Low Molecular Weight Chitosan—Preparation with the Aid of Pepsin, Characterization, and Its Bactericidal Activity

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Pepsin (EC 3.4.4.1) from porcine stomach mucosa caused depolymerization of a chitosan sample (a copolymer of glucosamine and *N*-acetylglucosamine linked by β-1−4-glycosidic bonds). N-terminal sequence and zymogram analyses confirmed dual (proteolytic and chitosanolytic) activities of pepsin. Optimum depolymerization occurred at pH 5.0 and 45 °C with an activity of 4.98 U. Low molecular weight chitosan (LMWC), the major depolymerization product, was obtained in a yield of 75−82%, the degree of polymerization of which depended on reaction time. The LMWC showed a nearly 10−14-fold decrease in the molecular mass as compared to native chitosan, which was also confirmed by GPC and HPLC analyses. IR and ^{13}C NMR spectra indicated a decrease in the degree of acetylation (DA, ∼13.4−18.8%) as compared to native chitosan (∼25.7%), which was in accordance with the CD analysis. Native chitosan had a crystallinity index (CrI) of ∼70%, whereas there was a decrease in the CrI of LMWC (∼61%). The latter showed a better bactericidal activity toward both *Bacillus cereus* and *Escherichia coli*, which was more toward the former. The bactericidal activity was essentially due to the lytic and not static effect of LMWC, as evidenced by the pore formation on the bacterial cell surface when observed under SEM. This study suggests the possible use of pepsin in place of chitosanase, which is expensive and unavailable in bulk quantities for the production of LMWC of desired molecular mass that has diversified applications in various fields.

Introduction

Chitosan, an *N*-deacetylation product of chitin (commercially found in the offal of the marine food processing industry), is shown to be a valuable biopolymer because of its widespread applications in biomedical, food, and chemical industries.[1] However, a high molecular mass and thus high viscosity of chitosan limit its applications. On the other hand, the chitosan depolymerization products, viz., low molecular weight chitosan (LMWC) and chitooligomers, overcome these limitations and hence find much wider applications in diversified fields.[2] It is reported that the LMWCs with a molecular mass ranging between 5 and 10 kDa are strong bactericidal, fungicidal, hypolipidemic as well as hypocholesterolemic agents.[3] According to Kondo et al., 20 kDa chitosan prevents the progression of diabetes mellitus and exhibits a higher affinity for lipopolysaccharides than 140 kDa chitosan.[4] Tsai et al. proposed the practical use of LMWC in milk preservation and oral hygiene.[5] LMWCs of 5−10 kDa were also shown to have potential as a DNA delivery system.[6] Varlamov et al.[7] showed bactericidal and antifungal activities associated with LMWC of 4.6 kDa and its *N*-2(3)-(dodec-2-enyl)succinoyl derivatives. Recently, Babak et al.[8] demonstrated the use of LMWC (5 kDa of degree of acetylation 3%) grafted with *N*-2(3)-(dodec-2-enyl)-succinoyl groups as potential nonviral vectors for gene therapy.

LMWC can be prepared by acid or enzymatic depolymerization of chitosan, of which the latter is preferred as the process can be easily controlled and monitored, and the products can be obtained without any modifications, which is normally seen with acid hydrolysis.[9−11] Chitinase and chitosanase are the specific enzymes, but their utility in such hydrolysis is limited because of escalating costs and the unavailability of bulk quantities.[11] Pantaleon et al. have reported nonspecific catalytic activity of various enzymes on chitosan, although the purity of enzyme preparations used was doubtful.[12] Among the nonspecific enzymes, pepsin from porcine stomach mucosa was found to depolymerize chitosan efficiently, and in our earlier study, we determined pepsin purity, effect of different acids as solvents, molecular mass, and degree of acetylation (DA) of chitosan during pepsin activity and kinetic details of chitosanolysis by pepsin.[13] In here, we have further confirmed the dual activities of purified pepsin and detailed its use in obtaining LMWC of a desired molecular mass in higher yields. Information on the structural characterization and its bactericidal activity toward *Bacillus cereus* and *Escherichia coli* have also been provided.

Materials and Methods

Materials. Pepsin (from porcine stomach mucosa, EC 3.4.4.1) was obtained from Sigma Chemical Co., St. Louis, MO. Shrimp chitin was from CFTRI Regional Center, Mangalore, India. Dextran standards (1−70 kDa) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Chitosan standards (50−600 kDa) were from Fluka Chemika, St. Louis, MO, and other chemicals used were of highest purity available.

Preparation of Chitosan. Shrimp chitin subjected to heterogeneous de-N-acetylation with hot NaOH[14] was further purified by dissolving in 1% acetic acid, filtering through a plug of glass wool to remove the insoluble portions, and precipitating with 2% Na_2CO_3 and freeze-drying, so as to get pure chitosan that was designated as native chitosan.

