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# Low molecular weight chitosans—preparation by depolymerization with *Aspergillus niger* pectinase, and characterization

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**Abstract**

The viscosity of a chitosan solution was rapidly lowered in the presence of pectinase from *Aspergillus niger* at pH 3.0 and 37 °C. The low molecular weight chitosans (LMWC) had a molecular weight in the range 20,000–5000 Da. Circular dichroism spectra showed a decrease in the segment of acetylated glucosamine units, whereas X-ray diffraction and CP-MAS $^{13}C$ NMR indicated higher crystallinity and polymorphism in LMWC. The latter on thermal drying resulted in structural alterations, and yielded an insoluble product. FT-IR and X-ray diffraction showed no evidence of either Schiff's base linkage or any annealed polymorph. CP-MAS $^{13}C$ NMR showed marked changes in the chain conformations of LMWC, which are believed to be responsible for its loss of solubility and functionality.


*Keywords:* Chitosan; Low molecular weight chitosan; Pectinase; Solubility; Conformation

## 1. Introduction

Chitosan, a linear polymer composed of β-1,4-linked glucosamine (GlcN) with various degrees of *N*-acetylated GlcN residues, is a deacetylated derivative of chitin extracted from an abundant source of shellfish exoskeletons.[1] The amino polysaccharide and its derivatives have many diverse applications in agriculture, medicine, and cosmetics.[2,3] Fairly recently, low molecular weight chitosans (LMWC) with an average MW in the range of 5000–20,000 Da were shown to possess superior biological activities compared to chitosan.[4] Jeon and coworkers[5] reported that of the three fractions produced by an enzymatic reactor system, LMWC with MW in the range 5000–10,000 had highest bactericidal activity towards pathogenic bacteria. LMWC were also shown to modulate plant resistance to disease[6] and to stimulate murine peritoneal macrophages, killing the tumor cells.[7] LMWC of 20 kDa were shown to prevent progression of *Diabetes mellitus*[8] and they had high affinity for some bacterial lipopolysaccharides. Evidence is beginning to accumulate, to show that LMWC are more biologically active than chitooligomers and than high molecular weight chitosan itself.

Chitosan can be depolymerized by acid[9,10] or enzymatic[11,12] hydrolysis. In the former, large amounts of free GlcN are produced.[10] Further disadvantages are the need to remove strong acid and browning. The use of dilute acids, such as 0.6 M HCl would require almost 20 days to reach a MW of 12,000 Da.[13] Enzymatic methods, which are easier to control and specific, are therefore preferred. Moreover, enzymatic hydrolysis may lend itself to large-scale production of less polymerized chitosan products, which retain their original biological properties. The more frequently used enzymes for such purposes are chitinases and chitosanases. For commercial utilization, however, their use is limited due to prohibitive cost and limited availability.

Pantaleone and coworkers[14] examined the feasibility of chitosan hydrolysis by food grade enzymes, including glycanases, lipases and proteases. Among them papain from *Carica papaya* and hemicellulase and lipase from *Aspergillus niger* were reported as effective enzymes to hydrolyze chitosan. The observation of chitosanolytic

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activity for such a wide array of enzymes is highly unusual, particularly in view of narrow substrate specificity of established chitosanases. However, irrespective of the catalytic mechanism, the observed lytic activities are of interest, because they open up the prospect of industrially viable modification of chitosan with low cost enzymes.

In our earlier study, we investigated chitosanolytic properties of commercial enzymes and found that some enzymes, such as pepsin, papain, and pectinase were better lytic agents than chitinase and lysozyme (Vishu and coworkers, unpublished results). In addition, we found that a pectinase from *A. niger* rapidly depolymerized chitosan to LMWC and chitooligosaccharides, the former being the major degradation product.[15] In this paper, we report preparation of LMWC from pectolytic hydrolyzates of chitosan and their structural characterization. In addition, the effect of heat drying on functionality of LMWC is also described.

## 2. Experimental

### 2.1. Materials

All chemicals used in this study were obtained from commercial sources and were of highest purity available. Pectinase from *A. niger* was from Sigma Chemical Co., USA.

