Low molecular weight chitosans—Preparation with the aid of pronase, characterization and their bactericidal activity towards *Bacillus cereus* and *Escherichia coli*

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Abstract

The homogeneous low molecular weight chitosans (LMWC) of molecular weight 9.5–8.5 kDa, obtained by pronase catalyzed non-specific depolymerization (at pH 3.5, 37 °C) of chitosan showed lyses of *Bacillus cereus* and *Escherichia coli* more efficiently (100%) than native chitosan (<50%). IR and 1H-NMR data showed decrease in the degree of acetylation (14–19%) in LMWC compared to native chitosan (∼26%). Minimum inhibitory concentration of LMWC towards 10^6 CFU ml^-1 of *B. cereus* was 0.01% (w/v) compared to 0.03% for 10^4 CFU ml^-1 of *E. coli*. SEM revealed pore formation as well as permeabilization of the bacterial cells, as also evidenced by increased carbohydrate and protein contents as well as the cytoplasmic enzymes in the cell-free supernatants. N-terminal sequence analyses of the released proteins revealed them to be cytoplasmic/membrane proteins. Upon GLC, the supernatant showed characteristic fatty acid profiles in *E. coli*, thus subscribing to detachment of lipopolysaccharides into the medium, whereas that of *B. cereus* indicated release of surface lipids. The mechanism for the observed bactericidal activity of LMWC towards both Gram-positive and Gram-negative bacteria has been discussed. © 2006 Elsevier B.V. All rights reserved.

Keywords: Low molecular weight chitosan; Pronase; Structure; Bactericidal activity; Mechanism

1. Introduction

Chitin, next to cellulose is the most abundant natural amino-polysaccharide on Earth. Commercially it is found in the offal of marine food processing industry [1], and as only a small quantity of the offal is utilized for animal feed, its disposal is of environmental concern. In the past two decades, chitosan, a β 1 → 4 copolymer of glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) residues obtained by partial de-N-acetylation of chitin, drew much attention owing to its better solubility and reactivity compared to chitin, and thus exhibiting enhanced bio-functionalities such as antimicrobial, antitumor, hypolipidemic, hypocholesterolemic, and immuno-stimulating activities [2,3].

Recent studies have revealed the antimicrobial potential of chitosan to be dependent on its *M* r as well as DA [4,5]. However, high *M* r and high viscosity of chitosan solutions have restricted its multidimensional utility. On the other hand, the LMWC obtained by physical, acidic or enzymatic depolymerization of chitosan, due to its ready solubility in water, is better amenable for a wide variety of biomedical applications. It was reported that LMWC (5–10 kDa) had highest bactericidal activity towards pathogenic bacteria [5], whereas a 20 kDa product prevented progression of *Diabetes mellitus* and showed a higher affinity for lipopolysaccharides (LPS) than the native chitosan of ∼140 kDa [6]. The practical use of LMWC in milk

Abbreviations: *M* r, Molecular weight; DA, Degree of acetylation; DP, Degree of polymerization; CFU, Colony forming units; MIC, Minimum inhibitory concentration; HPSEC, High performance size exclusion chromatography; SEM, Scanning electron microscopy; GPC, Gel permeation chromatography; GLC, Gas–liquid chromatography; LPS, lipopolysaccharides

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preservation and oral hygiene is also reported [7]. Of late, LMWC of 5–10 kDa was also shown to have potential as DNA delivery system [8].

Chitosan could be depolymerized by physical, chemical or enzymatic methods [9–11]. The former (e.g., sonication, shearing, etc.) requires special equipments and the chemical hydrolysis using HCl, H2SO4, H2O2, HNO2 do not lend themselves to easy reaction control and often results in the modification of the depolymerization products. Enzymatic depolymerization based either on specific or non-specific enzymes finds advantages over other methods, as it overcomes the cited drawbacks and also the reaction will be under facile control. Chitosanase, the specific enzyme for chitosanolysis is expensive, unavailable in bulk and results in a preferential formation of chitooligomers-monomers due to its specificity, whereas the non-specific enzymes, which are inexpensive and commercially available, result mainly in the formation of LMWC [12,13]. By varying the reaction conditions such as pH of the reaction medium, temperature and time, it was possible to obtain LMWC of Mw in the range 9.0±0.5 kDa by depolymerizing chitosan using pronase, a non-specific enzyme [14]. In the present study, further characterization of LMWC and the effect of its Mw and DA on bactericidal activity towards Gram-positive (Bacillus cereus) and Gram-negative (Escherichia coli) bacteria as well as the mechanism of their action are reported.

