Depolymerized products of chitosan as potent inhibitors of tumor-induced angiogenesis

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Abstract

Water-soluble low-molecular weight chitosan (LMWC) and chitooligosaccharides (COs) were obtained from chitosan (16% N-acetylation) by depolymerization induced by potassium persulfate under nitrogen atmosphere for 2 h. They were characterized by IR, X-ray, HPLC and $^{13}$C-NMR. Splitting of C3/C5 signals in the latter indicated a newer conformation, and also showed prominence of acetyl groups in LMWC, may be due to cleavage between two consecutive deacetylated residues. Molecular weight of LMWC, determined by HPSEC, showed a single peak of ~37 kDa. HPLC analysis of the solvent-extracted fraction revealed COs enriched with pentamer, hexamer and higher oligomers. The effect of LMWC and COs on the growth of Ehrlich ascites tumor (EAT) cells and tumor-induced neovascularization was studied. COs (50 μg) were more effective compared to LMWC (100 μg) and proved to be potent angioinhibitory and antitumor compounds, as shown by inhibition of angiogenesis and inducing apoptosis as a function of DNA fragmentation.

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1. Introduction

Chitosan, a linear-abundant polysaccharide composed mainly of β-(1–4)-linked 2-deoxy-2-amino-d-glucopyranose and partially of β-(1–4) linked 2-deoxy-2-acetamido-d-glucopyranose, is derived from N-deacetylation of chitin. Due to its biocompatibility and nontoxic nature, there is a growing interest on the potential of biologically active chitooligosaccharides (COs) and low-molecular weight, acid-free water-soluble chitosans (LMWC), which are depolymerized products of chitosan. They have received increased attention for their interesting properties, including their inhibitory effects on the growth of fungi/bacteria [1–3], their ability to induce disease resistance–response genes in higher plants and as an elicitor of defense mechanisms in plants [4,5]. COs also affect the mitogenic response and chemotactic activities of animals cells. In clinical situations, with advanced carcinogenesis, accumulation of ascites occurs, while sufficient amount of antitumor drugs cannot be administered due to poor general condition of the patients. Hence, development of new effective therapeutic approaches to treat cancer patients bearing ascites is in a continuous progress [6]. Attempts in this direction have recently been focused on angiogenesis, which leads to growth and metastasis of tumors [7]. N-Acetyl chitooligosaccharides, particularly the hexamer and heptamer, display notable antitumor activity against Sarcoma 180 solid tumors in BALB/C mice as well as in MM-46 solid tumor implanted in C3H/HC mice [8]. It is now well established that both free and conjugated oligosaccharides play a key role both in immunological and biochemical recognition [9]. Biologically active oligosaccharides are usually obtained from natural sources in fairly low yields and it is a major challenge in carbohydrate chemistry to provide sufficient amounts of well-characterized oligomeric products needed for fundamental research and their potential biomedical...
applications. Although several synthetic/chemical methods are available for the preparation of LMWC/COs, these methods are laborious as they still need many protection and deprotection steps and often the yields are low. LMWC/COs are generally prepared by partial hydrolysis of chitosan using hydrochloric acid [10], nitrous acid [11], phosphoric acid [12], hydrogen fluoride [13] or radiation [14] and also by thermal depolymerization [15] methods. Nevertheless, enzymatic methods are becoming more popular because they allow regioselective depolymerization under mild conditions [16–20]. The drawback of using such enzymatically hydrolyzed chitosan preparations for biochemical and food purposes is the undesirable level of pyrogenicity caused by the presence of protein admixtures. Also, use of chitosanase, the specific enzyme, is too expensive to be commercialized for the production of LMWC/COs.

Contrary to these, there have been very few reports on the degradation of chitosan by free radicals. It was demonstrated that viscosity of chitosan solution decreased rapidly in the presence of hydrogen peroxide [21] or potassium persulfate [22]. Although the kinetics of persulfate-induced degradation of chitosan have been worked out to some extent [23], no data is available on the nature and fate-induced degradation of chitosan have been worked out under mild conditions [16–20]. The drawback of using such enzymatically hydrolyzed chitosan preparations for biochemical and food purposes is the undesirable level of pyrogenicity caused by the presence of protein admixtures. Also, use of chitosanase, the specific enzyme, is too expensive to be commercialized for the production of LMWC/COs.

2. Materials and methods

2.1. Materials

Shrimp chitin was procured from CFTRI Regional Center at Mangalore, India. It was deacetylated and purified to chitosan (MW 96000 Da, 16\% N-acetylated) [24]. Dextran (T-10 to T-2000) were from Pharmacia Fine Chemicals (Uppsala, Sweden). N-acetyl chitooligosaccharides (DP 2 to 7) were the product of selective enzymatic hydrolysis of chitosan with *Aspergillus niger* pectinase isozyme. Female Wistar mice weighing 22–24 g were obtained from Animal House Facility of CFTRI, Mysore. Fertilized eggs were purchased from local poultry farm. All chemicals used were of the highest grade commercially available.