Molecular Mass Determination. Molecular mass (M_r) was determined by (i) viscometry using Ostwald's viscometer and Mark−

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Table 1. M_r and DA of LMWC Determined by Various Methods

		M_r (kDa)[a]			DA (%)	
sample	reactiontime (h)[b]	viscometry	GPC	HPSEC	IR	^{13}C-NMR
chitosan		72.0	70.8	71.0	25.7	26.1
LMWC	1	10.0	9.8	10.0	18.8	18.9
	3	8.2	8.0	8.1	15.6	15.7
	5	4.8	4.7	4.7	13.4	13.5

[a] Values are mean ±0.3 SD. [b] LMWC obtained after 1−5 h incubation of chitosan solution with pepsin.

Houwink's equation, $(\eta) = KM_r^a$, where (η) = intrinsic viscosity, $K = 0.076 \times 10^5$, and $a = 0.76$, wherein 0.3 M acetic acid + 0.2 M sodium acetate served as the solvent;[15] (ii) GPC on Sepharose CL-4B (Sigma; bed volume 180 mL);[16] and (iii) HPSEC (high-performance size exclusion chromatography) on E-linear and E-1000 columns connected in series. GPC and HPSEC columns were precalibrated with dextran/chitosan standards using acetate buffer (0.5 M acetic acid + 0.2 M sodium acetate, pH 4.5) as the solvent/eluant.

Infrared (IR) Spectroscopy. FT−-IR spectra (4000−400 cm^{-1}) were taken on a Perkin-Elmer Spectrum 2000 spectrometer under dry air at room temperature using KBr discs. The degree of acetylation (DA) was calculated using the formula $(A_{1655}\ cm^{-1}/A_{3450}\ cm^{-1}) \times 100/1.33$, where A is the absorbance.[17]

Circular Dichroism (CD). The CD measurements (between 200 and 240 nm) were carried out on Jasco J-810 spectropolarimeter using 5 mg samples dissolved in 1 mL of 0.1 M perchloric acid. The baseline was obtained using 0.1 M perchloric acid, and the mean residual ellipticity was calculated by taking the mean residual weight of GlcNAc.[18]

X-ray Diffractometry. X-ray diffractometry was performed using a EG-7G solid-state germanium liquid nitrogen-cooled Scintag XDS-2000 instrument equipped with a $\theta-\theta$ goniometer at 30 kV and 25 mA at a Cu Kα radiation of λ 1.54184 Å. The relative intensities were recorded between 0−45°. Crystallinity index (CrI; %) was obtained using the formula $(I_{110} - I_{am})100/I_{110}$, where I_{110} is the maximum intensity at 20° and I_{am} is the intensity of amorphous diffraction at 16°.[19]

Solid-State 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 4 and 7 kHz, respectively, for native chitosan and LMWC. 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. Approximately 300 mg of freeze-dried sample was inserted into a 7 mm ceramic rotor. The DA was calculated according to Ottey et al.[20] using the equation $DA = I_{CH3}/(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6$, where I_{C1} to I_{C6} are the intensities of C1−C6 ring carbons and I_{CH3} is intensity of the methyl carbon.

Scanning Electron Microscopy (SEM). Samples, spread on a double-sided conducting adhesive tape pasted on a metallic stub, were coated with gold (100 μg) in a sputter coating unit for 5 min and observed under SEM (LEO 435 VP, LEO Electron Microscopy, Cambridge, UK) at 20 kV.

Indicator Bacteria and Inoculum Preparation. The indicator bacterial cultures included were strains of *B. cereus* F4810, *E. coli* D 21, *Listeria monocytogenes* Scott A, *Yersinia enterocolitica* MTCC 859, *Staphylococcus aureus* FRI 722, and *Bacillus licheniformis* CFR 1621, which were obtained from the culture collection maintained in the Food Microbiology Department of this Institute.

The cultures were maintained at 6 °C on brain heart infusion (BHI, HiMedia, Mumbai, India) agar slants and subcultured at 15 day intervals. Prior to use, the cultures were successively propagated twice in BHI broth at 37 °C. Cell suspension of the culture, individually, was prepared from 20 h old BHI culture broth with appropriate dilution in 0.85% saline, giving an individual count of 10^{2-} to 10^6 colony forming units (CFU) mL^{-1}.[21]