2. Experimental procedures

2.1. Materials

Chitosan standards (Mw, 150–600 kDa) were obtained from Fluka Chemical Corp., Switzerland. Chitosan 10 (Mw, 60 kDa) was from Wako Chemical, Osaka, Japan. Pronase (Type XXV protease from Streptomyces griseus, EC. 3.4.24.4) was from Sigma Chemical Co., St. Louis, MO, USA. Dextran standards of Mr 10–70 kDa were from Pharmacia Fine Chemicals, Uppsala, Sweden and 1–5 kDa were from Fluka Chemika, USA. Shrimp chitin was from CFTRI Regional Center at Mangalore, India. Other chemicals used were of highest purity available.

2.2. Preparation of chitosan

Shrimp chitin was subjected to heterogeneous N-deacetylation to obtain chitosan [15], which was further purified by dissolving in 1% acetic acid, filtered and precipitated with 2% sodium carbonate. The precipitate was water washed and freeze-dried (native chitosan).

2.3. Isolation of LMWC

Chitosan solution (1%, in 1% acetic acid and pH adjusted to 3.5 with 0.1 N HCl/NaOH) was treated with pronase in the ratio 100:1 (w/w), incubated for different periods (1, 3 and 5 h) at 37 °C followed by arresting the reaction by heat denaturing the enzyme (100 °C, 5 min) and adding equal volume of 2 N NaOH. The precipitate (LMWC) obtained after centrifugation (3000 rpm, 10 min) was dialyzed against deionized water using a membrane (Sigma Chemicals Co, USA) having Mw cut-off 2 kDa at ambient temperature and freeze-dried.

2.4. Determination of the Mw

Viscometric measurement: The viscosity of chitosan dissolved in sodium acetate buffer (0.5 M acetic acid+0.2 M sodium acetate, pH 4.5) was measured using an Ostwald’s viscometer. The average Mw was deduced using the Mark–Houwink’s equation, \( \eta = K M_w^a \), where \( \eta \) = intrinsic viscosity, \( K \) = 3.5×10^4, \( a \) = 0.76 [16].

2.5. GPC and HPSEC

The Mw of chitosan was determined by GPC on a Sepharose CL-4B column (Sigma, bed volume—180 ml) [4]. LMWC was analyzed by HPSEC on E-linear-E-1000 columns (Waters Associates, Milford, Massachusetts, USA) connected in series to a RI detector (Shimadzu LC-6A system) and GPC on Biogel P30 (Bio Rad laboratories, CA, Bed volume—100 ml). Acetate buffer (pH 4.5) was used as the eluant and both the columns were pre-calibrated with dextran and chitosan Mw standards.

2.6. IR spectroscopy

IR spectral studies were performed in a Perkin Elmer spectrometer (Connecticut, USA) under dry air at room temperature using KBr pellets. Chitosan and LMWC (4 mg each) were mixed thoroughly with 200 mg KBr; 40 mg of the mixture was peltetted and subjected to IR spectroscopy. Reproducibility of the spectra was verified on three preparations and DA was determined using the formula, \( A_{1655 \text{ cm}^{-1}}/A_{3450 \text{ cm}^{-1}} \times 100 + 1.33 \), where \( A \) — absorbance at these wavelengths, calculated from baseline drawing [17].

2.7. Liquid state 1H-NMR

Native chitosan and LMWC (50 mg each) dissolved in 1 ml solvent mixture of D2O+DCl (0.98+0.02 ml, respectively) were subjected to 1H-NMR on a JEOL 300 MHz spectrometer at 23 °C. Degree of deacetylation (DDA, %) was calculated using integrals of the peaks of proton H1 of deacetylated monomer (H,D) and the three protons of acetyl group (H-Ac), i.e., DDA (%) = H1D/(H1D+H-Ac)×100 [18].

2.8. Chitosan/LMWC—solubility study

Solubility of chitosan/LMWC was determined according to the method of Qin et al. with slight modification [19]. Sample (chitosan/LMWC, 0.1 g) was suspended in 10 ml solvent mixture of D2O+DCl (0.98+0.02 ml, respectively) subjected to 1H-NMR on a 300 MHz spectrometer. The precipitate was washed thoroughly with ethanol, collected and weighed after drying over phosphorous pentoxide in vacuum. The solubility of chitosan/LMWC (average of five trials) was determined by the percent of chitosan/LMWC dissolved.

2.9. Indicator bacteria and inoculum preparation

Strains of Bacillus cereus F4810 (courtesy, Dr. J.M. Kramer, Central Public Health Laboratory, United Kingdom), Escherichia coli D21 (courtesy, Dr. M.A. Linggood, Unilever Research, United Kingdom), Listeria monocytogenes Scott A (courtesy, Dr. Arun K. Bhunia, Purdue University, USA), Yersinia enterocolitica MTCC 859 (courtesy, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India), Staphylococcus aureus FRI 722 (courtesy, Dr. E. Notermans, Public Health Laboratory, The Netherlands) and Bacillus licheniformis CFR 1621 (Native food isolate) were obtained from the culture collection maintained in the Institute.

