 26.2 |
| | 4 | 7.6 | 91.7 | 47.8 | 84.61 | 4.2 | 26.9 |
| PB 08 - 1% protein | 0 | 11.10 | 63.2 | 39.2 | -- | -- | -- |
| | 1 | 11.20 | 62.2 | 38.3 | 49.16 | 3.3 | -0.9 |
| | 2 | 10.70 | 60.1 | 37.5 | 41.97 | 5.6 | 3.6 |
| | 3 | 10.51 | 65.9 | 39.7 | 112.00 | 8.8 | 5.3 |
| | 4 | 10.29 | 60.0 | 37.5 | 126.54 | 8.8 | 7.3 |
| PB 08 - 1% water | 0 | 12.10 | 101.2 | 49.6 | -- | -- | -- |
| | 1 | 11.65 | 88.2 | 44.7 | 42.52 | 3.0 | 3.7 |
| | 2 | 11.01 | 89.2 | 44.8 | 30.00 | 4.2 | 9.0 |
| | 3 | 10.08 | 67.8 | 40.4 | 54.28 | 4.8 | 16.7 |
| | 4 | 9.90 | 88.1 | 46.8 | 56.11 | 6.1 | 18.2 |
| PB 08 - 2% ethanol | 0 | 15.19 | 139.2 | 57.9 | -- | -- | -- |
| | 1 | 13.46 | 101.3 | 49.7 | 47.69 | 8.1 | 11.4 |
| | 2 | 10.30 | 94.1 | 48.5 | 30.37 | 8.6 | 32.2 |
| | 3 | 8.92 | 93.6 | 48.3 | 52.14 | 8.6 | 41.3 |
| | 4 | 6.38 | 97.1 | 49.3 | 90.59 | 8.6 | 58.0 |
| PB 08 - 2% protein | 0 | 14.61 | 63.2 | 39.2 | -- | -- | -- |
| | 1 | 13.55 | 88.2 | 46.9 | 53.26 | 0.3 | 7.3 |
| | 2 | 14.86 | 80.6 | 44.6 | 31.73 | 3.6 | -1.7 |
| | 3 | 14.90 | 74.4 | 42.7 | 95.62 | 4.5 | -2.0 |
| | 4 | 16.14 | 63.8 | 39.0 | 103.25 | 6.1 | -10.5 |
| PB 08 - 2% water | 0 | 18.66 | 109.2 | 52.0 | -- | -- | -- |
| | 1 | 17.80 | 93.2 | 48.6 | 29.22 | 3.4 | 4.6 |
| | 2 | 16.27 | 88.2 | 46.9 | 57.40 | 4.0 | 12.8 |
| | 3 | 16.34 | 73.8 | 42.5 | 75.36 | 4.5 | 12.4 |
| | 4 | 15.20 | 0.0 | 0.0 | 144.31 | -- | 18.5 |

Table 79. Biodegradation of chitosan films PB10 in the presence of *A. fumigatus*

| Sample | Time | Intrinsic viscosity | WRV | Wc | Reducing sugar content (mg of reducing sugar/g of chitosan films) | Weight loss of film | Change in viscosity |
|---|---|---|---|---|---|---|---|
| | (week) | (dL/g) | (%) | (%) | | (%) | (%) |
| PB 10 - 1% ethanol | 0 | 11.93 | 111.2 | 52.9 | -- | -- | -- |
| | 1 | 11.19 | 66.6 | 40.0 | 30.79 | 3.4 | 6.2 |
| | 2 | 10.18 | 70.2 | 41.2 | 41.99 | 5.0 | 15.3 |
| | 3 | 8.65 | 66.1 | 39.8 | 58.89 | 5.6 | 27.5 |
| | 4 | 8.54 | 70.3 | 27.3 | 111.46 | 9.1 | 28.4 |
| PB 10 - 1% protein | 0 | 11.82 | 40.5 | 29.3 | -- | -- | -- |
| | 1 | 11.00 | 59.8 | 37.4 | 33.23 | 1.1 | 6.9 |
| | 2 | 10.8 | 55.8 | 35.8 | 44.75 | 3.2 | 8.6 |
| | 3 | 10.81 | 57.4 | 36.5 | 63.90 | 7.6 | 8.5 |
| | 4 | 10.93 | 51.4 | 33.2 | 135.75 | 7.7 | 7.5 |
| PB 10 - 1% water | 0 | 13.89 | 104.3 | 51.0 | -- | -- | -- |
| | 1 | 13.18 | 75.3 | 43.0 | 24.98 | 2.3 | 5.1 |
| | 2 | 14.59 | 64.4 | 39.2 | 33.35 | 4.4 | -5.0 |
| | 3 | 14.44 | 62.7 | 38.5 | 88.18 | 10.4 | -4.0 |
| | 4 | 14.40 | 81.4 | 44.9 | 94.76 | 34.2 | -3.7 |
| PB 10 - 2% ethanol | 0 | 8.18 | 97.2 | 48.7 | -- | -- | -- |
| | 1 | 7.36 | 80.0 | 44.6 | 45.47 | 4.8 | 10.0 |
| | 2 | 7.01 | 74.6 | 42.7 | 50.96 | 5.1 | 14.3 |
| | 3 | 4.95 | 77.8 | 43.7 | 97.03 | 7.2 | 39.5 |
| PB 10 - 2% protein | 0 | 8.61 | 86.2 | 45.9 | -- | -- | -- |
| | 1 | 8.76 | 67.4 | 40.3 | 27.90 | 2.9 | -1.7 |
| | 2 | 8.20 | 60.1 | 37.5 | 45.41 | 2.4 | 4.8 |
| | 3 | 7.80 | 77.5 | 43.7 | 72.02 | 4.0 | 9.4 |
| | 4 | 7.60 | 74.8 | 42.8 | 164.72 | 4.2 | 11.7 |
| PB 10 - 2% water | 0 | 13.35 | 153.2 | 60.7 | -- | -- | -- |
| | 1 | 13.10 | 105.6 | 51.4 | 30.52 | 3.8 | 1.9 |
| | 2 | 12.24 | 93.4 | 48.0 | 21.98 | 4.5 | 8.3 |
| | 3 | 12.00 | 91.7 | 47.8 | 53.57 | 4.2 | 10.1 |
| | 4 | 11.8 | 78.2 | 43.0 | 123.89 | -- | 11.6 |