Bactericidal Activity. This was studied against indicator bacterial strains individually in nutrient broth following the method of Chen et al.[22] To 10 mL aliquots of nutrient broth (HiMedia, Mumbai, India) of pH 6.0 supplemented with 0.5% dextrose, a cell suspension of specific bacterial strain was added, giving individual cell numbers of 10^2 to 10^6 CFU mL^{-1}. To the same tubes were added 0.1−0.5% (w/v in 0.01% acetic acid, pH adjusted to 6.0) of LMWC of 4.8−10.0 kDa. Acetic acid (0.01−0.1%) served as controls. The contents of tubes were mixed well and incubated for ∼20 h at 37 °C. From the incubated tubes, 1 mL aliquots were transferred into fresh 9 mL nutrient broth tubes, and the remaining broth aliquots were pour-plated with BHI agar (HiMedia) and incubated for 24 h at 37 °C, after which the plates were observed for the bacterial colonies and the tubes for turbidity. Bactericidal activity was calculated using $[(C - T)/C]100$, where C and T were the colony numbers in the control and chitosan sample plates, respectively.[22]

Results and Discussion

Native Chitosan. The molecular mass (M_r) of native chitosan was found to be ∼71 ± 2 kDa by viscometry, which was in accordance with the value calculated by GPC on the Sepharose CL-4B column (Table 1), and the DA was ∼25.7% as determined by FT−-IR, which was in good agreement with that calculated using solid-state ^{13}C NMR (Table 1). Like chitin, the presence of a single broad band near 3371 cm^{-1} in the IR spectra (Figure 1) and a single sharp peak at 78.781 ppm corresponding to the C3−C5 ring carbon atoms in ^{13}C NMR (Figure 2) indicated the possibility of β-conformation of the native chitosan, resulting in their susceptibility for depolymerization, due to parallel arrangement of the chains.[19,23] The X-ray diffraction pattern (Figure 3) was of a typical hydrated form with a reflection at 10.32° (*d*-spacing, 8.571 Å), a characteristic of tendon polymorphs.[24]

Pepsin Purity. Although many workers demonstrated chitosanolysis by several commercial nonspecific enzymes, the purity of the enzyme was in doubt.[12,25] Earlier, we showed the association of chitosanolytic activity with purified preparations of an isozyme of pectinase from *Aspergillus niger*, pepsin from porcine stomach mucosa, papain from *Carica papaya* latex, and Pronase from *Streptomyces griseus*.[13,26] To establish pepsin purity beyond ambiguity, the GPC purified pepsin[13] was subjected to acid-PAGE and zymogram analyses, wherein it showed both proteolytic and chitosanolytic activities (data not shown). Identification of the first 15 amino acid residues in the N-terminus (performed on Applied Biosystems Model 789, Foster City, CA) followed by its BLAST analysis (http://www.expasy.org/tools/blast/, Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr-Leu-Asp-Thr-Glu-Tyr-Phe) confirmed pepsin purity beyond ambiguity.

Chitosanolytic Activity of Pepsin. Kinetic data of the chitosanolytic activity of pepsin are detailed in our earlier publication.[13] In brief, pepsin showed optimum chitosanolysis at pH 5.0 and 45 °C with an activity of 4.98 U, in contrast to its proteolytic activity (800−2500 U) around pH 1.5−2.0 and 37 °C. The effect of varying substrate concentration on chitosanolytic activity by pepsin displayed Michaelis−Menten kinetics with K_m and V_{max} values of 3.27 mg mL^{-1} and 4520