The cultures were maintained at 6 °C on brain heart infusion (BHI) agar (HiMedia, Mumbai, India) slants and sub-cultured at 15-day intervals. Prior to use, the culture was successively propagated twice in BHI broth at 37 °C. Cell suspensions of the culture, individually, were prepared from 20 h old BHI culture broth with appropriate dilution in 0.85% saline, giving individual counts of 10^6–10^8 CFU ml^{-1} [20].

2.10. Bacterial growth inhibitory assay

To 9 ml aliquots of nutrient broth (HiMedia, Mumbai, India) containing 0.5% dextrose, added 1 ml of 0.1% chitosan and LMWC dissolved in 0.1 and 1% acetic acid, respectively and the broth pH was adjusted to 6.0 with sodium acetate, so as to get final chitosan/LMWC concentrations between 0.01–0.1%
(1–10 mg/10 ml broth solution). Nutrient broths containing 0.01 and 0.1% acetic acid and pH adjusted to 6.0 served as controls. To the broth, added a cell suspension (100 μl) of specific bacterial strain in the concentration range of 10^3–10^9 CFU/ml. The contents were mixed well and incubated for ~20 h at 37 °C and 1 ml aliquots were transferred into fresh 9 ml nutrient broth tubes, whereas the remaining aliquots were poured plated with BHI agar and incubated for 24 h at 37 °C. The plates were observed for the bacterial colonies and the tubes for turbidity [21]. Growth inhibitory activity was calculated using [(C – T)/C] × 100, wherein C and T were the colony counts in the ‘control’ and ‘test’ plates, respectively [5].

2.11. SEM

After 20 h incubation at 37 °C, 0.5 ml aliquots from the above assay tubes were transferred to micro-centrifuge tubes and centrifuged. The pellets obtained were treated with phosphate buffer (pH 7.0, 0.3 M), fixed with glutaraldehyde (1%) for 1 h at 4 °C and further treated with 10–96% alcohol in a sequential manner and dried. They were spread on double-sided conducting adhesive tape pasted on a metallic stub and coated with gold (100 μm) in a sputter coating unit for 5 min and observed under SEM (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) at 20 kV [22].

2.12. Characterization of cell-free supernatant

(A) Cultures of B. cereus (10^6 CFU ml^-1) and E. coli (10^6 CFU ml^-1), individually suspended in saline, were subjected to sonication (Julabo model USR-1, West Germany; 51 kHz, 6 × 30 and 3 × 20 s, respectively) and incubated at 37 °C for 24 h followed by centrifugation (10000 rpm, 4 °C for 15 min). The supernatant was dialyzed (cut-off value 10 kDa) and freeze-dried (positive control), (B) The cultures suspended in saline were centrifuged after incubation at 37 °C for 24 h, the supernatant was subjected to dialysis against saline and freeze-dried (control) and (C) to one set of cultures in saline, was added LMWC (1%) for 1 h at 4 °C and further treated with 10 96% alcohol in a sequential manner and dried. They were spread on double-sided conducting adhesive tape pasted on a metallic stub and coated with gold (100 μm) in a sputter coating unit for 5 min and observed under SEM (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) at 20 kV [22].

3. Results and discussion

3.1. Characterization of native chitosan

The M_r of native chitosan was 71 ± 2 kDa as determined by GPC on Sepharose CL-4B, which was in accordance with the values calculated by viscometry. The DA calculated from IR and 1H-NMR data was in good agreement with each other (Figs. 1 and 2, Table 1). The presence of a single broad peak centered at 3371 cm^-1 in the IR-spectrum of native chitosan was indicative of its β-conformation [28]. The latter is more susceptible for degradation due to parallel arrangement of the individual chains, resulting in weak inter-chain hydrogen bonding network and an easy penetration of the enzymes [3].

