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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 ^{1}H-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.

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, H_2SO_4, H_2O_2, HNO_2 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 M_r 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 M_r 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 (M_r, 150–600 kDa) were obtained from Fluka Chemical Corp., Switzerland. Chitosan 10 (M_r, 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 M_r 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 M_r cut-off 2 kDa at ambient temperature and freeze-dried.

2.4. Determination of the M_r

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 M_r was deduced using the Mark–Houwink's equation, $(\eta)=KM_r^a$ where (η)=intrinsic viscosity, $K=3.5\times10^4$, $a=0.76$ [16].

2.5. GPC and HPSEC

The M_r 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-8A 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 M_r standards.

2.6. IR spectroscopy

IR spectral studies were performed in a Perkin Elmer spectrum 2000 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 palletized and subjected to IR spectroscopy. Reproducibility of the spectra was verified on three preparations and DA was determined using the formula, $(A^{-1}_{1655\ cm}/A^{-1}_{3450\ cm})\times100\div1.33$, where A—absorbance at these wavelengths, calculated from baseline drawing [17].

2.7. Liquid state ^{1}H-NMR

Native chitosan and LMWC (50 mg each) dissolved in 1 ml solvent mixture of D_2O+DCl (0.98+0.02 ml, respectively) were subjected to ^{1}H-NMR on a Jeol 300 MHz spectrometer at 23 °C. Degree of deacetylation (DDA, %) was calculated using integrals of the peaks of proton H_1 of deacetylated monomer (H_1D) and the three protons of acetyl group (H-Ac), i.e., DDA (%)=$H_1D/(H_1D+H\text{-}Ac/3)\times100$ [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 (distilled water, 0.01% and 1% acetic acid) at 25 °C for 2 h under constant stirring. Soluble chitosan/LMWC was removed by centrifugation at 5000 rpm for 15 min. 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 10^2–10^6 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%