Chitosan films regenerated in an ethanolic solution of sodium hydroxide as the coagulation bath indicated a better susceptibility to decomposition, which was especially visible for films showing the lowest DD. This observation probably resulted from the type of the regeneration medium, which modified the structure of films. Prolonged regeneration in a solution of sodium hydroxide containing casein showed a stronger decrease in viscosity during biodegradation. A 2-4 times higher weight loss of films was observed than degradation using lysozyme as was compared in literature[261].

4.14.2. Incubation of MCChB films with or without proteins by *Aspergillus fumigatus*

The study aimed at the estimation of the effect of protein addition during the coagulation of MCChB gel-like dispersion on the susceptibility to the biodegradation of MCChB films using fungus *Asp. fumigatus*.

A relative fast biodegradation was shown by MCChB films containing of 20 wt% casein or 33 wt% keratin in a coagulation bath. The increase in protein content did not lead to faster decomposition of films (Table 80, Figure 175).

Table 80. Degradation yield of MCChB films with or without protein, calculated on the basis of the amounts of reducing sugar

| Sample | Concentration of protein[a] (wt%) | Yield of biodegradation [mol of reducing sugars / mol of monomer content (GlcN + GlcNAc) of the respective chitosan film (%)] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Incubation time (days) | | 4 | 8 | 10 | 13 | 16 | 22 | 25 | 28 |
| Ca 0.12 | 11 | 2.55% | 4.62% | 4.55% | 4.64% | 4.46% | 3.68% | 4.43% | 4.64% |
| Ca 0.25 | 20 | 2.44% | 5.34% | 5.72% | 5.69% | 5.47% | 7.09% | 6.66% | 6.80% |
| Ca 0.5 | 33 | 2.16% | 4.38% | 4.77% | 5.19% | 5.32% | 5.64% | 5.71% | 5.86% |
| Kr 0.12 | 11 | 1.93% | 5.14% | 5.06% | 4.62% | 5.16% | 4.24% | 4.41% | 4.75% |
| Kr 0.25 | 20 | 2.54% | 6.49% | 7.22% | 6.41% | 6.52% | 7.06% | 6.72% | 7.07% |
| Kr 0.5 | 33 | 4.03% | 7.78% | 7.91% | 7.83% | 7.71% | 7.76% | 8.07% | 8.98% |
| Kr 1.0 | 50 | 2.71% | 5.46% | 5.52% | 5.37% | 5.31% | 5.28% | 5.51% | 6.03% |
| Kr 1.5 | 60 | 2.90% | 5.93% | 5.96% | 5.56% | 5.67% | 5.55% | 5.72% | 6.33% |
| MCChB | 0 | 1.88% | 5.24% | 5.61% | 4.60% | 4.81% | 4.89% | 4.86% | 5.20% |