3.2. Pronase activity

The purity of pronase was established unambiguously by SDS-PAGE, capillary electrophoresis and also by reverse-phase HPLC, wherein only a single peak was observed [14,29]. In addition, N-terminal sequence analysis of pronase was found to be Ser–Gln–Gly–Ser–Val–Tyr–XX–Pro–Tyr–Ala–Asp, which was in harmony with the reported sequence data for pronase. Optimum chitosan depolymerization by pronase occurred at pH 3.5 and 37 °C and the reaction obeyed Michaelis–Menten kinetics with K_m and V_max of 5.71 mg ml^-1 and 288.26 nmol min^-1·mg^-1, respectively [14]. The activity of pronase towards chitosanolsysis was 0.12 Unit (Unit=μmol of reducing equivalents released min^-1·mg^-1) [30], whereas towards proteolysis, it was 4 Units (Unit=enhanced absorbance at 280 nm, due to the release of TCA soluble peptides from hemoglobin–reaction time × mg protein in the reaction mixture), indicating its preferential proteolysis. The enzyme was stable up to 4 h at optimum conditions. Although pronase showed increase in the activity, chitosan concentration above 20 mg ml^-1 (substrate: enzyme ratio of 200:1) could not be used owing to its high viscosity, which affects enzyme penetration and thus depolymerization.

3.3. Characterization of LMWC

The M_r values calculated by HPLC (Table 1) and GPC were in good agreement with each other, and varied between 9.5 and 8.5 kDa depending on the reaction time (i.e., 1–5 h). The use of acetate buffer minimized interaction between the column material and the free –NH₂ groups on LMWC. Appearance of a single symmetrical peak (Fig. 3) was indicative of molecular homogeneity of the LMWC preparations.

3.3.1. IR spectroscopy

In the IR spectra of both chitosan and LMWC, the band around 2900 cm^-1 due to the –CH vibration represented a good internal reference for comparison with other band absorbance (Fig. 1). The absence of a strong absorption band above 3500 cm^-1 indicated the involvement of both 3-OH and primary hydroxyl group (6-CH2OH) in the intra- and inter-chain hydrogen bonding [28]. A slight downward shift in the absorption at around 3369 cm^-1 for LMWC (1 h) was indicative of its disorderliness, whereas an upward shift observed in 5 h sample indicated its orderliness. This could be explained based on a fold-decrease in the DA although M_r of 1 and 5 h samples was comparable (Table 1). In case of 1 h LMWC, fold-decrease in the M_r was ~7.5, whereas that of DA,
~1.35 compared to native chitosan, accounting to a decrease of 0.181 DA for every fold decrease in the $M_r$. Accordingly, if pronase is specific for a type glycosidic linkage, then 5 h LMWC with 8.35-fold decrease in the $M_r$ was supposed to show ~1.51-fold decrease in the DA, but practically it showed 1.81-fold decrease, indicating that though pronase can act on all the four types of glycosidic linkages in chitosan (–GlcN–GlcN−, –GlcN–GlcNAc–, –GlcNAc–GlcN– as well as –GlcNAc–GlcNAc–), it has more chitinase than chitosanase-like activity, releasing GlcNAc/GlcNAc-rich oligomers, leaving more GlcN in the LMWC. The latter was also evident from our earlier data [29], wherein there was the release of only GlcNAc and not GlcN after chitosanolysis by pronase. To confirm chitinase-like activity of pronase, we made use of chitosan samples of $M_r$ 71±2 kDa having DA 15, 19, 26 and 40% with which pronase showed activity of 0.71, 0.86, 1.12 and 1.52 Units, respectively.

Intensity of the peak near 1650 cm$^{-1}$ (amide I) in LMWC decreased due to decrease in acetyl content. The absorbance near 1320 cm$^{-1}$, corresponding to N-acetylglucosamine residues gradually decreased in LMWC (Absorbance= log % T, 0.0668, 0.0290 and 0.0230, respectively for native chitosan, LMWC-1 and 5 h) confirming a decrease in the DA. The band around 1623 cm$^{-1}$ is expected to be associated with an intra-

Fig. 1. IR spectra of native chitosan (A) and LMWC obtained after 1 h (B) and 5 h (C) of pronase catalyzed chitosanolysis.
molecular hydrogen bond between –C=0 and –NH₂ groups. In the present study, a decrease in the peak height near 1650 cm⁻¹ in LMWC (1 h) compared to native chitosan indicates a decrease in the intra-molecular hydrogen bonding. In the 750–500 cm⁻¹ region, thought to be more sensitive to changes in crystallinity, a shoulder at 662.62 cm⁻¹ in case of 5 h LMWC is assigned to out-of-plane bending of –NH and –OH groups and participation in hydrogen bonding [28]. The region between 1460 and 1420 cm⁻¹ is considered for polysaccharides as conformation sensitive and a shift of the same in LMWC is attributed to modification in the environment of –CH₂OH group, although the high degree of band coupling in this region makes it difficult to assess the kind of chain packing or hydrogen bonding network [28].