### 2.2. Preparation of chitosan

Chitosan was prepared by heterogeneous alkaline deacetylation of shrimp chitin. It was further purified by dissolving in 1% aq AcOH (v/v) followed by filtration to remove insoluble materials. The soluble chitosan was precipitated with 2 M NaOH, washed thoroughly with double distilled water and lyophilized. The viscosity average molecular weight ($M_v$) of chitosan was calculated using the Mark–Houwink equation; $[\eta] = K_m M_v^a$, where $K_m = 3.5 \times 10^4$, $a = 0.76$.[16]

### 2.3. Enzyme assay

The pectinase activity of *A. niger* pectinase was assessed by the dinitrosalicylicylate method using sodium pectate (0.25% solution of pectic acid in 0.1 M AcONa buffer, pH 3.0) as the substrate. The chitosanolytic activity was determined by treating chitosan (1% solution in 1% AcOH and pH adjusted to 3.0) with pectinase at 25 °C for 1 h, inactivating the enzyme at 100 °C for 10 min followed by the addition of equal volume of 2 M NaOH and estimating the reducing sugars released in the supernatant after centrifugation of the reaction mixture.[17]

### 2.4. Production of chitosan hydrolyzate

Commercial pectinase from *A. niger* showed three isozymes on native-polyacrylamide gel electrophoresis (PAGE), of which, the one with $R_f$ 0.92 showed both pectinase and chitosanase activities as evidenced by the zymogram analysis.[17] This isozyme, upon extraction from the gel using the modified electro-elution set-up (Vishu and coworkers, corresponded) was subjected to sodium dodecylsulfate-PAGE.[17]

The isozyme thus purified by electro-elution was added to 200 mL of 1.0% (w/v) chitosan solution, pH 3.0 in a conical flask with the enzyme/chitosan ratio of 0.003 (w/w). The reaction mixture was incubated at 37 °C for 6 h with constant stirring. After hydrolysis, the enzyme was inactivated at 100 °C for 10 min and the pH adjusted to 12 with 2 M NaOH to precipitate products with a high degree of polymerization (DP). The suspension was centrifuged, the insoluble residue was washed with double distilled water and lyophilized to give LMWC.

### 2.5. CD spectroscopy

Circular dichroism was carried out using a JASCO-J20C automatic recording spectrometer. The instrument was continuously purged with $N_2$ before and during the experiment. Slits were programmed to yield 10 Å bandwidth at each wavelength so that the resolution was more or less constant. The far UV spectra were recorded between 200 and 240 nm. Chitosan concentration of 5.0 mg $mL^{-1}$ in 0.1 M perchloric acid was used in a cell of path length 1 cm. The baseline for each spectrum was obtained using 0.1 M perchloric acid. The mean residual ellipticities were calculated by taking mean residue weight of D-GlcN.

### 2.6. X-ray diffractometry

Powder X-ray diffraction patterns were obtained using a EG-7G solid state Germanium liquid nitrogen cooled detector Scintag XDS-2000 instrument equipped with a $\theta$–$\theta$ goniometer, with following operation conditions: 30 kV and 25 mA with a Cu $K_{\alpha 1}$ radiation at $\lambda$ 1.54184 Å. The relative intensity was recorded in the scattering range ($2\theta$) of 4–60°. The crystallinity index (CrI) was determined by the method of Struszezyk.[18]

### 2.7. CP-MAS $^{13}$C NMR

The spectra were recorded at 75.3 MHz on a Bruker $dsx_{300}$ spectrometer. The cross polarization pulse sequence was utilized for all samples, which were spun at the magic angle of 5 kHz. A contact time of 1 ms and a pulse repetition time of 5 s were used, and more than 2000 scans were accumulated for each run. Approxi-

mately, 300 mg of freeze–dried samples were inserted into a 7 mm ceramic rotor. The degree of acetylation (DA) was derived from the relative intensities of the resonances of the ring carbons ($I_{C-1}$, $I_{C-2}$, $I_{C-3}$, $I_{C-4}$, $I_{C-5}$, $I_{C-6}$) and the methyl carbon ($I_{CH_3}$) from the following equation.[19]

$$DA = \frac{I_{CH_3}}{(I_{C-1} + I_{C-2} + I_{C-3} + I_{C-4} + I_{C-5} + I_{C-6})/6}$$

### 2.8. Infrared spectroscopy

IR spectra were recorded in KBr discs on a Impact 410 Nicolet FT-IR spectrometer under dry air at room temperature. The DA of chitosan was determined following the method of Miya and coworkers[20] and correlated with that deduced from CP-MAS $^{13}C$ NMR.