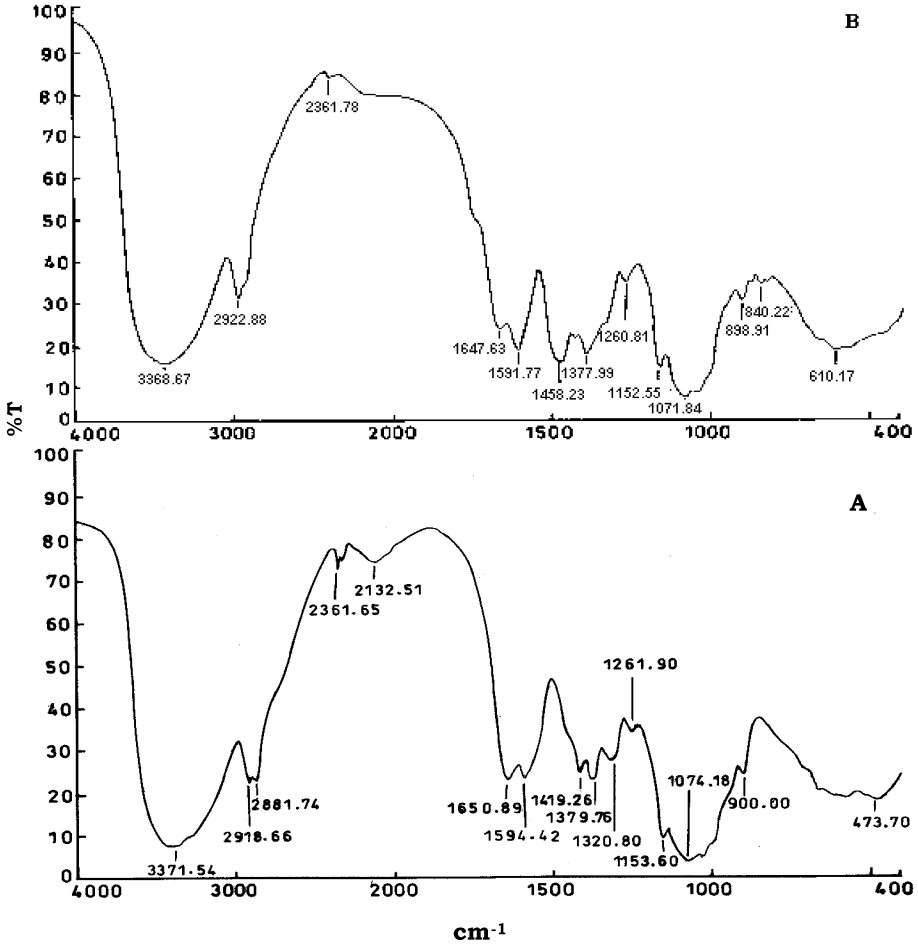

Figure 1. FT-IR spectra of (A) native chitosan and (B) LMWC.

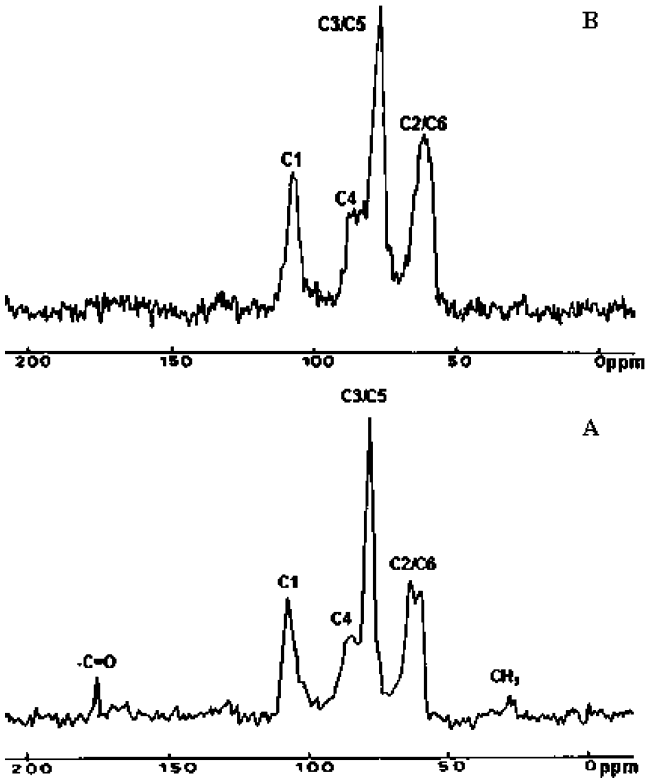

Figure 2. ^{13}C NMR spectra of (A) native chitosan and (B) LMWC.

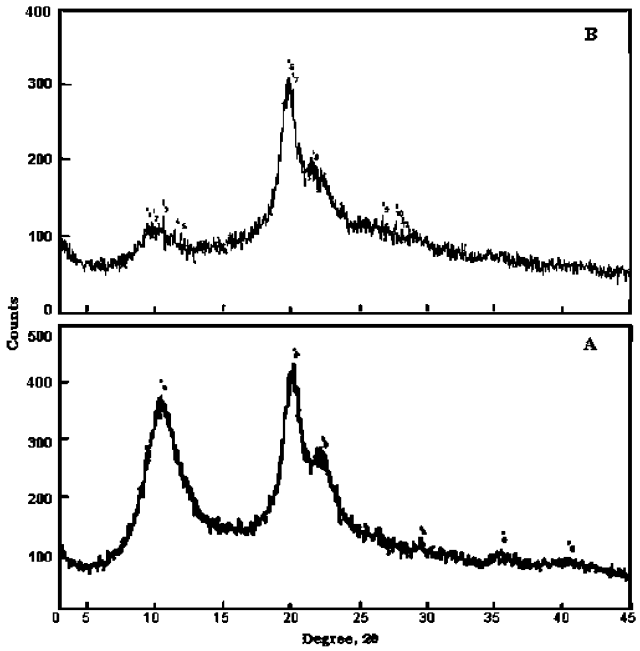

Figure 3. X-ray diffraction patterns of (A) native chitosan and (B) LMWC.

nmol min^{-1} mg^{-1}, respectively. The activation energy (E_a) toward chitosanolysis was 56.03 kJ mol^{-1} as compared to 124−130 kJ mol^{-1} toward proteolysis, indicating the enzyme's preference toward a specific substrate rather than chitosan. At optimum conditions, pepsin showed sustained chitosanolytic activity up to 5 h, after which there was a gradual decline.