[a] - concentration of protein in a coagulation bath

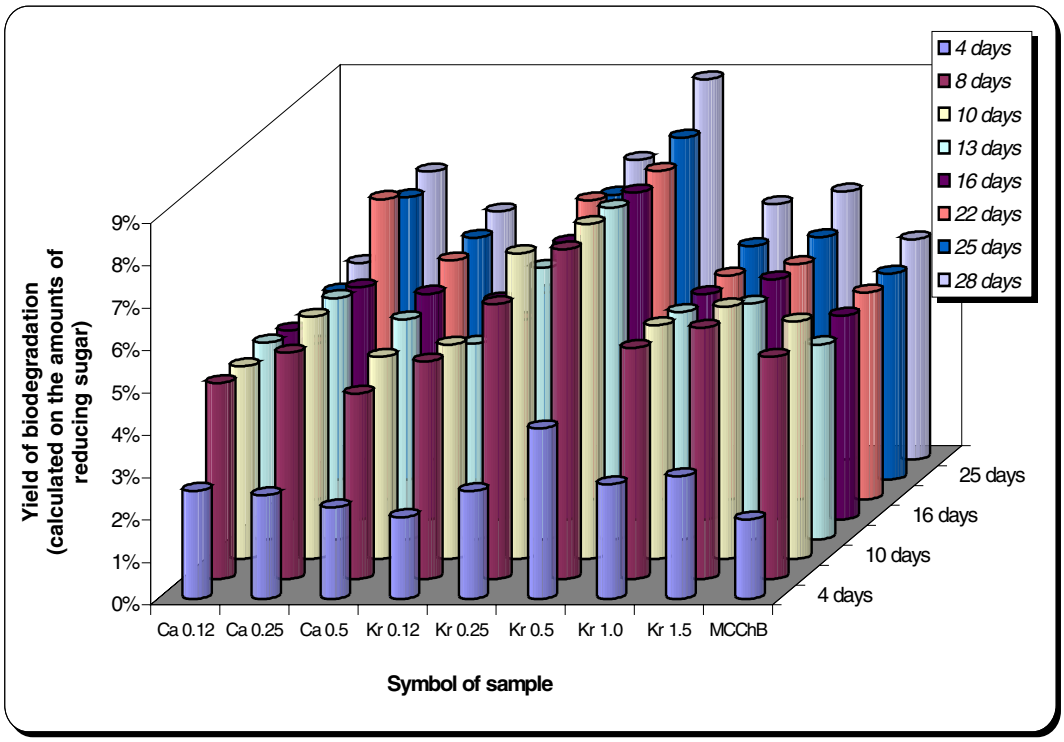



Figure 175. Yield of biodegradation [mol of reducing sugars / mol of monomer content (GlcN + GlcNAc) of the respective MCChB film (%)]

The highest amount of reducing sugars was detected in a medium containing films prepared in the presence of 20 wt% (casein) or 33 wt% (keratin) proteins. This fact suggests that the introduction of protein caused more rapid decomposition of films. Films containing lower amounts of keratin decomposed faster. Decolouration was found for MCChB films containing higher amounts of keratin (concentrations of protein in the coagulation bath in the range from 33 wt% to 60 wt%). It was observed that the fungi grew on the film and its medium.

4.15. Biological decomposition of MCChB films and paper sheets containing MCChB with or without proteins in communal waste (mineralization test)

The aim of this investigation is to determine the susceptibility for the biodecomposition of MCChB films with or without proteins as well as paper sheets prepared by the direct introduction and precipitation of MCChB with or without proteins.

During biological decomposition of MCChB films, a significant effect of the protein addition on the decomposition velocity was observed (Table 81, Figure 176).
Moreover, the highest swelling showed an increase in the biodegradation rate, as noted by the application of casein. Introduction of keratin caused a 5-10% faster decomposition compared to the MCChB film without protein. However, the WRV and Cs of MCChB films with keratin were considerably lower than those investigated for MCChB film without protein. It could be concluded that the introduction of proteins, especially casein, gave a better swelling behaviour and caused a strong increase in the susceptibility for biodegradation of MCChB films.

Table 81. Biochemical oxygen demand for MCChB films possibly prepared in the presence of 20 wt% proteins (keratin or casein)

| Decomposition time (hours) | Biochemical oxygen demand (mg $O_2$/100 mg of investigated sample) | | | |
|---|---|---|---|---|
| | Inoculum[1] | MCChB film[1] | Casein-MCChB film[1] | Keratin-MCChB film[1] |
| 24 | 4.5 | 6.0 | 10.0 | 6.0 |
| 48 | 6.5 | 17.0 | 26.0 | 20.0 |
| 72 | 8.0 | 28.0 | 37.0 | 29.0 |
| 96 | 10.0 | 32.0 | 45.0 | 33.5 |
| 120 | 11.5 | 38.0 | 53.0 | 40.5 |
| 144 | 13.0 | 43.0 | 61.0 | 46.0 |
| 168 | 13.5 | 45.0 | 67.0 | 49.0 |
| 192 | 15.0 | 47.0 | 71.0 | 52.5 |
| 216 | 16.5 | 49.0 | 75.0 | 54.5 |
| 240 | 16.5 | 50.0 | 79.0 | 56.5 |
| 264 | 18.0 | 50.5 | 81.0 | 58.0 |
| 288 | 19.0 | 52.5 | 85.0 | 59.5 |
| 312 | 20.0 | 54.0 | 87.0 | 61.0 |
| 336 | 21.0 | 55.0 | 89.0 | 62.0 |
| 360 | 22.0 | 56.0 | 93.0 | 63.0 |
| 384 | 23.0 | 57.0 | 93.0 | 63.0 |
| 408 | 23.0 | 57.0 | 95.0 | 63.5 |
| 432 | 23.0 | 57.0 | 97.0 | 65.0 |
| 456 | 23.0 | 57.5 | 99.0 | 65.5 |

[1] – average value of two repetitions

The increase in decomposition of paper sheets caused the application of MCChB (65%-83%) significantly higher than was observed for the films and cellulose paper used as a control (Table 82, Figure 177).