2.2. Preparation of LMWC and COs

Briefly, chitosan solution (1\%, in 0.5\% acetic acid solution), taken in a three-necked flat-bottomed flask, was purged with nitrogen at 60 °C under stirring (200 rpm/4 g). Subsequently, potassium persulfate (KPS, 0.8 mM) was added to the solution and the reaction was completed in 2 h. The reaction mixture was precipitated with alcohol (3 volumes) to get LMWC [25], redissolved in deionized water, dialyzed (using 12-kDa cutoff dialysis membrane, Sigma Chemical Co., MO, USA) overnight and lyophilized (VirTis, Gardiner, NY, USA). The alcoholic supernatant was concentrated by rotary flash evaporation (Buchi, Flawil, Switzerland), extensively dialyzed and lyophilized to get COs. The chitooligomeric mixture (10 mg) was N-acetylated [26] by dissolving in water (2 ml), and adding slowly with constant stirring at room temperature distilled acetic anhydride (0.1 ml) in methanol (2 ml). After 30 min, the product was concentrated by flash evaporation, dialyzed (~2 kDa cutoff benzoylated dialysis membrane, Sigma) and lyophilized.

2.3. Characterization

(i) Infrared spectra were recorded in KBr discs on a Perkin Elmer 2000 FTIR spectrometer (Norwalk, USA) under dry air at room temperature. The native chitosan and lyophilized LMWC and COs samples (6 mg) were blended with 200 mg of KBr (IR grade) and about 40 mg of the mixture was used to prepare a pellet for IR spectral measurement.

(ii) Solid state NMR measurements were carried out with Bruker DSX 300 spectrometer (Munic, Germany). Spectra were acquired at 75 MHz with cross-polarization magic-angle spinning (CPMAS) technique, which were spun at the magic angle at 5 kHz. A contact time of 1 ms and a pulse repetition time of 5 s were used and more than 3000 scans were accumulated for each sample. LMWC (readily water soluble unlike native chitosan) was dissolved in D$_2$O in 5-mm tube (100 mg/ml) and its NMR spectrum was recorded with Bruker AMX 400 spectrometer using 32k points, spectral width 30 000 Hz and pulse angle of 52° with a recycling time of 2 s.

(iii) X-ray analysis was done using Sintag XDS-2000 instrument fitted with a 0–0 goniometer and EG-7G solid state germanium liquid nitrogen cooled detector, under following conditions; 30 kV and 25 mA with CuK$_\alpha$ radiation at $\lambda$1.54184 Å. The relative intensity was recorded in the scattering range (20) of 4–40°.

(iv) Molecular weight of LMWC by high-performance size exclusion chromatography (HPSEC) was performed on Shimadzu HIC 6A system controller and CR-4A Chromatopack integrator (Shimadzu Corp., Kyoto, Japan) unit fitted with columns E-linear and E-1000 μ-Bondapak (30 cm×3.9 mm, Waters Associates, Milford, USA) and a RI detector connected in series with a guard column. The columns were eluted with distilled water at a flow rate of 1 ml/min. The operating temperature was 27 °C and the injection volume was 10 μl. A calibration curve was made by using standard dextrans (T-10 to T-2000) of known MW.
2.4. Growth of EAT cells in vivo

EAT cells (5 x 10⁶/mouse) were injected intraperitoneally (i.p.) into 6–8-week-old Swiss Albino mice, kept under normal diet. The tumor growth was followed by recording the animal weights until they succumb to the ascites tumor burden on 12th or 14th day after injection.

2.5. In vivo treatment and peritoneal angiogenesis

For in vivo treatment, LMWC and COs (50–100 μg/mouse) were injected into the EAT bearing mice every alternate day for 6 days, and after 4 days of cell growth in vivo the mice were sacrificed on the 11th day. An equal volume of saline-treated animals served as the control. Native chitosan could not be used as control as its solution in 0.5% acetic acid was very viscous, unlike LMWC and COs that were readily water soluble. An incision was made in the abdominal region and EAT cells along with ascites fluid were harvested into a beaker containing 2 ml saline and centrifuged at 3000 rpm (805 x g) for 10 min at 4 °C. Contaminating RBCs (if any) in EAT cells were lysed with 0.8% (w/v) ammonium chloride. The cells were resuspended in saline or HBSS (Hanks balanced salt solution, pH 7.4) and placed on ice, and used for further experiments. After harvesting EAT cells, the peritoneum was cut open and the inner lining of the peritoneal cavity was examined for angiogenesis in both control and treated tumor-bearing mice and photographed.

