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Research paper

A method for direct preparation of chitosan with low molecular weight from fungi

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Abstract

By modifying the common method for the preparation of chitosan from fungi, low molecular weight chitosan with an average MW of 4.5×10^4 g/mol and a numerical MW of 1.7×10^4 g/mol can be directly extracted from the raw material without the need of thermal or chemical depolymerization. Based on the solubility of low molecular chitosan up to alkaline pH ranges, reprecipitation and washing with ethanol is required to keep the low molecular fraction within the preparation. The use of water for washing between the preparation steps would cause solving and discarding of the low molecular chitosan. The chitosan was analyzed by laser light scattering and ^{1}H-NMR spectroscopy.

Keywords: Chitosan; Low molecular weight; Preparation; Fungi; Mucorales; Laser light scattering

1. Introduction

Chitosan is a well studied linear polysaccharide, polymerized from the monomers 2-acetoamido-2-desoxy-β-D-glycopyranose (GlcNac) and 2-amino-2-desoxy-β-D-glycopyranose (GlcN), which contains a higher part of GlcN causing the solubility of chitosan in aqueous media.

Chitosan is industrially produced from crab shell waste, but research has been carried out on the use of alternative sources for chitosan [1–7]. The studies were focused mainly on chitosan from fungi of the order *Mucorales*, which contain chitin and chitosan in vivo in their cell walls. The advantage of using fungi is the easy handling, harvesting and controlling to produce high quality chitosan. Furthermore, the manipulation of characteristics of chitosan, such as molecular weight (MW) and grade of deacetylation (DD), through fungi culturing were discussed. The MW of the chitosan produced so far from fungi is high, indicating that fungi produce these high polymerized carbohydrates naturally. In this point, the chitosan from fungi is comparable with chitosan from crab shells, which is also normally produced with a high MW [8].

The use of chitosan for pharmaceutical and medical applications, based on the polycationic nature of this material, has been studied for different fields. Chitosan stimulates the immune system [9] and has an antibacterial effect [10]. Therefore it can be used as part of wound healing devices, such as bandaging material and sponges [11] and also as an artificial skin [12]. For these applications the MW of the used chitosan either does not matter at all or is important because of a given high viscosity.

However, there are potential applications of chitosan in which a low MW would be essential. Given a low MW the polycationic characteristic of chitosan can be used together with a solubility at physiological pH ranges and a lower antigene effect. On this basis low molecular weight chitosan (LMWCh) could be used as a parenteral drug carrier. Gene therapy research is aiming for non-viral gene delivery systems, for which chitosan showed promising results in former studies [13]. Other studies based on animal testing showed the possibilities of LMWCh for treatment of type 2 diabetes, gastric ulcer and caries prophylaxe [14–17].

LMWCh has been produced by degradation of chitosans with high MW up to present. This depolymerization can be carried out either with a chemical method, often supported thermically, or biochemically with enzymes. Studies recommended nitrous acid, hydrochloric acid, phosphoric acid and hydrogen peroxide [18–20]. An enzymatic

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degradation can be achieved with glycosyl hydrolases, such as chitinase and chitosanase [21,22]. These enzymes have been cloned and expressed to study their use for scale-up production of LMWCh. Lysozyme, which hydrolyses the glycosidic bonding between GlcNac and *N*-acetyl-muraminic acid (MNac), is also able to divide the bonding between GlcNac–GlcNac or GlcNac–GlcN alternatively [23]. A direct preparation of LMWCh from the raw material has not yet been reported up to present.

2. Materials and methods

2.1. Cell cultivation and harvest

Absidia coerulea(DSM 1143, DSM 3018) was maintained at 4 °C on agar containing malt extract and transferred monthly. For cultivation spores were germinated for 48 h in a 200-ml medium containing 40 g/l glucose, 10 g/l peptone, 1 g/l yeast extract, 0.5 g/l $MgSO_4$, 0.1 g/l $CaCl_2$, 10 g/l $(NH_4)_2SO_4$, 1 g/l K_2HPO_4, 1 g/l NaCl. The pH was adjusted to 4.5 with HCl and the culture grown on a shaker at room temperature; 20 ml of this culture were used as an inoculum for final cultivation in 1-l baffle bottomed culture flasks containing the same medium composition. Standard growth time was 48 h.

The grown biomass was washed with distilled water and the cells disrupted with an ultra-turrax to isolate the cell wall material from cytosolic contents. The homogenized slurry was filtered and resuspended in distilled water. This procedure was repeated three times and the collected cell wall material was dried at 45 °C for 24 h.