## 3. Results and discussion

The specific activity of *A. niger* pectinase and its isozyme ($R_f$ 0.92) was found to be 9.85 and 3.31 units $mg^{-1}$, respectively before and after electro-elution and the chitosanolytic activity of the latter was 0.65 units $mg^{-1}$. The optimal pH and temperature for the latter were found to be 3.0 and 47 °C, respectively (data not shown). It is to be noted that to obtain LMWC the reaction mixture was incubated at 37 °C for 6 h with constant stirring, since the enzyme showed loss of activity at its optimum temperature during the course of time. The time dependent change of viscosity at enzyme:chitosan ratio of 0.003 was measured under optimal reaction conditions. As shown in Fig. 1, the rate of hydrolysis by pectinase was rapid and viscosity reduction was 50% during first 30 min, and it approached nearly zero after 12 h of incubation. The

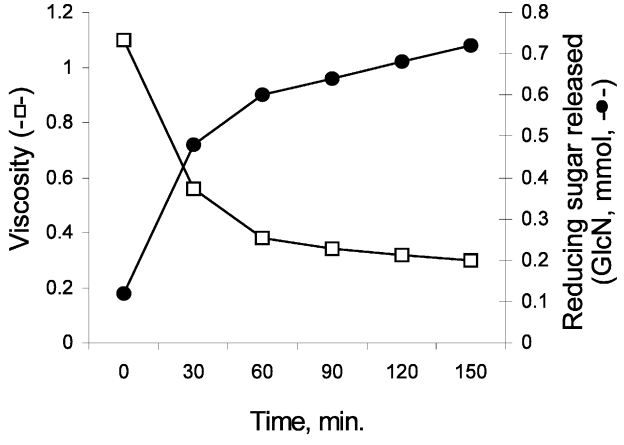


Fig. 1. Viscosity reduction of chitosan solution in the presence of *A. niger* pectinase at pH 3.0 and 37 °C.

viscosity loss correlated well with the reducing sugar released. In our preliminary experiments (Vishu and coworkers, unpublished results), porcine pepsin and papain from *C. papaya*, both showed higher initial rates, but it slowed down after 2 h and never approached zero even after 12 h. Surprisingly, the lytic activity of *A. niger* pectinase nearly equaled that observed with *Streptomyces griseus* chitinase, which possibly may be of use as a potential alternative to the more expensive chitinases and chitosanases.

After hydrolysis, the pH of the pectinase hydrolyzate was slowly raised by adding 2 M NaOH. High $M_v$ chitosan is soluble below pH 6.2 and above this pH it precipitates out of the solution. A decrease in DP, however, shifts this precipitation point to higher pH values. Many workers[14,21] exploited this property of chitosan to monitor its enzymatic depolymerization. In the present study, the precipitation point was shifted to higher pH values in a time dependent manner, indicating progressive decrease in the $M_v$ of chitosan. After approximately 6 h of hydrolysis, the yield of LMWC was over 70%.

The most commonly used equation relating intrinsic viscosity, $[\eta]$ values with $M_v$ is Mark–Houwink equation; $[\eta] = K_m M_v^a$, where $a$ and $K_m$ are constants that are independent of $M_v$ over a wide range of values.[16] Recently, Terbojevich and coworkers,[22] based on these constants, found good agreement in $M_v$ values obtained by viscometry and light scattering method. As can be seen from Table 1, the $M_v$ of chitosan dropped sharply from 90,000 to 20,000 Da within 3 h of contact with pectinase. After 6 h, it was around 6000 Da. Thus, it is obvious that LMWC or oligomers with higher DP values can easily be prepared by varying the contact time with pectinase.

Being a linear copolymer of 2-acetamido-2-deoxy-β-D-glucopyranoside (GlcNAc), and 2-amino-2-deoxy-β-D-glucopyranoside (GlcN), in chitosan four types of linkages, viz., GlcN–GlcN, GlcNAc–GlcN, GlcN–GlcNAc and GlcNAc–GlcNAc exist. Class I chitosanase cleaves the first two types of linkages, class II cleaves only the first, whereas class III cleaves both first and the third type of linkages. GlcNAc–GlcNAc linkage will be cleaved only by chitinase. Cleavage of second and third type of linkages results in GlcNAc either in reducing or non-reducing end of the products, respectively. The pectinase isozyme belonged to Class I chitosanase type, and resulted in the formation of products with GlcNAc in the reducing end, as evidenced by the MALDI-TOF-MS of the oligosaccharides[17] and their incubation with hexosaminidase, an exo-enzyme acting on non-reducing GlcNAc, which did not cause any change in the oligomeric fractions, thus confirming the cleavage pattern as depicted in Fig. 2. As a result, there was no appreciable decrease in the %DD of

Table 1
Intrinsic viscosity, $M_v$ and %DD for chitosan and LMWC

| Sample (reaction time, h) | Intrinsic viscosity [$\eta$] (g d l$^{-1}$) | $M_v$ (Da) | %DD [a] |
|---|---|---|---|
| Chitosan-0 | 2.00 | 90,600 | 84 |
| LMWC-3 | 0.86 | 20,000 | 85 |
| LMWC-6 | 0.26 | 6000 | 86 |

[a] By CP-MAS $^{13}$C NMR.