CDV

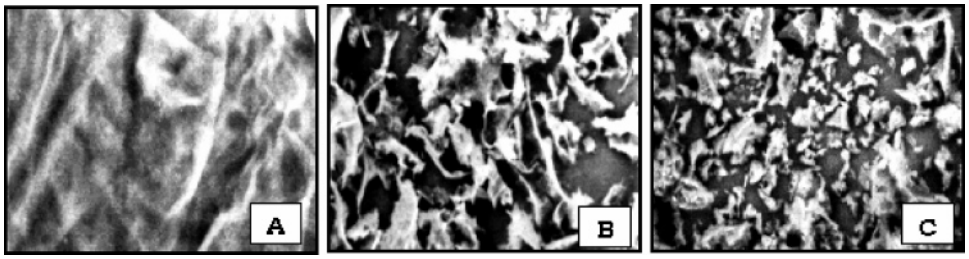

Figure 4. SEM [×500] of (A) native chitosan and (B) LMWC and (C) obtained after 1 and 5 h of reaction time, respectively.

LMWC—Isolation and Characterization. Enzymatic reaction was arrested adding 2 N NaOH and adjusting the pH to neutrality (pH 7.0). The precipitate (LMWC) was separated by centrifugation (3000 rpm, 10 min) and subjected to dialysis using a membrane of cutoff value of 2 kDa, so as to remove entangled chitooligomers during sedimentation. Unless stated otherwise, LMWC obtained after 1 h reaction time was used for further characterization.

Molecular Mass (M_r) and Size. The M_r of LMWC determined by viscometry was between 4.8 and 10.0 kDa depending on the reaction time (1−5 h), which was in good agreement with the values calculated by GPC as well as HPSEC (Table 1). The appearance of a single peak in the HPSEC profile was indicative of molecular homogeneity of the product (figures not shown). Maximum depolymerization occurred up to 5 h, after which a decrease in the M_r was not much appreciable. SEM also indicated a ∼7−14-fold decrease in the size as compared to native chitosan (Figure 4).

Degree of Acetylation (DA). Figure 1 represents the IR spectra, and the DA calculated is given in Table 1. LMWC showed 13.4−18.8% DA as compared to 25.7% in native chitosan. Like native chitosan, the absence of splitting of the bands around 3600−3000 cm^{-1} was indicative of a β-conformation of the LMWC preparation, which was further evidenced by a less resolved region near 2932 cm^{-1}.[15] The band near 1620−1650 cm^{-1} (amide I) is expected to be associated with the intramolecular hydrogen bonding between −−C═O and $-NH_2$ groups, and a decrease in its intensity in LMWC further supports a decreased DA.[27] According to Brugnerotto et al.,[28] a band near 1320 cm^{-1} corresponds to GlcNAc residues, and a decrese in its intensity in LMWC confirms the previous fact. The band around 1429 cm^{-1} is attributed to $-CH_2$ bending and orientation of the primary hydroxyl group in the molecule, which appears at a lower wavenumber for LMWC, due to multiple conformations of the molecule as a result of decreased DA.[27]

In the CD spectra (Figure 5), the peak near 211 nm due to the $\eta \rightarrow \pi^*$ transition corresponds to the GlcNAc residue, and Domard[18] correlated this peak height to the DA, which is independent of chain conformation, length, ionic strength, and pH. As depolymerization progresses, a gradual decrease in this peak height was observed, indicating decreased GlcNAc content, which further supports the results of IR spectra.

Crystallinity Index (CrI). Solid-state CP-MAS ^{13}C NMR is known to be very sensitive to perturbations in the local order structure. Broadening of the signals is likely due to the presence of both GlcN and GlcNAc residues in the macromolecule, which restricts packing of the molecule by a hydrogen-bonding network.[19,26,27] The line width in the spectra of LMWC (Figure 2) was broader than that of native chtosan, indicating the former to be less crystalline. The signal at 26.843 ppm, which corresponds to an acetamido-methyl group, showed a decrease in the intensity in LMWC, indicating a decrease in the DA. The latter calculated from the NMR data was in agreement with that derived from IR spectra (Table 1). The C1 and C4 signals

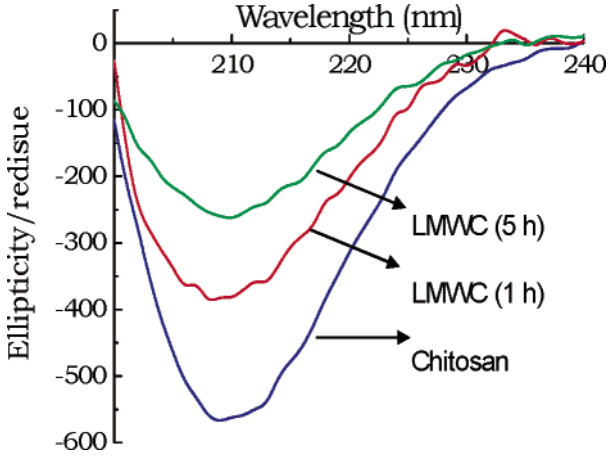

Figure 5. CD spectra of native chitosan and LMWC.