3.3.2. Liquid state ¹H-NMR

Lavertu et al. [18] performed spectral assay at 70 °C and found that the hydrolytic cleavage of acetyl groups of chitosan by dilute acid (i.e., DCl) at this temperature to be quite slow. However, in the present study, as the native chitosan showed observable cleavage of the acetyl group in the acidic medium above 60 °C, the spectral measurements were performed at 23 °C, which also showed the appearance of a solvent signal at around 4.37 ppm. The DA calculated from ¹H-NMR spectral data (Fig. 2) was in accordance with that obtained from IR-spectra (Table 1). In the spectra, the resonance near 1.8 ppm is assigned to the N-acetyl protons and a progressive decrease in its height for 1 h and 5 h LMWC indicated a decrease in DA. From the chemical shift values (Table 2), it was obvious that depolymerization was also associated with conformational changes, in accordance with the IR spectra. Compared to native chitosan, 1 h samples showed a downfield shift in the values, whereas 5 h LMWC showed an upfield shift substantiating the orderliness and altered conformation of the latter, as also explained by IR-spectra.

3.4. Solubility of chitosan and LMWC

Solubility of chitosan plays a crucial role for its application, which in turn depends on its Mᵣ and DA. Chitosan is soluble in

### Table 1

<table>
<thead>
<tr>
<th>Chitosan</th>
<th>Mᵣ (kDa)</th>
<th>DA (%)</th>
<th>Yield (%)</th>
<th>IR</th>
<th>¹H-NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>71.0±2.0ᵃ</td>
<td>25.78</td>
<td>25.53</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LMWC—1 h</td>
<td>9.50±0.15ᵇ</td>
<td>19.04</td>
<td>18.87</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>LMWC—3 h</td>
<td>9.10±0.12ᵇ</td>
<td>16.34</td>
<td>16.08</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>LMWC—5 h</td>
<td>8.50±0.12ᵇ</td>
<td>14.29</td>
<td>14.34</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Determined by GPC on Sepharose CL-4B column.
ᵇ Determined by HPLC.

### Table 2

<table>
<thead>
<tr>
<th>Chitosan</th>
<th>H-1(D)</th>
<th>H-2/6</th>
<th>H-3(D)</th>
<th>H-Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>4.65</td>
<td>3.53</td>
<td>2.92</td>
<td>1.84</td>
</tr>
<tr>
<td>LMWC—1 h</td>
<td>4.58</td>
<td>3.51</td>
<td>2.91</td>
<td>1.82</td>
</tr>
<tr>
<td>LMWC—5 h</td>
<td>4.73</td>
<td>3.61</td>
<td>2.93</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Fig. 2. ¹H-NMR spectra of native chitosan (A) and LMWC obtained after 1 h (B) and 5 h (C) of pronase catalyzed chitosanolysis.

Fig. 3. HPLC profiles of LMWC obtained after 1 h, 3 h and 5 h of pronase catalyzed chitosanolysis.
aqueous acidic medium below pH 6.2 and above this pH it precipitates out of the solution. In the present study, native chitosan was insoluble in aqueous medium (pH ~5.6), showed only 13% solubility in 0.01% acetic acid and its complete dissolution required 1% acetic acid, while LMWC was 66–74±2% soluble in aqueous medium and became completely soluble in 0.01% acetic acid. To precipitate out LMWC after pronase digestion, it was necessary to adjust the pH to 6.7 and 6.9 after 1 h and 5 h reaction time, respectively. Altogether, solubility of chitosan/LMWC was not only dependent on pH of the solvent used, but also the percent of acid. Surprisingly, the 5 h LMWC showed a decrease in solubility in aqueous medium (66±2%) compared to that obtained after 1 h (74±2% solubility). According to our earlier reports [9,29], observed difference in the solubility of 1 h and 5 h samples could be due to the removal of more of GlcNAc in 5 h sample, resulting in LMWC having more GlcN, the –NH₂ group of which undergo stronger intra- and inter-chain hydrogen bonding, affecting the solubility. Cheng and Li [9] made use of two volumes of acetonitrile instead of equal volume of 2 N NaOH as in the present study, to arrest the catalytic reaction. The use of acetonitrile gives a product readily soluble in water, contrary to the insoluble sodiated-form of LMWC. But the drawbacks of using acetonitrile are its cost, flash treatment at elevated temperature of the supernatant, which results in Maillard reaction products and the consequent difficulty in recovering both LMWC and chitooligomers-monomers in one step and the safety of the resulting product due to the presence of residual solvent.