LMWC compared to native chitosan, which was further confirmed by spectral data.

CP-MAS $^{13}$C NMR showed a slight increase in the %DD (degree of deacetylation = 100 − DA) value after depolymerization (see Table 1). A difference of just 1–2% was, however, insignificant and that CP-MAS $^{13}$C NMR suffers from lower sensitivity when the DA is low. To investigate whether the %DA was indeed lowered during depolymerization, CD spectra of chitosan (curve A) and depolymerized samples (curves B and C) were recorded (Fig. 3). The CD spectra of GlcNAc oligomers are characterized by a peak, corresponding to an n→π* transition at 211 nm and its peak height is related to the acetyl content of the chitosan,[23] which is independent of the α, β anomeric equilibrium, the chain length beyond two residues, ionic strength and pH. This means that any difference in the peak height between chitosan substrate and depolymerized sample should reflect a difference in the acetyl content. A decrease in the peak height was attributed to the cleavage of GlcNAc–GlcN linkage, which was confirmed by characterization of heterooligosacchardies obtained.[17]

Fig. 4 gives the powder X-ray diffraction patterns of chitosan and LMWC. Chitosan showed a diffraction pattern 'tendon' typical of a hydrated polymorph, except for the 020 reflection, which appeared at 11.4°. After depolymerization, this reflection was shifted to a higher $2\theta$ value (see Table 2) with consequent decrease in the intensity, whereas the intensities of 100 and 110 reflections increased considerably. The former is attributed to a larger $d$-spacing, which is likely due to an increase in the unit cell dimension. Reflection at 020 is associated with the most ordered regions involving

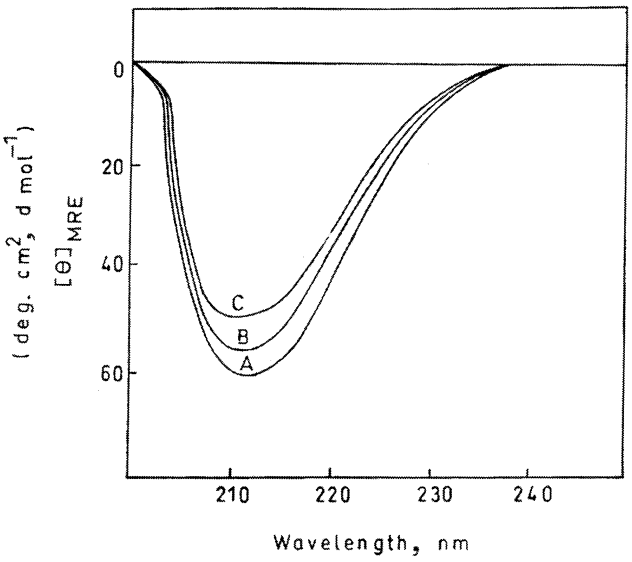


Fig. 3. CD spectra of chitosan (A) and lyophilized LMWC, reaction time: 3 (B), 6 h (C).