Table 2. ^{13}C NMR Spectra—Chemical Shift Values (ppm)

sample	CH_3	C2/C6	C3/C5	C4	C1	—C═O
chitosan	26.843	60.906	78.703	84.703	108.103	176.524
LMWC	26.084	62.516	78.671	87.568	107.111	174.668

are believed to be highly sensitive to the molecular conformation, as they are the ring carbons involved in the glycosidic linkages and in LMWC, and there was a slight shift, indicating conformational heterogeneity, which was further confirmed by a slight splitting of C2/C6 signals (Table 2).

Figure 3 represents X-ray diffractogram, and the reflection around 9−11° is associated with the most ordered region involving the acetamide groups, and its intensity reflects the hydrated crystal content. A decrease in its intensity in LMWC supports a decreased DA, thus indicating the presence of fewer hydrated crystals. LMWC showed a decrease in the CrI as compared to native chitosan (∼61 and 70%, respectively), contrary to the report of Ogawa,[24] showing a decrease in M_r, resulting in increased CrI. This could be attributed to the higher mobility of the molecules owing to their reduced size. Thus, depolymerization, in addition to decrease in the M_r, size, and DA, caused more random distribution of GlcNAc residues on the chitosan (LMWC) chain, resulting in lower crystallinity and higher solubility.

Solubility of LMWC. Solubility plays an important role in the bio-applications of LMWC, which in turn depends on both DA and molecular structure.[29] Annealed chitosan polymorphs (anhydrous form) are usually associated with decreased solubility and thus loss of functionality.[24] Although the M_r of LMWC was <10 kDa and the DA was in the range of 13.4−18.8%, they were not readily soluble in aqueous medium (water solubility of LMWC, ∼63−72%), instead it required a very dilute acidic medium for complete solubilization (LMWC, 100% solubility in 0.01% acetic acid as compared to 1% acetic acid for chitosan; in 0.01% acetic acid, only 13% chitosan was soluble). This observation was in contrast to the earlier reports

Table 3. Growth Inhibitory Effect of LMWC toward *B. cereus* (10^6 CFU mL^{-1}) and *E. coli* (10^4 CFU mL^{-1})

				indicator bacteria	
				B. cereus F4810	*E. coli* D21
chitosan	M_r (kDa)	DA (%)	concentration (%, w/v)	percent inhibition[a]	
native	71 ± 2	26.1	0.1	60 ± 1	NI[b]
			0.2	53 ± 2	NI
			0.3	50 ± 1	NI
			0.4	28 ± 3	NI
			0.5	10 ± 2	NI
LMWC (1 h)	10.0	18.9	0.1	100 ± 0[ac]	32 ± 2[c]
			0.2	98 ± 1[b]	56 ± 3[c]
			0.3	97 ± 1[c]	100 ± 0[c]
			0.4	90 ± 1[c]	84 ± 1[c]
			0.5	74 ± 2[c]	48 ± 2[c]
LMWC (3 h)	8.15	15.7	0.1	98 ± 1[a]	50 ± 2[c]
			0.2	94 ± 1[b]	85 ± 1[c]
			0.3	86 ± 2[a]	100 ± 0[c]
			0.4	78 ± 2[c]	88 ± 1[c]
			0.5	62 ± 3[c]	72 ± 2[c]
LMWC (5 h)	4.74	13.5	0.1	97 ± 1[a]	60 ± 1[c]
			0.2	88 ± 2[a]	74 ± 2[c]
			0.3	76 ± 2[d]	100 ± 0[c]
			0.4	68 ± 1[c]	87 ± 2[c]
			0.5	57 ± 1[c]	62 ± 1[c]

[a] Mean ± SD, average of five trials; inhibition was determined by colony (plating) count. [b] NI: no inhibition. [c] Lowercase superscripts within each column indicate significant differences between trials when the same concentrations of native chitosan and LMWC were used as antibacterial agents (*P*-values, $a \leq 0.005$, $b \leq 0.001$, $c \leq 0.0001$, and $d \leq 0.05$), simple interactive sample analysis (SISA).