3.5. Bactericidal activity

Compared to native chitosan, LMWC showed better growth inhibitory effect towards both Gram-positive and Gram-negative bacteria such as B. cereus, E. coli, Y. enterocolitica and B. licheniformis. However, for further study, B. cereus and E. coli, one each from Gram-positive and Gram-negative bacteria, were chosen. The effects of $M_r$, DA and the concentration of LMWC on growth inhibitory action are given in Table 3. The growth inhibitory effect of LMWC was more towards B. cereus as against E. coli. As could be seen from Table 3, LMWC with $M_r$ 9.5 kDa and 19% DA (1 h sample) caused complete growth inhibition of B. cereus whereas for E. coli, it was the 5 h sample (8.5 kDa and 14% DA) that was more effective, indicating a differential role played by both $M_r$ and DA of LMWC. According to Chen et al. [21], chitosans with lower DP and DA were more effective as microbial growth inhibitory agents. The enhanced growth inhibition and bactericidal activity associated with LMWC was attributed to lower DA (~14–19%), as a result of which, the number of GlcN residues increases and at the assay condition (pH 6.0), their amino group on C2 acquires a net positive charge (–NH₂→–NH₃⁺) and preferably bind to the negatively charged microbial cell-surface causing its permeabilization. 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, towards B. cereus, confirming the dependency of inhibition on the DA, $M_r$ as well as the functional groups present [2,4]. In the present study, the LMWC had a $M_r$ ranging between 9.5 and 8.5 kDa, which was comparable to those used by the earlier workers, but even at 0.01% level, they showed 100% inhibition, which could be attributed to their relatively low DA (~14–19%), thus establishing its definite role for the bactericidal activity [31].

From Table 3, it was clear that increase in the concentration of LMWC showed a linear inhibitory effect on bacterial growth. With B. cereus, LMWC obtained after 1 h reaction time showed optimum lytic activity compared to 3 h and 5 h samples. Unlike with B. cereus, 5 h LMWC showed better antibacterial activity towards E. coli compared to 1 h and 3 h samples. This could be due to the fact that 5 h sample (8.5 kDa, 13.4% DA), owing to its smallest size and decreased DA can slide, so as to form hydrogen bonding easily with the electronegative atoms of the E. coli cell membrane that are present on the surface, unlike with Gram-positive bacteria, wherein LMWC has to penetrate the cell-wall in search of electronegative groups.

The MIC, defined as the lowest concentration of test samples at which the cell growth is neither visible to naked eye (turbidity) nor measurable by plating (viable counts), was found to be 0.01% towards 10⁶ CFU ml⁻¹ of B. cereus and 0.03% towards 10⁴ CFU ml⁻¹ of E. coli. Dilution of broth containing the organism and test samples did not show any growth even after prolonged incubation (for 96 h), indicating bactericidal

| Table 3 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Inhibitory effect of chitosan and LMWC towards B. cereus and E. coli** |
| **Chitosan** | **$M_r$ (kDa)** | **DA (%)** | **Concentration (%)** | **Indicator bacteria** |
| Native | 71.0 | 26 | 0.01 | B. cereus F4810 | 62±2 |
| | | | 0.03 | | 61±3 |
| | | | 0.05 | | 60±2 |
| LMWC-1 h | 9.50 | 19 | 0.005 | E. coli D21 | 94±1 |
| | | | 0.01 | | 100±0 |
| | | | 0.02 | | 100±0 |
| | | | 0.03 | | 99±1 |
| | | | 0.04 | | 98±2 |
| | | | 0.05 | | 97±2 |
| | | | 0.10 | | 98±1 |
| LMWC-3 h | 9.10 | 16 | 0.005 | E. coli D21 | 88±2 |
| | | | 0.01 | | 98±1 |
| | | | 0.02 | | 97±1 |
| | | | 0.03 | | 96±2 |
| | | | 0.04 | | 97±1 |
| | | | 0.05 | | 97±1 |
| | | | 0.10 | | 96±2 |
| LMWC-5 h | 8.50 | 14 | 0.005 | E. coli D21 | 86±2 |
| | | | 0.01 | | 97±1 |
| | | | 0.02 | | 95±2 |
| | | | 0.03 | | 94±2 |
| | | | 0.04 | | 95±3 |
| | | | 0.05 | | 96±2 |
| | | | 0.10 | | 94±2 |

NI—no inhibition.

* Inhibition was effective against a cell population of 10⁶ CFU/tube.

* Inhibition was effective against a cell population of 10³ CFU/tube.

* Values of mean of five trials ± standard deviation.
effect of the LMWC, which was further confirmed by SEM studies (Fig. 4), wherein disruption/disintegration of cell surface could be seen.

Analyses of the cell-free supernatants of the sonicated and LMWC treated cultures showed the presence of carbohydrates and proteins (Table 4), in support of the observation of SEM. The latter in the LMWC treated as well as sonicated culture supernatants was several folds higher than that in the untreated cultures. Similarly, increased levels of cytoplasmic enzymes such as maltase, lactase and protease in the LMWC treated and sonicated culture supernatants substantiated release of cytoplasmic contents (Table 4). Protein profiles (cell-free supernatants) on PAGE (Fig. 5) as well as HPLC (data not shown) also supported the damaged cell wall/outer membrane, evidenced by the release of proteins.