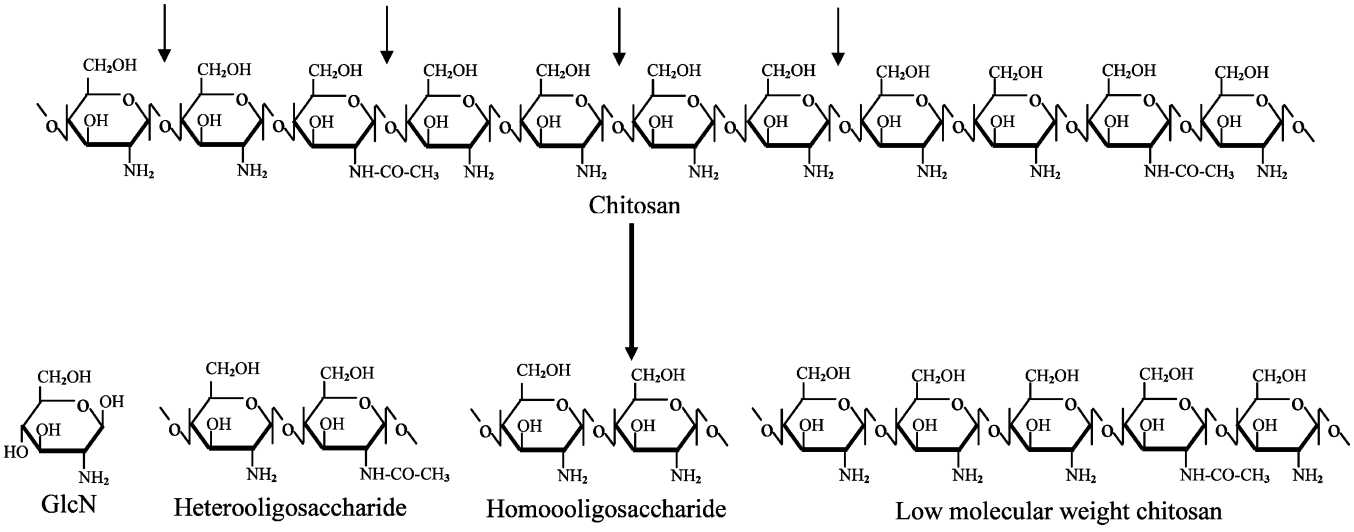


Fig. 2. Action of chitinase/chitosanase on the hypothetical fragment of chitosan.

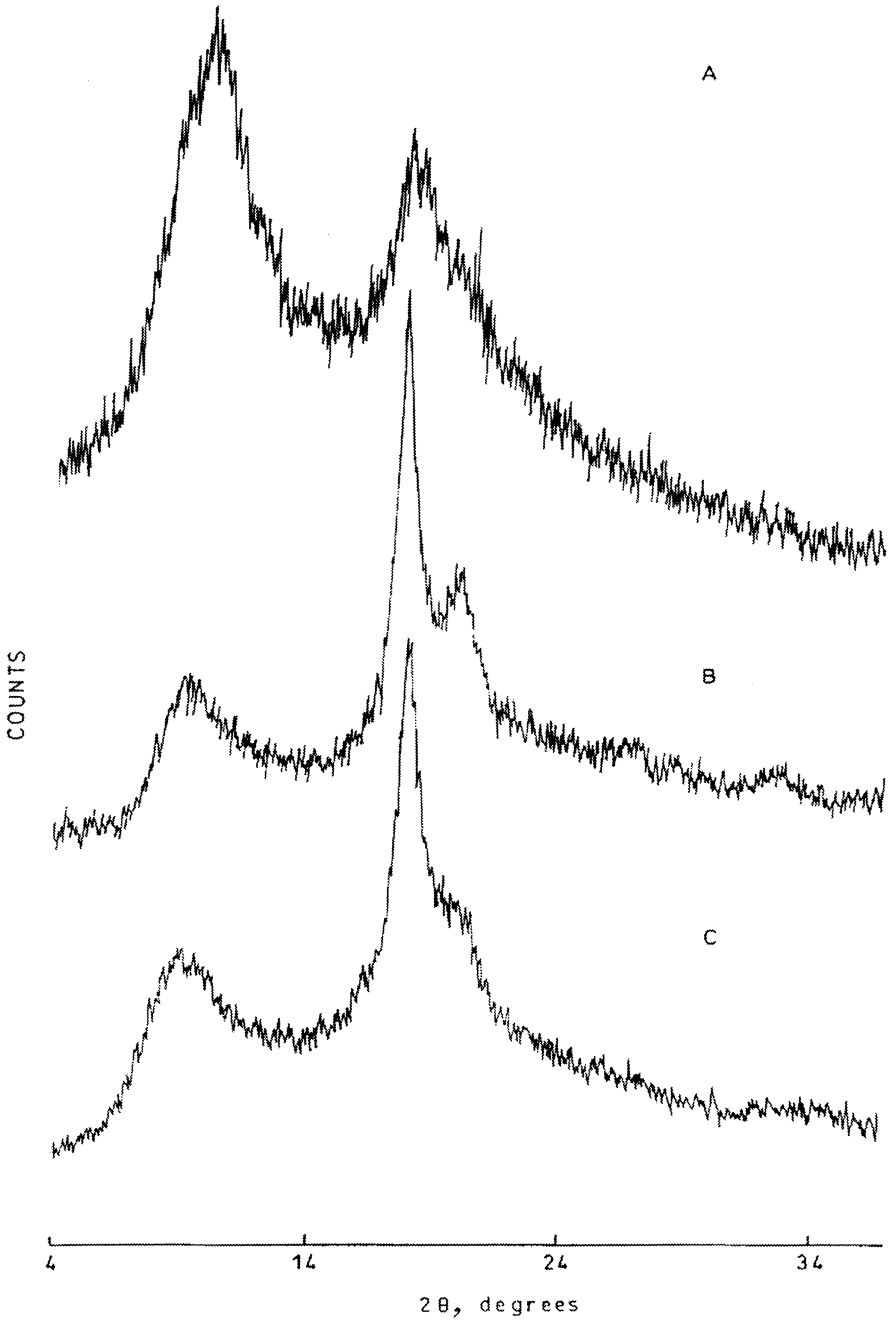


Fig. 4. X-ray diffractogram of chitosan (A), lyophilized (B) and oven-dried (C) LMWC.