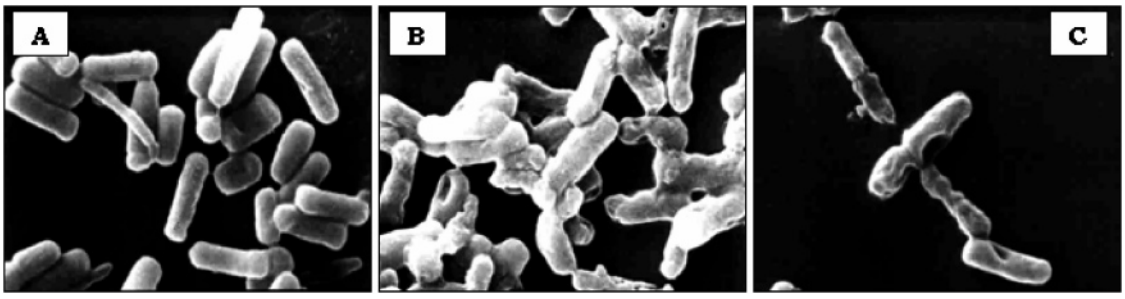

Figure 6. SEM (×10 000) of *B. cereus* before (A) and after 12 and 24 h treatment with LMWC (B and C, respectively).

that a decrease in the M_r of chitosan is associated with increased solubility.[30] However, there was no evidence for the annealed polymorphism in the LMWC obtained as evidenced by the absence of a reflection at 15° in the X-ray diffraction pattern, a characteristic of annealed polymorphs.[24] In the present study, the chitosanolytic activity of pepsin was associated with a decreased DA as evidenced by the spectral data, and hence, the observed poor solubility could probably be due to the exposure followed by conversion of $-NH_2$ groups on LMWC to the sodiated form (Na^+, added to terminate the reaction), which could result in an altered molecular conformation.[31] This was obvious from the *d*-spacing of LMWC, which was neither typical of tendons nor of L-2 polymorphs. The observed difference in the solubility could also be due to the higher probability of LMWC to undergo Schiff's base formation resulting in the decreased solubility.[32]

Bactericidal Activity of LMWC. As compared to native chitosan, LMWC (0.1−0.5%, w/v) markedly inhibited the growth of *B. cereus*, *E. coli*, *Y. enterocolitica*, and *B. licheniformis*. However, both native chitosan and LMWC did not inhibit the growth of *L. monocytogenes*, *S. aureus*, *Y. enterocolitica*, and *B. licheniformis* tested. However, for a detailed study, only strains of *B. cereus* F4810 and *E. coli* D21, one each from Gram-positive and -negative strains, respectively, were selected. The effect of M_r, DA, and the concentration of LMWC on bactericidal action are given in Table 3. Both native chitosan and LMWC generally showed potent bactericidal effects for Gram-positive bacteria, as also observed by Jeon et al. and No et al.[33,34]

The minimum inhibitory concentration (MIC, the lowest concentration of test samples at which the cell growth is neither visible to the naked eye (turbidity) nor measured by plating (viable counts)) was found to be 0.1% (w/v) and 0.3% (w/v) toward *B. cereus* and *E. coli*, respectively (Table 3). Dilution of broth containing the organism and test sample did not show further growth even after prolonged incubation (for 96 h), indicating a lytic effect of the latter, which was confirmed by SEM (Figures 6 and 7), wherein complete disappearance of the cells was observed.

From Table 3, it was clear that an increase in the concentration of chitosan did not show linear inhibitory effect, especially in *E. coli*; 100% inhibition was shown by 0.3% (w/v) LMWC. On either side of the concentration, there was a decrease in the inhibitory effect, as also observed in its application in agriculture.[35] This could be due to the fact that at lower concentrations, LMWC acts rather as an activator than an inhibitor. At higher concentrations, binding of chitosan to the microbial cell surface provides a net positive charge and hence segregates the individual cells or there may be self-aggregation of the LMWC.[31,36]

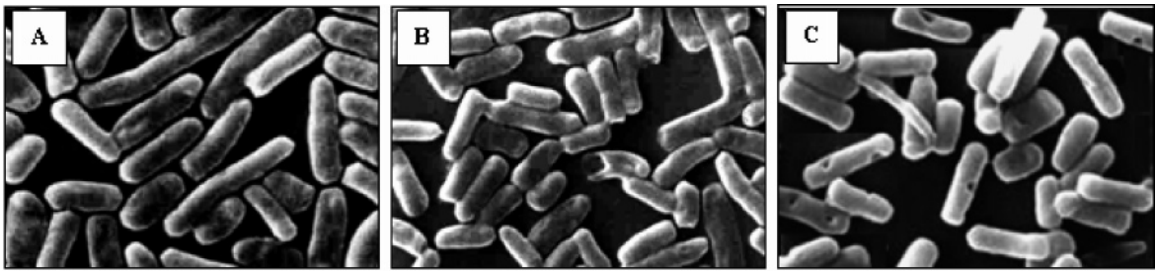

Figure 7. SEM (×10 000) of *E. coli* before (A) and after 12 and 24 h treatment with LMWC (B and C, respectively).