Gram-negative bacteria are known to have LPS and protein in the outer membrane, whereas Gram-positive ones generally have surface lipids [31]. Identification by GLC of fatty acids in the LMWC-treated culture supernatants (Table 5) further confirmed the dissociation and thus permeabilization of surface lipids of B. cereus and the outer membrane of E. coli. The release of saturated fatty acids in E. coli in abundance (14:0, 16:0 and 18:0 followed by 18, 20-series unsaturated fatty acids) confirmed disruption of cell wall/membrane.

The amino acid sequence of the protein (electrophoretic mobility, Rf=0.15) from the supernatant of B. cereus treated

Table 4

<table>
<thead>
<tr>
<th>Samples</th>
<th>Carbohydrate (μg/ml)</th>
<th>Protein (μg/ml)</th>
<th>Maltase (Units)</th>
<th>Lactase (Units)</th>
<th>Protease (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control (A)</td>
<td>49.8</td>
<td>124.8</td>
<td>0.16</td>
<td>0.28</td>
<td>0.30</td>
</tr>
<tr>
<td>Control (B)</td>
<td>0.74</td>
<td>23.58</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Test (C)</td>
<td>44.1</td>
<td>109.8</td>
<td>0.14</td>
<td>0.26</td>
<td>0.28</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control (A)</td>
<td>52.3</td>
<td>179.7</td>
<td>0.18</td>
<td>0.30</td>
<td>0.32</td>
</tr>
<tr>
<td>Control (B)</td>
<td>0.92</td>
<td>31.24</td>
<td>0.06</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Test (C)</td>
<td>47.8</td>
<td>160.8</td>
<td>0.16</td>
<td>0.27</td>
<td>0.29</td>
</tr>
</tbody>
</table>

A—cultures subjected to sonication. B—cultures without any treatment. C—cultures treated with LMWC.

Fig. 4. SEM of B. cereus and E. coli—before (A and C) and after (B and D) treatment with LMWC, respectively (magnification, ×10000). E and F represent the SEM immediately after the addition of LMWC to the test cultures (E—B. cereus and F—E. coli, magnification ×5000).
with LMWC was found to be –Ala–Gly–Lys–Thr–Phe–Pro–Asp–Val– and this was identified as a fragment of the surface-layer (S-layer) protein of *B. cereus* (–X–Gly–Lys–Thr–Phe–Pro–Asp–Val–). S-layer protein is a para-crystalline mono-layered assembly of proteins, which coat the cell-surface of *B. cereus*. Sequence of the protein from *E. coli* (Rf=0.23) determined by Edman degradation was –Ala–Pro–Val–Gly–Ala– and this showed homology with a fragment of regulator of nucleoside diphosphate kinase (rnk protein). Shankar et al. [32] reported that the latter, a 14.0 kDa cytoplasmic protein with no homology to any other protein, is involved in the restoration of nucleoside diphosphate kinase activity. Identification of S-layer and rnk proteins in the cell-free supernatants once again confirmed pore formation and permeabilization of the bacterial cell wall by the action of LMWC.

Further, the protein of Rf 0.5 of both sonicated and LMWC treated *B. cereus* culture showed identical N-terminal sequences (–Ser–Lys–Phe–Gly–Leu–Pro–). BLAST analysis ([www.expasy.org](http://www.expasy.org)) of this sequence indicated it to be a fragment of nucleosomal enzyme, methionyl t-RNA formyltransferase [33]. The N-terminal sequence of the protein with Rf of 0.42 from sonicated *E. coli* culture was –Gln–Gly–Pro–Glu–Gln– and that of LMWC treated was –Tyr–Asp–Pro–Glu–Ala–. Both these proteins were fragments of the hypothetical cytoplasmic protein ECs [34]. The appearance of different fragments of the same protein could be attributed to the damage caused by sonication and proteolysis.

### 3.6. Mechanism of bactericidal action of LMWC

Till date, the exact mechanism of growth inhibitory activity of chitosans is yet to be elucidated. Stacking of chitosan molecules over the microbial cell surface, thus blocking the transport of nutrients [2] or binding to DNA and thus inhibiting transcription or permeabilization of the microbial cell wall/membrane or binding of trace metals or water binding and the activation of several defense mechanisms in the host tissue, etc. [3] have been postulated for antibacterial activity of chitosan. Helander et al. [35] showed the binding of chitosan to the outer membrane of Gram-negative bacteria forming a vesicular structure, causing disruption and extensive alteration in the outer membrane surface and resulting in the loss of its barrier property. It is also possible that, due to its ability to bind LPS, chitosan causes permeabilization of microbial cell membrane and thus enhancing the non-protein nitrogenous substance uptake and releasing the LPS from the cell surface [36]. We could observe a sudden increase in the turbidity of the test cultures immediately after the addition of LMWC and a steady clearing of the solution, indicating initial interactions between the two followed by cellular/membrane disintegration. The former is also evident from the SEM of the cells immediately after the addition of LMWC, wherein we could see irregularities on the cell surface and aggregation of the cells (Fig. 4E and F).