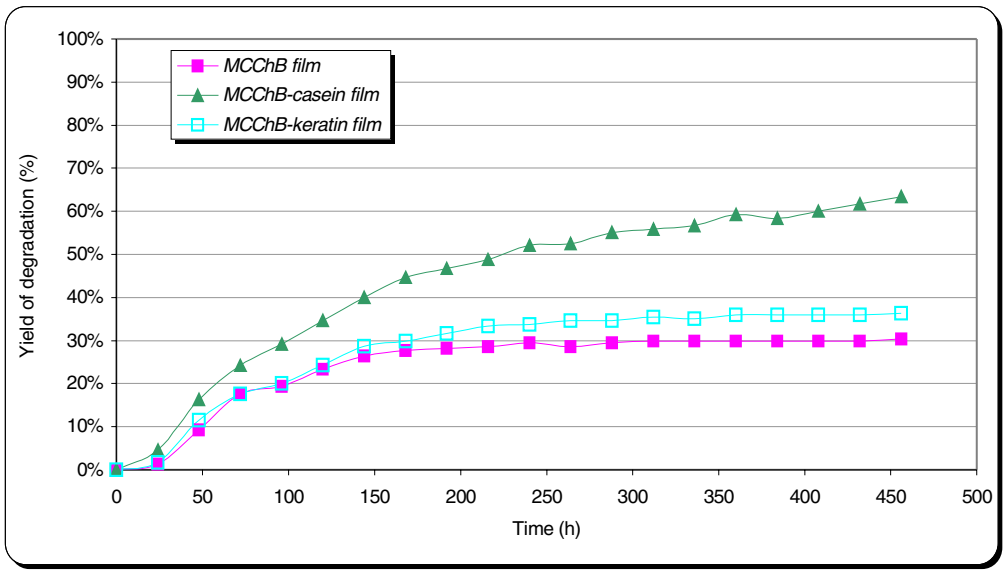



Figure 176. Biological decomposition of MCChB films with or without proteins (casein or keratin)

Table 82. Biochemical oxygen demand for paper sheets modified by MCChB by the precipitation or direct introduction method

| Decomposition time (hours) | Biochemical oxygen demand (mg $O_2$/100 mg of investigated sample) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Inoculum[1] | Control (paper) [1] | Paper-MCChBP[1] | Paper-MCChB-caseinP[1] | Paper-MCChB-keratinP[1] | Paper-MCChBD [1] | Paper-MCChB-caseinD[1] | Paper-MCChB-keratinD[1] |
| 24 | 4.5 | 5.0 | 4.5 | 3.5 | 3.5 | 5.0 | 4.0 | 5.0 |
| 48 | 6.5 | 7.5 | 7.0 | 8.0 | 7.0 | 8.0 | 7.0 | 8.5 |
| 72 | 8.0 | 9.5 | 8.0 | 9.5 | 9.5 | 10.5 | 9.0 | 10.5 |
| 96 | 10.0 | 12.0 | 10.5 | 10.5 | 11.5 | 13.0 | 13.0 | 13.5 |
| 120 | 11.5 | 16.5 | 12.0 | 13.5 | 14.5 | 24.5 | 20.0 | 18.5 |
| 144 | 13.0 | 25.0 | 15.0 | 13.5 | 17.5 | 39.0 | 29.5 | 26.5 |
| 168 | 13.5 | 40.0 | 19.0 | 17.0 | 25.0 | 57.5 | 42.5 | 41.0 |
| 192 | 15.0 | 57.5 | 27.0 | 17.0 | 40.0 | 73.5 | 56.5 | 58.0 |
| 216 | 16.5 | 73.5 | 38.5 | 21.0 | 53.5 | 84.0 | 70.5 | 74.0 |
| 240 | 16.5 | 83.5 | 52.5 | 29.0 | 71.0 | 92.0 | 81.5 | 85.0 |
| 264 | 18.0 | 91.0 | 67.0 | 36.5 | 84.5 | 97.0 | 89.0 | 92.0 |
| 288 | 19.0 | 97.5 | 79.0 | 48.0 | 93.0 | 101.0 | 94.5 | 97.5 |
| 312 | 20.0 | 102.0 | 87.0 | 58.5 | 99.5 | 105.0 | 99.5 | 101.5 |
| 336 | 21.0 | 105.5 | 93.0 | 70.0 | 102.5 | 107.5 | 103.5 | 105.0 |
| 360 | 22.0 | 109.0 | 97.0 | 80.5 | 108.5 | 110.0 | 106.5 | 108.0 |
| 384 | 23.0 | 111.0 | 101.0 | 87.0 | 110.5 | 111.5 | 108.5 | 110.0 |
| 408 | 23.0 | 113.0 | 103.0 | 92.5 | 112.0 | 113.0 | 110.5 | 112.5 |
| 432 | 23.0 | 115.5 | 105.0 | 96.0 | 115.0 | 115.0 | 113.0 | 114.0 |
| 456 | 23.0 | 117.5 | 108.0 | 99.0 | 116.5 | 115.5 | 115.0 | 115.0 |

[1] – average value of two repetitions; P – precipitated MCChB, D – direct introduced MCChB