2.6. Morphological analysis

The EAT cells taken from untreated tumor-bearing mouse were treated with LMWC/COs for 1 h and were fixed in a solution of methyl alcohol/acetic acid (3:1). The fixed cells were mounted over the glass slides, stained with 1% Wright’s Giemsa stain and identified by their morphological characteristics of apoptosis.

2.7. DNA isolation

The EAT cells (1 ml) were incubated with LMWC/COs (50 μg) for 1 h at 37 °C, terminated with ice-cold HBSS (2 volumes) and the supernatant was removed by centrifugation. The cells were lysed by treatment with buffer containing 50 mM Tris, pH 8.0 and 0.5% SDS, and incubated for 30 min at 37 °C. The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 h at 4 °C and later centrifuged (7000 rpm/4383 x g). The supernatant was subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction and the DNA was precipitated by adding 2 volumes of ice-cold ethanol and centrifuged at 10,000 rpm (8944 x g) at 4 °C for 10 min and the sediment was dissolved in Tris–EDTA buffer (pH 8.0). The content of DNA was deduced by reading the absorbance at 260 nm, and later it was resolved by electrophoresis on 1.5% agarose gel (50 V, 2 h) for visualization by ultraviolet illumination and subjected to gel documentation after staining with ethidium bromide (0.05 μg/ml), destained in water.

2.8. Chorioallantoic membrane (CAM) assay

Fertilized chick eggs were incubated at 37 °C in a humidified and sterile atmosphere for 5 days, after which, a window (0.5 cm) was cut opened (in the egg shell) to check for the normal development of embryo. The window (beneath which is situated CAM) was resealed and incubation was continued until the 9th day for the embryo to develop further. When the window was reopened, either LMWC, COs (50 μg) (along with cell-free EAT ascites fluid taken out in saline, 2 μl) or saline (control) was applied on a cover slip, air dried and inverted over the CAM and the window was closed. The CAM was visualized after 2 days, i.e., on the 11th day, for changes in the microvessel density in the area under cover slip and photographed [27].

3. Results and discussion

The functional properties of chitosan and its derivatives are mainly dependent upon their MW and the consequent ease of solubility in aqueous media. To overcome these problems, depolymerized chitosan products (LMWC/COs) are increasingly gaining importance in many of the biomedical applications. LMWC will allow the successful application of the most known regioselective reactions whereas the use of high-MW water-insoluble chitosan results in very few modifications [28]. Nevertheless, LMWC having complete solubility in neutral aqueous medium is preferred for better physiological, therapeutic applications. LMWC and COs are prepared by depolymerization of native chitosan by various methods. Use of KPS was shown to be very effective in depolymerizing chitosan [23,25]. The depolymerization products were evaluated by various spectroscopy data. FTIR spectra (Fig. 1a) of LMWC showed broad absorption above 3500 cm⁻¹, centered around 3401 cm⁻¹ indicating retainment of intra- and intermolecular –OH hydrogen bonding. The spectrum of LMWC was different from that of native chitosan (inset in Fig. 1a), the amide I and amide II bond...
absorptions were observed at 1629 and 1525 cm\(^{-1}\) rather than at 1654 and 1596 cm\(^{-1}\), respectively, for native chitosan.

Solid-state \(^{13}\)C-NMR spectrum of LMWC (Fig. 2) revealed some structural changes of chitosan after depolymerization. There was a major upfield shift of C1 signal from 109 to 100 ppm, strongly suggesting that during degradation the electron density around C1 is changed, and therefore it is reasonable to believe that the persulfate free radical would attack sequentially C4 and C1 and break away the adjacent C–O–C glycosidic bond in the linear polymeric chain. Though the electron density around C4 should also have been changed, it was not clearly observed because the peaks (C3/C5) were overlapping. Nevertheless, the liquid state \(^{13}\)C-NMR of LMWC (Fig. 1c) showed all the ring carbons including C4 and those due to acetamido groups. The upfield shift from 86 to 76 ppm of C4 was attributed to depolymerization effect. The increased intensities of signals at 23 and 176 ppm indicated LMWC to comprise more of GlcNAc residues, and that the GlcNH\(_2\) residues are much more susceptible to free radical induced degradation than GlcNAc moieties in the chain [29].