Table 2
*d*-Spacing and CrI values of chitosan and LMWC

| Sample | Reflection (°) | | | *d*-Spacing (Å) | | | CrI (%) |
|---|---|---|---|---|---|---|---|
| | 020 | 100 | 110 | 020 | 100 | 110 | |
| Chitosan | 11.40 | 19.80 | 22.52 | 7.75 | 4.48 | 3.94 | 37 |
| LMWC, lyophilized [a] | 10.78 | 19.80 | 22.16 | 8.20 | 4.48 | 4.0 | 71 |
| LMWC, oven-dried [a] | 11.06 | 19.86 | 22.30 | 7.99 | 4.46 | 3.98 | 68 |

[a] Chitosan–pectinase reaction time, 6 h. Oven dried at approx 100 °C for 3 h.

acetamido groups. The GlcNAc residues in chitosan are distributed so as to form a highly ordered structure through hydrogen bonding with GlcNAc residues of neighboring chain, facilitating the incorporation of water molecules into such a network and forming a hydrated crystal. The latter gave an intense crystalline peak in the range 9–11° (20). Depolymerization, however, resulted in a fewer but unfavorable distribution of GlcNAc residues in LMWC, which failed to interact with each other, and hence showed reduction in the intensity of 020 reflection. This, together with changes in the intensities of 100 and 110 reflections suggest different packing of chains and/or different hydrogen bonding network in LMWC. Crystallinity measurements showed higher CrI for depolymerized chitosan (Table 2), in good agreement with that of Ogawa,[24] who also reported increase in crystallinity with decrease in the $M_v$ of chitosan. This was attributed to the higher mobility of chitosan chains as a consequence of decreased chain length.

Solid state CP-MAS $^{13}C$ NMR is known to be very sensitive to changes in the local order structure. The line widths in the spectrum of LMWC (Fig. 5(B)) were smaller than chitosan, indicating the latter to be more crystalline,[25] as also indicated by X-ray-diffraction data.

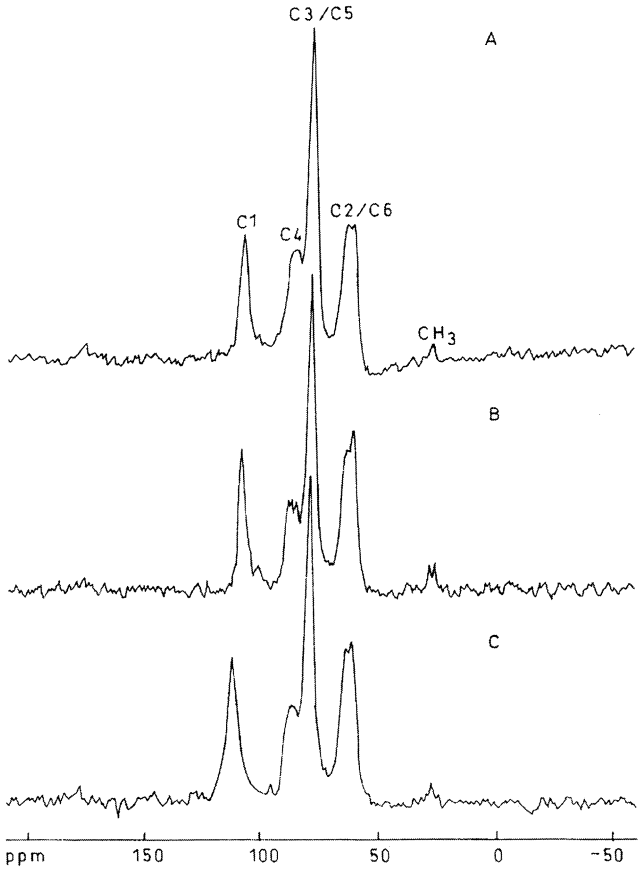


Fig. 5. CP-MAS $^{13}C$ NMR of chitosan (A), lyophilized (B) and oven-dried (C) LMWC.