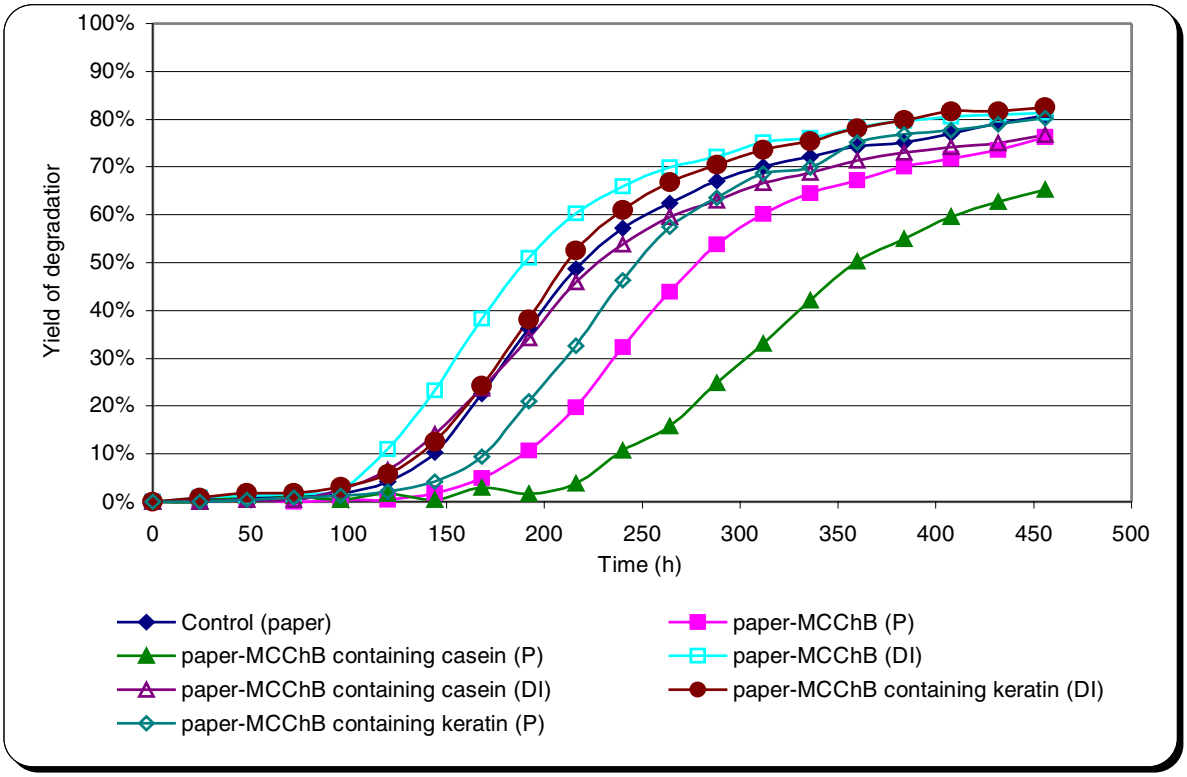



Figure 177. Biological decomposition of paper sheets prepared by the direct introduction (DI) or precipitation (P) of MCChB with or without proteins

The susceptibility to biodecomposition depended mainly on the method of MCChB addition applied. Moreover, the effect of the increase in swelling behaviour was also shown for acceleration of the degradation of the unmodified MCChB. Paper sheets prepared by the direct introduction of MCChB, having higher WRV and Cs, showed greater susceptibility. This phenomenon is confirmed by the

observation carried out by optical microscope. The coat-like composition of precipitated MCChB onto cellulose fibres affected a decrease in the decomposition, but web-like structure caused more rapid biodegradation. The addition of protein to coagulation bath caused increase in the decomposition time of paper sheets especially for the precipitated MCChB containing casein.

Direct introduction of MCChB caused an increase in the degradation level of 5-10% as compared to cellulose paper (reference) and precipitated MCChB reduced it by 10-20% from that of the reference sample. The MCChB films showed a composting rate at least 40-78% of that calculated for cellulose.

## 5. Conclusions

1. Chitosan films prepared in sodium hydroxide solution in ethanol show better solubility in aqueous acetic acid than films regenerated with aqueous NaOH. The concentration of the initial chitosan affects the properties of films, except those films regenerated with an ethanolic alkali solution as a coagulation bath (Chapter II, section 4.1.).
2. Coagulation of films in aqueous media containing sodium hydroxide results in an increase in tensile strength. However, the elongation at break of films regenerated in ethanol media is significantly better. Their mechanical strength depends on the methods of chitosan preparation: films prepared from insects microcrystalline chitosan showed significantly higher tensile strength and elasticity than films prepared from crustacean chitosan of similar DD and $\overline{M}_v$ (Chapter II, section 4.1.1.).
3. MCChB gel-like dispersions, as well as MCChB films show an increase in WRV and Wc as compared to chitosan film formed from the MCChA gel-dispersion. The modification of MCChB films by protein causes a higher increase in swelling beyond the level obtained for MCChA films. In the case of modification of MCChB gel-like dispersion by keratin, WRV and Cs are decreased with increases in the keratin amount (Chapter II, section 4.7.).
4. The addition of 23 wt% sodium chloride in the coagulation bath microcrystalline chitosan gel-like dispersion results in gels and films showing higher swelling behaviour and a decrease in the depolymerization. The parameters of initial chitosan affect the properties of the MCChB films. Films prepared from MCChB gel-like dispersion possess the highest WRV and Wc as compared to initial chitosan and standard films prepared from acidic solution by drying (Chapter II, section 4.7.).
5. The properties of casein as well as keratin chitosan films prepared by addition of glycerol show lower WRV but better mechanical properties than chitosan film without glycerol (Chapter II, section 4.8.).
6. The tensile strength of MCChB films increase with the increase in keratin concentration to 20 wt% in the coagulation bath. The same observation is found for changes in the crystallinity index. The elongation at break increases with rise in protein concentrations in the coagulation bath. However, MCChB gel-like dispersion prepared in presence of > 33 wt% casein gave films which shrunk and adhered to the PTFE surface (Chapter II, section 4.8.).
7. The IR spectra of films formed from MCChB powder with casein indicate shift of the absorption of amide I to a higher wavenumber and the strong absorption at 3290 $cm^{-1}$ responsible for N-H stretch. The intensity of C-O stretch, ranging from 1150 $cm^{-1}$ to 1050 $cm^{-1}$, decreased with the increase in casein content in the bath. The addition of keratin causes an increase in the amide I band as well as CH stretching intensity at 2800 $cm^{-1}$ with a rise in keratin amount. An increase of hydrogen bond energy is found with an increase in the protein content (Chapter II, section 4.9.).
8. It was found that MCChB films prepared in presence of 20 wt% casein with the introduction of 23 wt% sodium chloride show the highest mechanical strength with an accompanying increase in crystallinity index. The introduction of glycerol (0,7 g per 1g dry weight of MCChB with or without protein) increases the mechanical properties, especially elongation at break (Chapter II, sections 4.8. and 4.9.).
9. During preparation of MCChB gel-like dispersion, a strong decrease in $\overline{M}_v$ for highly viscous chitosan with relatively low DD was particularly found. No high differences in the DD of chitosan and MCChB were observed (Chapter II, section 4.10.).
10. The swelling parameters of MCChB gel-like dispersion increase with the decrease in its DD as well as $\overline{M}_v$. The introduction of casein strongly increases the WRV and Cs coefficients of gels and films with low DD and high $\overline{M}_v$. The addition of keratin favors the increase of these parameters for gel-like dispersion with high DD and low $\overline{M}_v$ (Chapter II, section 4.10.).
11. The presence of proteins in the coagulation bath significantly increases the mechanical properties of MCChB films. This observation is visible for films from MCChB having low $\overline{M}_v$ with keratin. The optimal mechanical properties result for films prepared from chitosan with low DD and high $\overline{M}_v$. The increase in the mechanical strength of MCChB films containing protein in contrast with the films without protein is observed for DD < 80%. An increase in the mechanical strength of MCChB films containing keratin formed from high-deacetylated chitosan was also observed (Chapter II, section 4.10.).