2.2. Chitosan extraction

To exclude thermal effects on the MW of the produced chitosan all preparation steps were carried out at room temperature. Only the drying of the biomass and the chitosan was carried out at 45 °C, which is known to have no influence on chitosan stability.

To evaluate the modified method reference chitosan was quantitatively prepared from biomass of *A. coerulea* as previously described in the literature [1–7]. First, a deproteinization was carried out with 2% (w/v) NaOH for 3 h. After subsequent washing with distilled water up to pH-neutrality the remaining alkali-insoluble material (AIM) was dried. For extraction of chitosan, AIM was treated with 4% (w/v) acetic acid overnight. Through centrifugation the acid-insoluble fraction was precipitated and the supernatant, containing the chitosan, was isolated. The clear solution was alkalized with NaOH and the chitosan flocculated. The chitosan was precipitated by centrifugation, the supernatant discarded and the pellet resuspended in distilled water. This washing cycle was repeated until pH 7 was reached.

For the preparation of LMWCh biomass of *A. corulea* was deproteinated as described above. The alkali biomass suspension in the deproteinization step was now mixed with ethanol to reprecipitate soluted material, which would otherwise be discarded. Here, the alkali soluble material was now united with alkali insoluble material to assure a complete extraction of chitosan. The alkaline–ethanolic insoluble material (AEIM) was centrifuged, the supernatant discarded and resuspended in ethanol. This washing cycle was repeated until pH neutrality was reached, followed by drying at 45 °C. The AEIM was then treated as above with 4% acetic acid overnight and centrifuged to obtain the chitosan solution. This solution was alkalized with NaOH and ethanol was added, until flocculation occurred. Subsequent precipitation of the acid-soluble material by centrifugation with resuspension in ethanol and final drying followed.

For final purification dialysis of an aqueous solution of LMWCh was carried out in a dialysis tube with a MWCO of 3.000 g/mol.

2.3. Analysis of identity and molecular weight

The identity of samples was proven by ^{1}H-NMR. Samples in a final concentration of 2% (m/v) were solved in deuteric hydrochloric acid (0.5% v/v in D_2O). Measurement was carried out with an ARX300 (Bruker, Rheinstetten) at 300–320 K using the sodium salt of 3-(trimethylsilyl)-propionsulfonic acid.

Gel permeation chromatography was carried out with a system of PL aquagel-OH 30 (Polymer Laboratories, MW: 1×10^2 to 3×10^4 g/mol) and TSKgel GMPWxl (Tosohaas, MW: 5×10^4 to 1×10^6 g/mol) columns. MW of divided chitosan fractions was measured with laser light scattering (miniDawn Tristar, Wyatt Technology) connected to a RI-detector (Shodex RI SE-51, Showa Denko KK). Refractive index measurement was carried out to analyze the distribution of concentration of molecular fractions. The average MW (Mw) and the numerical MW (Mn) can be calculated by setting off these data with laser light scattering.

3. Results and discussion

The yield of AIM was 39% of dry cell mass (DCM) compared with a yield of AEIM of 59%. At the next step the extraction of chitosan with water yielded 6.2% of DCM. On the other hand, LMWCh reached 2.9% of DCM.

When comparing AEIM and AIM, 20% of alkaline-soluble material was reprecipitated through the admixture of ethanol. Given a protein–chitosan matrix in cell walls of fungi, deproteinating of the biomass sets chitosan free and enables the solubility of LMWCh, which would otherwise be discarded without the adding of ethanol. The lower yield of the chitosan extracted with ethanol could be based either on a structural change with the following complexation of chitosan and proteins through the adding of ethanol in

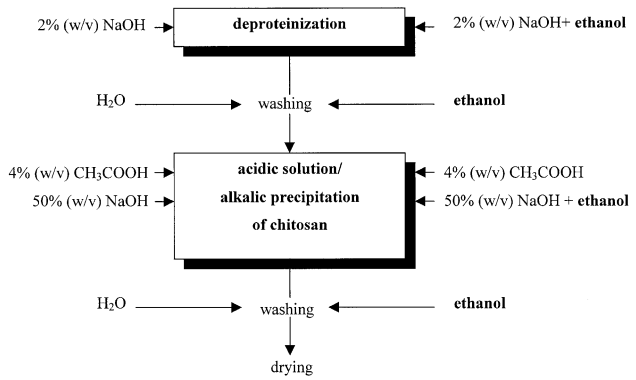

Fig. 1. Overview of the preparation of chitosan from fungi with water as washing detergent and ethanol as precipitation/washing detergent.