Gram-negative bacteria contain an outer membrane wherein LPS and proteins are held together by electrostatic interactions with divalent metal ions, 1–2 layers of peptidoglycans (cell-wall) and a cell membrane (containing lipid bilayer, transmembrane proteins and inner/outer membrane proteins). Additionally there are present phospholipids, proteins and lipoproteins. LPS are structurally characterized by three independent regions namely, Lipid A, Core oligosaccharide and O-specific chains. Lipid A contains repeating units of (GlcN)₂ to which fatty acids (mainly C10–C16) and also phosphate groups are attached [37]. The Core region is characterized by the presence of 2-keto-2-deoxyoctanoic acid and heptoses. The O-specific antigenic oligosaccharide-repeating units, are made up of glucose, galactose including uronic acid and some unusual sugars such as 2-O-acetyl abequose, 3-deoxyoctulosonic acid, 2-amino-2-deoxy-hexuronic acid, 4-O-lactylhexose, etc., which are negatively charged. These negatively charged groups in *E. coli* cell wall/out membrane get involved in ionic-type of binding with –NH₃⁺ groups of LMWC (which are cationic in nature below pH 6.2) with concomitant release of the LPS from the outer membrane, distorting the electrostatic interactions of the latter with metal ions, resulting in the exposure of cell-wall and cell membrane to osmotic shock and thus spillage of the cytoplasm (Fig. 6A).

Earlier, we showed that the chitooligosaccharides (DP, 4–8) do show bactericidal action towards Gram-negative bacteria owing to their deposition on the bacterial cell-surface because of their ionic interaction, thus blocking the nutrient flow [29]. Unlike chitooligosaccharides, LMWC are strong enough to bind and release the LPS from the Gram-negative bacterial cell wall, which is also evident from the SEM (Fig. 4D) and this could be due to the higher molecular structure of the LMWC.

In Gram-positive bacteria, the cell membrane is covered by a cell-wall made up of 30–40 layers of peptidoglycans, which

### Table 5

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th><em>B. cereus</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>12.04</td>
<td>1.90</td>
</tr>
<tr>
<td>16:0</td>
<td>27.22</td>
<td>40.34</td>
</tr>
<tr>
<td>16:1</td>
<td>1.96</td>
<td>33.32</td>
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<tr>
<td>18:0</td>
<td>26.54</td>
<td>6.46</td>
</tr>
<tr>
<td>18:1</td>
<td>9.64</td>
<td>15.33</td>
</tr>
<tr>
<td>18:2</td>
<td>12.31</td>
<td>1.34</td>
</tr>
<tr>
<td>20:4</td>
<td>10.29</td>
<td>1.31</td>
</tr>
</tbody>
</table>
Fig. 6. Site of attachment of chitosan to outer membrane of Gram-negative bacterium (A) and cell wall of Gram-positive bacterium (B).
contain repeating units of GlcNAc, N-acetylmuramic acid as well as D and L-amino acids including isoglutamate and sometimes toichoic acid, to which positively-charged –NH\textsuperscript{+} groups of LMWC could bind, resulting in cell-wall distortion–disruption and exudation of the cytoplasmic contents (Fig. 6B). This was evident from the SEM data (Fig. 4B), where one could see pores along with irregularities on the cell surface of B. cereus and E. coli after treatment with LMWC. The mode of action of cationic antibacterial agents is widely believed to be due to interacting with and disrupting the wall/membrane structure [29,38].

In conclusion, depolymerization of chitosan, associated with decrease in \( M_\text{r} \) and DA, with the aid of pronase can be exploited commercially for the bulk production of LMWC of desired molecular weight. Pronase is easily available at a low cost compared to chitosanase. LMWC of \( M_\text{r} \) varying between 9.5 and 8.5 kDa and DA between 14 and 19% showed better bactericidal activity towards both B. cereus and E. coli compared to chitosan of much higher \( M_\text{r} \). Bactericidal activity was essentially due to pore-formation on the cell surface and thus permeabilization of the bacterial cell contents. Being non-toxic LMWC could be of potential use as a food preservative.

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References