12. MCChB films with or without protein prepared from crustacean chitosan M3A showed high swelling together with strong mechanical properties. They are improved by the introduction of protein, particularly casein (Chapter II, section 4.10.).
13. Cross-linking of MCChB films using GA causes a decrease in the swelling parameters, principally measured in acetic acid solution (Chapter II, sections 4.11. and 4.12.).
14. Paper sheets prepared by MCChB direct introduction possessed a reduction in swelling parameters with an increase in chitosan content. Direct introduction of MCChB gel-like dispersion at a concentration > 4 wt% does not cause significant changes in the swelling of paper sheets. A strong increase in mechanical properties, especially measured at high rel. humidity, is observed for excesses of MCChB higher than 4 wt%. Introduction of protein in MCChB favors a stronger reduction in the swelling parameters of paper in contrast with the paper sheets containing MCChB without protein. The precipitation method of MCChB containing proteins yields better mechanical strength of paper sheets than for the direct introduction method. However, MCChB gel-like dispersion without proteins direct introduced, more strongly affects the mechanical strength of paper sheets. An increase in relative humidity causes a decrease in the tensile strength, Young's modulus and increase in elongation at break of paper sheets containing MCChB. However, a lower decrease in tensile strength as well as Young's modulus is noted for paper sheets containing MCChB with protein, introduced by the precipitation method (Chapter II, section 4.13.).
15. "Coat"- or "web"-like fiber structures of MCChB are detected by the optical microscopy of paper sheets. Direct introduction of MCChB resulted more in "web"-like structure of MCChB among the fibres and the precipitation of MCChB resulted in formation of coated fibres. The coat-like fibres are often found for paper sheets prepared with introduction of protein, but the layers of MCChB are thicker (Chapter II, section 4.13.).
16. The susceptibility to biodegradation of films decomposed in the presence of *Aspergillus fumigatus*, prepared from acidic solution of chitosan by drying, is significantly increased for films showed relatively high swelling parameters. A high reduction in viscosity of solution obtained from degraded films is obtained from films formed from chitosan with low DD. Introduction of protein in MCChB gel-like dispersion influence decomposition of the MCChB films by *A. fumigatus*: a higher yield of biodegradation was found for MCChB films prepared in presence of 20 wt% or 33 wt% proteins as was measured by the value of reducing sugars in fermentation medium (Chapter II, section 4.14.).
17. The type of protein introduced affects the susceptibility to biodecomposition of MCChB films. Faster biodegradation is found for films containing casein, distinguished by the highest swelling parameters. The modification of MCChB by keratin shows at least 10% higher decomposition of films, even those with lower WRV, Wc and Cs, than films without proteins (Chapter II, section 4.14.).
18. The biodecomposition rate of paper sheets is correlated with the method of MCChB addition. The direct introduction method, resulting from 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 (Chapter II, section 4.15.).