Rhoades and Roller[37] observed that chitosan hydrolysate obtained after chitosanolysis using papain was more active as an antibacterial agent than native chitosan and chitooligomers. It has been reported earlier that chitosan of 3.1 kDa (DA, 52%) and 7.4 kDa (DA, 45%) showed only 58 and 71% inhibition, respectively, toward *B. cereus*, and that the inhibition was dependent on the DA and M_r as well as the functional groups in the chitosan sample.[16] In the present study, the LMWC used had M_r ranging between 4.6 and 10.0 kDa, which were in between the one used by the earlier workers, and at the 0.01% level, they showed 100% inhibition toward *B. cereus*, which could be attributed to its low DA (~13.4−18.8%) as compared to the one used early (45−52%).

The mechanism of antimicrobial activity differs with the M_r as well as DA of the chitosan sample. Chitosan and LMWC, having high M_r/$-NH_3^+$ groups, cannot pass through the microbial membrane and hence stack to the cell surface, which blocks nutrient transport or permeabilizes the microbial cell membrane, resulting in cell lysis.[31] While chitooligosaccharides show a similar effect,[38] owing to their small size and being water soluble, they can traverse the microbial membrane and may bind and regulate DNA transcription.[3] In addition, chitosan could also bind to trace metals, distorting the membrane structure or its water binding capacity may deprive water availability for microbes causing cell death.[3] In the present study, the blockage of the transport system looks more practical because, when chitosan samples were added to the test cultures in the nutrient broth, there was the formation of turbidity, which could be due to the binding of positively charged chitosan onto the negatively charged cell surface thus causing microbial aggregation.[31] According to Chen et al.,[22] chitosans with a lower degree of polymerization and with a lower DA were more effective as antimicrobial agents. The enhanced antimicrobial activity associated with the LMWC could be attributed to their lower DA (~13.4−18.8%), as a result of which, the number of GlcN residues in the molecule increases and at the assay conditions, their amino group acquires a positive charge ($-NH_2 \rightarrow -NH_3^+$) and binds to the negatively charged microbial cell surface.[31]

Further, SEM studies indicated pore formation on the bacterial cell surface, confirming lytic rather than static activity of LMWC toward bactericidal activity. As depicted in Figure 6, after 12 h of LMWC treatment, there were irregularities on the *B. cereus* cell surface, which could be due to the deposition of LMWC, and we could also see cell aggregation. After 24 h of LMWC treatment, there were pores on the cell surface. Similar results were obtained when *E. coli* was subjected to LMWC treatment (Figure 7); however, the cell damage was not as prominent as in *B. cereus* within 24 h, in support of the earlier reports.[33]

In Gram-positive bacteria, the cell membrane is covered by a cell wall made up of 30−40 layers of peptidoglycans, which contain GlcNAc and *N*-acetylmuramic acid as well as D- and L-amino acids including isoglutamate and teichoic acid,[39] to which the positively charged amino groups of chitooligomers/GlcN can bind, resulting in cell wall distortion−disruption, exposure of the cell membrane to osmotic shock, and exudation of the cytoplasmic contents. Gram-negative bacteria, on the other hand, contain an outer membrane wherein lipopolysaccharide and proteins are held together by electrostatic interactions with bivalent metal ions, one to two layers of peptidoglycans (cell wall), and a cell membrane (containing lipid bilayer, transmembrane proteins, and inner/outer membrane proteins). The negatively charged O-specific antigenic oligosaccharide repeating units of the *E. coli* lipopolysaccharide form an ionic-type of binding with the amino groups of chitooligomers (which are cationic in nature below pH 6.2), thus blocking the nutrient flow with concomitant bacterial death due to depletion of the nutrients. Here, the deposition of cationic oligomers on to the cell surface is more prominent than membrane disruption as in the case of Gram-positive bacteria, owing to a stronger association of O-chains to the outer membrane structure. The smaller molecular mass of the chitooligomers/GlcN and more electronegativity on Gram-negative bacteria facilitate effective binding and aggregation of the former, blocking the nutrient flow and ultimately leading to cell lysis.

In conclusion, the depolymerization of chitosan with the aid of pepsin can be exploited commercially for the bulk production of LMWC. Pepsin is easily available at a low cost as compared to chitosanase. Depolymerization was associated with decrease in M_r, DA, and CrI. LMWC of M_r, varying between 4.6 and 10.0 kDa, showed lytic activity toward food-borne pathogens, including *B. cereus* and *E. coli*.

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