the deproteination step. This would prevent the following acidic extraction. On the other hand, the ability of chitosan molecules for agglomeration is known. In this case the water preparation concentrates chitosan molecules and enables agglomeration and precipitation to occur, which would cause higher extraction yields. This would also lead to a higher average MW, if these complexes cannot be dissolved through GPC. Using ethanol instead of water would prevent association of these molecules and would leave molecules still dissolved in the alkaline deproteination medium. This could lead to less yield of precipitated chitosan and a lower average MW of LMWCh because of non-associated single molecules.

Typical signals of the RI-detector for separated chitosan samples produced with described methods are shown in Fig. 1. The graphs are marked into two sections, of which section I stretches from 8 to 16 ml and section II from 16 to 20 ml elution volume.

The dotted line reveals the fractional distribution of a chitosan sample prepared with water. For section I the distribution stretches from 9 to 14 ml, whereas in section II a small amount can be detected between 18 and 19 ml. Chitosan samples produced with ethanol show a typical elution pattern, which is represented by the full line. A small fraction is seen in section I from 11.5 to 15 ml and for section II a three-peaked area exists from 17 to 19.5 ml.

The calculated molecular weights of these fractions are shown in Table 1. In section I samples prepared with ethanol have a low molecular weight and the three-peaked area consists of material with MW of monomers. In section I chitosan prepared with water has a high molecular weight as

Table 1
Molecular weight of fractions of chitosan samples shown in Fig. 1

Section	Mn (g/mol)	Mw (g/mol)
Ethanol I	1.7×10^4	3.7×10^4
Ethanol II	1.7×10^2	4.2×10^2
Water I	2.4×10^5	8.6×10^5
Water II	2.4×10^3	7.3×10^3

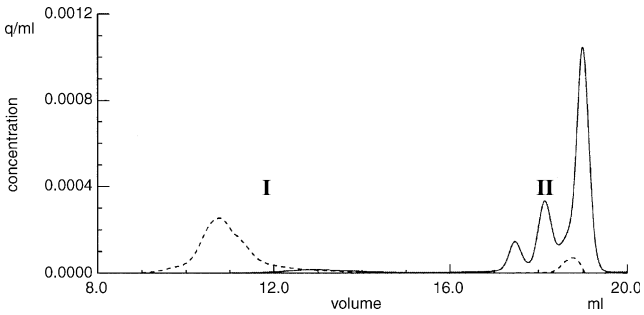

Fig. 2. Internal concentration distribution of chitosan samples analyzed by RI-detector (dotted line, chitosan produced in aqueous suspension; full line, chitosan produced in ethanolic suspension).

described in former studies [6] and shows low molecular oligomer-like parts in section II.

Fraction II of the chitosan prepared with ethanol must for the most part consist of the alkaline-soluble material, which was reprecipitated by ethanol. This material was discarded in the water preparation, although a small amount is still detectable. It can be assumed that this material consists of degraded proteins and amino acids. LMWCh could be part of section I, therefore dialysis was carried out to divide these two fractions from each other.

^{1}H-NMR-spectra of these samples generally show typical peak distributions of chitosan as described before [24]. Differences can be noted comparing the baselines, as spectrum 2 shows a high background signal, which is caused by impurities within the sample. Furthermore, in spectrum 1 the peak area between 3.1 and 4.4 ppm is split, which correlates with the superimposition of the resonance signals of the ring protons in high polymerized chitosans. On the opposite spectrum 2 has a one peak signal in this area, which shows reduced peak broadening and some spin–spin-coupling of the protons and which gives identification of a chitosan with a lower MW than in spectrum 1. Between 3.6 and 3.7 ppm and 1.1 ppm and 1.3 ppm resonance signals of ethanol can also be detected.

The major difference in the RI diagrams of the two preparations is the three-peaked area in section II.