**Thesen zur Dissertation mit dem Thema: Herstellung von Chitosan und einige Anwendungen**

Marcin H. Struszczyk, Institut für Organische Chemie und Strukturanalytik, Uni. Potsdam, 23.11.2000

1. Die Deacetylierung von crabshell – Chitosan führte gleichzeitig zu einem drastischen Abfall der mittleren viscosimetrischen Molmasse ( $\overline{M}_V$), insbesondere wenn die Temperatur und die Konzentration an NaOH erhöht werden. Diese Parameter beeinflussten jedoch nicht den Grad der Deacetylierung (DD).
   Wichtig ist jedoch die Quelle des Ausgangsmaterials: Chitin aus *Pandalus borealis* ist ein guter Rohstoff für die Herstellung von Chitosan mit niedrigem DD und gleichzeitig hoher mittlerer $\overline{M}_V$, während Krill-Chitin (*Euphausia superba*) ein gutes Ausgangsmaterial zur Herstellung von Chitosan mit hohem DD und niedrigem $\overline{M}_V$ ist. Chitosan, das aus Insekten (*Calliphora erythrocephala*), unter milden Bedingungen (Temperatur: 100°C, NaOH-Konzentration: 40 %, Zeit: 1-2h ) hergestellt wurde, hatte die gleichen Eigenschaften hinsichtlich DD und $\overline{M}_V$ wie das aus Krill hergestellte Chitosan. Der Bedarf an Zeit, Energie und NaOH ist für die Herstellung von Insekten-Chitosan geringer als für crabshell-Chitosan vergleichbare Resultaten für DD und $\overline{M}_V$.
2. Chitosan wurde durch den Schimmelpilz *Aspergillus fumigatus* zu Chitooligomeren fermentiert. Die Ausbeute beträgt 25%. Die Chitooligomere wurden mit Hilfe von HPLC und MALDI-TOF-Massenspektrmetrie identifiziert. Die Fermentationsmischung fördert die Immunität von Pflanzen gegen Bakterien und Virusinfektion. Die Zunahme der Immunität schwankt jedoch je nach System Pflanze-Pathogen. Die Fermentation von Chitosan durch *Aspergillus fumigatus* könnte eine schnelle und billige Methode zur Herstellung von Chitooligomeren mit guter Reinheit und Ausbeute sein. Eine partiell aufgereinigte Fermentationsmischung dieser Art könnte in der Landwirtschaft als Pathogeninhibitor genutzt werden. Durch kontrollierte Fermentation, die Chitooligomere in definierter Zusammensetzung (d.h. definierter Verteilung des Depolymerisationsgrades) liefert, könnte man zu Mischungen kommen, die für die jeweilige Anwendung eine optimale Bioaktivität besitzen.
3. Die aus Chitosan-Dispersionen hergestellten MCChB-Filme weisen bessere mechanische Eigenschaften (Bruchfestigkeit, Dehnung) und eine höhere Wasseraufnahmefähigkeit auf als Filme, die nach herkömmlichen Methoden aus sauerer Lösung hergestellt werden. Die Einführung von Proteinen ändert die mechanischen Eigenschaften der MCChB-Filme abhängig von der Art, der Proteine sowie des DD und der $\overline{M}_V$ des eingesetzte Chitosan. Die Zugabe von Protein beschleunigt den biologischen Abbau der MCChB-Filme. Aus den untersuchten MCChB-Filmen mit Proteinzusatz können leichte, reißfeste und dennoch elastische Materialen hergestellt werden.
4. Mit Hilfe von MCChB-Dispersion kann Papier modifiziert werden. Dadurch werden die mechanischen Eigenschaften verbessert und die Wasseraufnahme wird verringert. Die Zugabe von Proteinen verringert das Wasseraufnahmevermögen noch weiter. Ein geringes Wasseraufnahmevermögen ist der bedeutendste Faktor bei der Papierherstellung. Auch Papier, das mit einem MCChB-Protein-Komplexe modifiziert wurde, zeigt gute mechanische Eigenschaften.
5. Wird Chitosan durch unmittelbare Einführung von MCChB auf Cellulose-Fasern aufgebracht, so erhält man eine netzartige Struktur, während durch Ausfällung aufgebrachtes Chitosan eine dünne Schicht auf den Cellulose-Fasern bildet. Die netzartige Struktur erleichtert die Bioabbaubarkeit, während die Schichtstruktur diese erschwert. Die guten mechanischen Eigenschaften, die geringe Wasseraufnahmefähigkeit und die mit Cellulose vergleichbare Bioabbaubarkeit von Papier, das mit MCChB modifiziert wurde, lassen MCChB für die Veredlung von Papier nützlich erscheinen.

**Ein Teil dieser Arbeit ist veröffentlicht:**

**A. Artikeln**

1. E. Galas, C. Kubik, M.H. Struszczyk, **Methods of the Immobilization of Enzymes on Chitosan Carrier**s, In.: *Progress on Chemistry and Application of Chitin and Its Derivatives Vol. 2*, H. Struszczyk ed., Lodz, Poland, 55-73, **1996**,
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**B. Abstract und Poster**

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16. M.H. Struszczyk, M.G. Peter, F. Loth, **Method for the Microcrystalline-Protein Films Preparation,** 6th Meeting of the Polish Chitin Society, Poznan, Poland, 19-20.10.1999,
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18. E. Zala, M.H. Struszczyk, M. G. Peter, **Effects of Preparation Methods of Chitosan Films on Their Properties,** Yamaguci, 09. 2000, Japan
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**Lebenslauf**