Fig. 5(A) and Table 3 showed the depolymerized chitosan to have some 'extra' peaks at 26.6 and 84.3 ppm, assigned to distinctly different chemical environments of methyl and *C*4 ring carbons, respectively.[26] The chemical shifts of C-1 and C-4 carbons in 1,4-linked polymers are believed to be highly sensitive to the conformation of the glycosidic linkage.[26] The difference in the C-4 shift between chitosan and depolymerized chitosan was 3 ppm (Table 3). The spectral data suggested the presence of chitosan chains in LMWC that may pack at least in two distinct ways.

Economic viability and the technical acceptability of large-scale production of LMWC are dependent on the ease of isolation and drying. The latter is a popular industrial process. The enzymatically-depolymerized chitosans, however, should be handled with due precautions, lest, they may change their properties and functionalities. A point of difference from native chitosan is the much higher number of reducing units present in LMWC, which have the higher probability to undergo Schiff's base formation, Amadori rearrangement, etc. They impart undesirable properties[27] such as brown colour and poor solubility to the product. Additionally, due to the higher chain mobility, LMWC may form annealed polymorph, which is known to destroy chitosan functionality.[24]

To investigate the effect of thermal drying on physicochemical properties, LMWC was dried in an oven at 100 °C for 3 h. The latter though showed little or no browning, however, became insoluble in aqueous acetic acid. FT-IR spectra showed no evidence of Schiff's linkage and that treatment with HCl had no effect on solubility. Hydrochloric acid is known to decompose Schiff's linkage back to free amine. The other possibility of the formation of an annealed polymorph was ruled out by X-ray diffraction pattern (Fig. 4(C)) showing no evidence of 15° reflection. Water is known to improve crystallinity by removing imperfections in the crystals. The diffraction patterns and the $2\theta$ values (Fig. 4(C) and Table 2) revealed no differences between oven-dried and lyophilized LMWC, except for the intensity of 110 reflection and CrI, which were reduced to 68% (Table 2), and likely due to the removal of water during oven drying. The fact that LMWC lost solubility upon oven drying despite the absence of Schiff's linkage and annealed polymorph is surprising. It is known that minor components ($<0.5\%$) in the mixture of crystalline solids are often undetected by powder X-ray diffraction.[28] In the present case, however, the content of minor component was expected to be larger than 0.5%, since a major portion of the sample was insoluble. However, no additional crystalline peaks were detected in the diffractogram of oven-dried LMWC. One plausible explanation is that there is a subtle change in the conformation, which may be difficult to detect by X-ray diffraction measurements.

Table 3
$^{13}C$ NMR chemical shifts (ppm) of chitosan and LMWC

| Sample | C-1 | C-4 | C-3 and C-5 | C-2 and C-6 | $CH_3$ |
|---|---|---|---|---|---|
| Chitosan | 107.9 | 84.6 | 78.4 | 63.6, 60.9 | 26.7 |
| LMWC, lyophilized [a] | 107.7 | 87.3, 84.3 | 78.4 | 62.6, 60.0 | 27.8, 26.6 |
| LMWC, oven-dried [a] | 109.4 | 87.8 | 79.4 | 63.8, 61.3 | 27.4 |

[a] Chitosan–pectinase reaction time, 6 h. Oven dried, approx 100 °C for 3 h.

The latter, though sensitive to changes in the long-range order, such as difference in *d*-spacing, is insensitive to local order changes, such as changes in the molecular conformation.

Keeping in mind these limitations the solid-state structure of oven-dried LMWC was further probed by CP-MAS $^{13}C$ NMR (Fig. 5 and Table 3), which revealed differences between lyophilized and oven-dried LMWC. The 'extra' peaks at 26.6 and 84.3 ppm disappeared upon oven drying and chemical shift values, however, showed downfield shift of resonance of all ring carbons. C-1 and C-4 of oven-dried LMWC were shifted downfield by 1.5 and 3.2 ppm, respectively. Nevertheless, distinct chemical shift values emerged for oven-dried sample suggesting significant differences in its molecular conformation. The loss of solubility of the latter is likely due to a unique conformation, hitherto unknown, wherein the chains are twisted in such way that they do not permit the redissociation of individual chains.

In conclusion, depolymerization of chitosan with the aid of pectinase is simple, easily controlled and quickly accomplished. Low cost, easy availability in large quantities and GRAS status for *A. niger* products make pectinase a suitable biocatalyst for the production of LMWC with defined MW values. Depolymerization brings about significant changes in the primary and higher order structures of chitosan, viz, distribution of GlcNAc, number of reducing ends, chain arrangement and chain conformations and crystallinity. Because of higher reducing end and chain mobility, LMWC need to be handled with care. Oven drying destroys chitosan functionality by changes in the chain conformations.