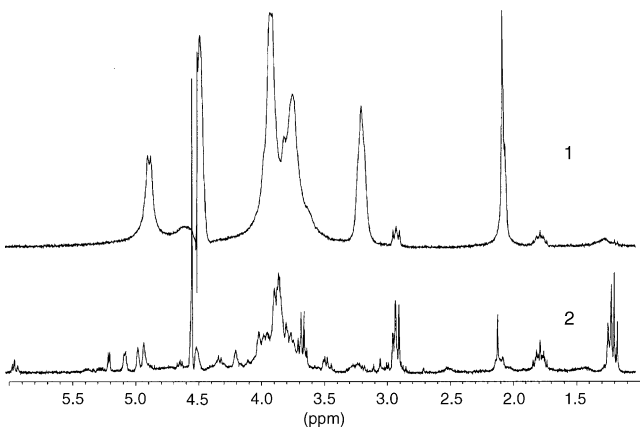

Fig. 2. ^{1}H-NMR-spectrums of chitosan samples prepared with (1) water and (2) ethanol.

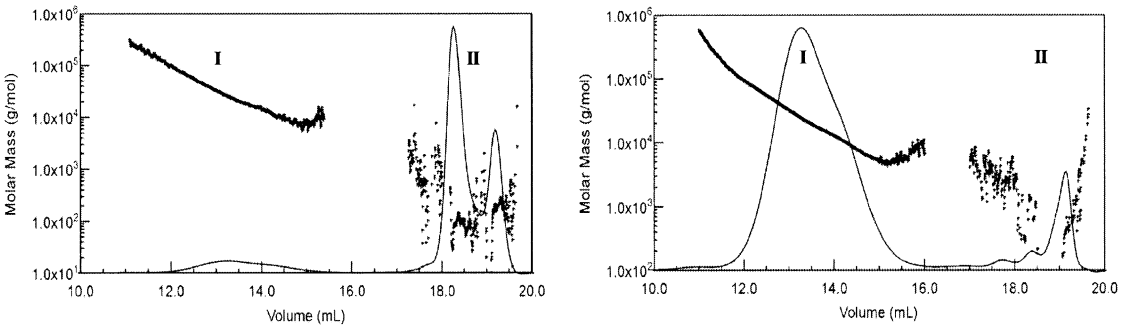

Fig. 4. Concentration distribution of ethanol prepared chitosan sample analyzed with RI-detector and calculated MW of these fractions (a) before dialysis and (b) after dialysis.

It can be assumed that the high background signal in ^{1}H-NMR-spectrum 1 is caused by this fraction, which in this case cannot consist of chitosan. To prove this point and to purify the ethanol prepared chitosan, dialysis was carried out.

Through dialyzing the chitosan samples the integral concentration ratio of section I to section II was reversed (Figs. 2–5). Although most of the monomer-like fraction content was removed, some small fraction was left because of precipitation of chitosan followed by clogging of the dialysis pores. Furthermore, some molecules in this fraction had a MW above the MWCO of the dialysis tube and therefore could not be removed. The resulting Mw of the dialyzed LMWCh is 4.5×10^4 g/mol and Mn is 1.7×10^4 g/mol. The average MW of the dialyzed sample is slightly higher than before dialysis, which could give evidence for agglomeration processes through the purifying in aqueous medium.

Comparing the ^{1}H-NMR spectrums before and after dialysis, it can be shown that the base line is now smoothened and peaks, which were caused by the removed low molecular material, disappeared. Some signals of ethanol are still detectable, although intensive thermal and vacuum drying was undertaken. In any case, the use of ethanol is essential for precipitation within the medium, because LMWCh will not precipitate in purely alkaline aqueous medium.

Low molecular weight chitosan can be easily produced with this method. Given the ^{1}H-NMR-spectra identity and purity is comparable to commercially available chitosans. The residual ethanol content can be eliminated by spray drying of a LMWCh solution, which would then lead to precipitation by evaporating the solvent.

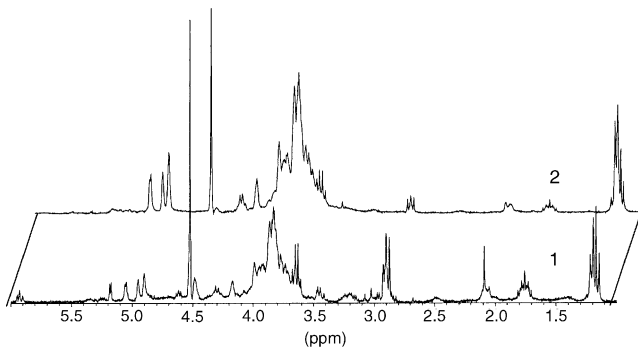

Fig. 5. ^{1}H-NMR-spectrums of ethanolic chitosan sample (1) before and (2) after dialysis.

Acknowledgements

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