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## LIST OF PUBLICATIONS

### A. Original Publications

1. E. Galas, C. Kubik, M.H. Struszczyk, **Methods of the Immobilization of Enzymes on Chitosan Carrier**s, In.: *Progress on Chemistry and Application of Chitin and Its Derivatives Vol. 2*, H. Struszczyk ed., Lodz, Poland, **1996**, pp. 55-73,
2. M. Ratajska, M.H. Struszczyk, S. Boryniec, M.G. Peter, F. Loth, **The Degree of Acetylation of Chitosan: Optimization of the IR Method**, *Polimery* 42, **1997**, pp. 576-579,
3. M. H. Struszczyk, G. M. Peter, M. Ratajska, S. Boryniec, **Selected Methods on Chitosan Deacetylation Degree,** In.: *Progress on Chemistry and Application of Chitin and Its Derivatives Vol. 3*, H. Struszczyk ed., Lodz, Poland, **1997**, pp. 28-50,
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7. M.H. Struszczyk, F. Loth, L.A. Köhler, M.G. Peter, **Characterization of Chitosan**, in: W. Praznik and A.Huber, eds.: *Carbohydrates as Organic Raw Materials*, Vol. IV, WUV Universitätsverlag, Wien, ISBN 3-85114-407-4, **1998**, 110-117
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14. M.H. Struszczyk, **Chitin and Chitosan I: Properties, Productions and Application**, In: Polimery, 2002 (in press)
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### B. Conference Presentation

1. M.H. Struszczyk, T. Antczak, **Medical Application of Chitosan**, in XXXIIth Polish Conference of Student Science Society Medical Academy, Lodz, Poland, 20-21.05.1994,

2. C. Kubik, M.H. Struszczyk, E. Galas, **Immobilization of Dextranase on Chitosan Beads**, Annual Meeting of the Polish Polymer Society, Poznan, Poland, 13.11.1995,
3. E. Galas, C. Kubik, M.H. Struszczyk, **Methods of the Immobilization of Enzymes on Chitosan Carrier**s, 3dn Meeting of the Polish Chitin Society, Lodz, Poland, 11-12.10.1995,
4. M.H. Struszczyk, F. Loth, M.G. Peter, **Methods of the Calibration of Chitosan Deacetylation Degree Determination,** 4th Meeting of the Polish Chitin Society, Poznan, Poland, 15-16.10.1996
5. M.H. Struszczyk, M.G. Peter, **Chemistry of Chitosan**, 4th Int. Workshop on Carbohydrates as Organic Raw Materials, Wien, 20.-21.3.1997 (Zuckerindustrie 122, 131 (1997)).
6. M.H. Struszczyk, F. Loth, M.G. Peter, **Analysis of Deacetylation Degree in Chitosans from Various Sources**, 7th Int. Congr. on Chitin and Chitosan, Lyon, France, 03.-07.09.1997
7. H. Pospieszny, M.H. Struszczyk und M.G. Peter, **Biodegradation of Chitosan**, 4th Meeting of the Polish Chitins Society, Poznan, Poland, 16-17.10.1997.
8. M.H. Struszczyk, F. Loth und M.G. Peter, **Methods for Calibration of the Determination of Chitosan Deacetylation Degree**, 4th Meeting of the Polish Chitin Society, Poznan, Poland, 16-17.10.1997.
9. M. H. Struszczyk, M.G. Peter, F. Loth, **Preparation of Chitosan Film,** Workshop on Organic and Biochemical Thin Films, Potsdam, Germany, 01.-03.04.1998,
10. M.H. Struszczyk, F. Loth, M.G. Peter, **Preparation of Chitosan Films Containing Protein,** 5th Meeting of the Polish Chitin Society, Poznan, Poland, 19-20.10.1998,
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16. M.H. Struszczyk, M.G. Peter, F. Loth, **Method for the Microcrystalline-Protein Films Preparation,** 6th Meeting of the Polish Chitin Society, Poznan, Poland, 19-20.10.1999,
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**C. Seminars**

1. M.H. Struszczyk, **Analytical Methods for Determination of Deacetylation Degree (DD) of Chitosan**, Potsdam, Germany, 15.05.1997,
2. M.H. Struszczyk, **Preparation of Chitosan Microcrystalline Films**, Potsdam, Germany, 07.06.1998,
3. M.H. Struszczyk, M.G. Peter, **Chitosan from Cuticles of Insects**, DBU-Statusseminar, Potsdam, Germany, 10.06.1999,

4. M.H. Struszczyk**, Preparation of chitosan from various sources and its selected application**, Potsdam, Germany, 26.06.2000,

**D. Unpublished reports**

1. M.H. Struszczyk, **Determination of the chitosan molecular weight and the deacetylation degree**, Potsdam, Germany, 16.09.1996,
2. M.H. Struszczyk, **Determination of the Acetylation Degree of Chitosan**, Potsdam, Germany, 10.12.1996,
3. M.H. Struszczyk, **Preparation of chitosan films,** Potsdam, Germany, 23.03.1998,
4. M.H. Struszczyk, **Effect of the molecular weight as well as deacetylation degree on the mechanical properties and film behaviour of MCCh gel likes dispersion or formed films**, Potsdam, Germany, 10.01.1999,
5. M.H. Struszczyk, **Preparation of chitin and chitosan from insects larval form of *Calliphora erythrocephala*,** Potsdam, Germany, 23.04.1999,
6. M.H. Struszczyk, **Preparation of chitin and chitosan from insects larval form of *Calliphora erythrocephala* – scale up,** Potsdam, Germany, 11.11.1999,
7. M. H. Struszczyk, **Preparation of chitosan derivatives such as: carboxymethylated chitosan and sulphonated chitosan from standard chitosan and its microcrystalline form. Application of their for film preparation**, Potsdam, Germany, 05.08.2000

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