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## References

1. Kittur, F. S.; Kumar, K. R.; Tharanathan, R. N. *Z. Lebensm. Unters. Forsch. A* **1998**, *206*, 44–47.
2. Hadwiger, L.A.; Fristensky, B.; Riggleman, R. C. In *Chitin, Chitosan and Related Enzymes*; Zikakis, J. P., Ed.; Academic Press: New York, 1984, p. 291.
3. Nagai, T.; Sawayangi, Y.; Namba, N. In *Chitin, Chitosan and Related Enzymes*; Zikakis, J. P., Ed.; Academic Press: New York, 1984, p. 21.
4. Muzzarelli, R. A. A. *Chitosan in Pharmacy and Chemistry*; Atech: Italy, 2002, pp 1–8.
5. Jeon, Y. J.; Park, P. J.; Kim, S. K. *Carbohydr. Polym.* **2001**, *44*, 71–76.
6. Vasyukova, N. I.; Zinov'eva, S. V.; II'inskoya, L. I.; Perekhod, E. A.; Chalenko, G. I.; Gerasimova, N. G.; II'ina, A. V.; Varlamov, V. P.; Ozeretskovskaya, O. L. *Appl. Biochem. Microbiol.* **2001**, *37*, 103–109.
7. Seo, W. G.; Pae, H. O.; Kim, N. Y.; Oh, G. S.; Park, I.; Kim, Y. H.; Kim, Y. M.; Lee, Y.; Jun, C. D.; Chung, H. T. *Cancer Lett.* **2000**, *159*, 189–195.
8. Kondo, Y.; Nakatani, A.; Hayashi, K.; Ito, M. *Biol. Pharmacol. Bull.* **2000**, *23*, 1458–1464.
9. Horowitz, S. T.; Roseman, R.; Blumenthal, H. J. *J. Am. Chem. Soc.* **1957**, *79*, 5046–5049.
10. Rupley, J. A. *Biochim. Biophys. Acta* **1964**, *83*, 245–250.
11. Aiba, S. *Carbohydr. Res.* **1994**, *265*, 323–328.
12. Muraki, E.; Yaku, F.; Kojima, H. *Carbohydr. Res.* **1993**, *239*, 227–237.
13. Terbojevich, M.; Cosanni, A.; Muzzarelli, R. A. A. *Carbohydr. Polym.* **1992**, *18*, 35–42.
14. Pantaleone, D.; Yalpani, M.; Scollar, M. *Carbohydr. Polym.* **1992**, *237*, 325–332.
15. Tharanathan, R. N.; Kittur, F. S.; Vishu Kumar A. B. Indian Patent, 0232/DEL/2002.
16. Rinaudo, M.; Milas, M.; Dung, L. P. *Int. J. Biol. Macromol.* **1993**, *15*, 281–285.
17. Kittur, F. S.; Vishu Kumar A. B.; Gowda, L. R.; Tharanathan, R. N. *Carbohydr. Polym*. **2003**, *53*, 191–196.
18. Struszezyk, H. J. *J. Appl. Polym. Sci.* **1987**, *33*, 177–189.
19. Ottoy, M. H.; Varum, K. M.; Smidsord, O. *Carbohydr. Polym.* **1996**, *29*, 17–24.
20. Miya, M.; Iwamoto, R.; Yoshikawa, S.; Mima, S. *Int. J. Biol. Macromol.* **1980**, *2*, 323–324.
21. Shin, S. S.; Lee, Y. C.; Lee, C. *J. Food Biochem.* **2001**, *25*, 307–321.

22. Terbojevich, M.; Cosanni, A.; Muzzarelli, R. A. A. *Carbohydr. Polym.* **1996**, *29*, 63–68.
23. Domard, A. *Int. J. Biol. Macromol.* **1987**, *9*, 98–104.
24. Ogawa, K. *Agric. Biol. Chem.* **1991**, *55*, 2375–2379.
25. Focher, S.; Naggi, A.; Terri, G.; Casanni, A.; Terbojevich, M. *Carbohydr. Polym.* **1992**, *18*, 43–49.
26. Tanner, S. F.; Chanzy, H.; Vincendon, M.; Roux, C.; Gaill, F. *Macromolecules* **1990**, *23*, 3576–3583.
27. Fujimaki, M.; Namiki, M.; Kato, H. *Amino-Carbonyl Reactions in Food and Biological Systems*; Elsevier: Amsterdam, Chapter 7, 1996.
28. Suryanarayanan, R. In *Physical Characterization of Pharmaceutical Solids*; Brittain, H. G., Ed.; Marcel Dekker: New York, 1995.