Another major observation (Fig. 2) noticed was the splitting of C5 and C3 signals, which indicated the probable conversion of chain orientation from parallel (beta) to antiparallel (alpha) pattern, as seen in chitin [30,31]. Polymorphism is a common phenomenon observed in crystalline polymers because there is relatively only a little difference in the potential energy between several modes of packing of the individual chains. The \(\alpha\)-form is unable to form a gel before it is completely soluble in water, in agreement with our observation wherein LMWC was found readily soluble in water without any visible swelling and gel formation, unlike the enzymatic depolymerized LMWC [32], which retains a \(\beta\)-form and swells in water with gradual solubilization. The latter shows crystalline hydrated structure. \(\beta\)-form can be converted to \(\alpha\)-form but there is no means by which this transformation can be reversed. The \(\alpha\) and \(\beta\) forms could also be distinguished by IR, and by their differences in the hydrogen bonding networks, especially in the frequencies of amide I, as discussed earlier.

X-ray diffraction pattern (Fig. 1b) indicated LMWC to be nearly completely amorphous, exhibiting absence of peak in the region 10–11° 2θ, which is a characteristic of hydrated “tendon form” of native chitosan [33]. The appearance of diffraction peaks at 9.46°, 11.9°, 16.7°, 20.76° and 21.86° indicated subtle structural modifications, presumably due to loss of ordered structure, in support of \(^{13}\)C-NMR data.
The SEC measures molecular size rather than molecular weight, and the factors such as shape of the molecule and degree of solvation are known to affect the apparent MW. This is true of LMWC, which is a linear molecule with overall net positive charge density, and was characterized by HPSEC (Fig. 1d) using T-series dextran standards, which gave a single peak indicating homogeneity and having a MW in the range 37–40,000 Da.

The alcoholic supernatant, after the precipitation of LMWC, contained a mixture of undissociated K$_2$S$_2$O$_8$, glucosamine and chitooligosaccharides (COs). However, its dialysis with a low-cutoff membrane (2 kDa) to remove salts and glucosamine [since it was found that compounds above 1 DP (degree of polymerization), MW ~200 Da, did not escape from this membrane] gave a product, which upon re-N-acetylation followed by HPLC revealed (Fig. 3) several N-acetylchitooligosaccharides, ranging from DP 5, 6 and higher oligomers. The latter, in comparison with the standard chitoigomeric mixture (Fig. 3a), appears to be mostly oligomers of DP 8 or 9. It is known that such oligomeric mixtures, especially N-acetylchitopentaose, N-acetylchitohexaose and higher oligomers, are of considerable biological value [34] and of therapeutic biomedical utility.

EAT cells, when injected intraperitoneally to mice, grow as tumor with accumulation of a large volume of ascites fluid in the peritoneal cavity [27]. The in vivo effect of LMWC and COs on the formation of ascites in EAT-bearing mice showed a significant decrease in the volume of ascites, in treated mice as compared to control mice. The ascites volume decreased to an extent of over 90%, with 7.5±0.5 ml ascites in control animals to 0.7±0.2 ml in treated animals. Prior to sacrificing the animals, their body weights were taken and monitored, which showed that during 11-day tumor growth period there was considerable decrease in the body weight of the treated mice. This effect was also evident when the EAT cell number of the control and LMWC/COs treated mice was considered, 56±5×10$^7$ cells/animal obtained in the control mice were reduced to 18±7×10$^7$ and 90±10×10$^6$ cells per animal for LMWC and COs treated mice, respectively (Fig. 4).

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LMWC (100 μg). Hence, an attempt was made to find out whether COs really induce apoptosis (morphological changes) in these EAT cells (which are nonpolar) [35] through nucleosomal DNA fragmentation. Apoptosis is gene-directed cell death with distinct morphological and biochemical features [36]. The EAT cells (Fig. 5a) treated with LMWC and COs for 1 h and stained with Wrights Giemsa stain showed distinct morphological changes, as evident from oozing out of cytosolic contents (Fig. 5b), characteristic nuclear condensation and membrane disruption (Fig. 5c) and membrane blebbing (Fig. 5d) [36]. Further, the DNA fragmentation data as seen by agarose gel electrophoresis (Fig. 6) revealed clear indication of apoptosis.

It was observed that the peritoneum of mice bearing EAT, when compared to that of normal mice (Fig. 7a), was rich in blood vessels (Fig. 7b) due to angiogenesis (formation new capillary blood vessels), which enables delivery of oxygen and nutrients, essential for growth and metastasis of tumor [37]. Angiogenesis, evident in the inner peritoneal lining of EAT bearing mice, has been shown to be a reliable model for in vivo angiogenesis assay. Hence, the peritoneal lining of LMWC- and COs-treated mice was checked for such an effect (Fig. 7c,d). A reduction due to inhibition of the neovasculature in peritoneal angiogenesis was observed in EAT-bearing mice that were treated with LMWC and COs. To verify that LMWC and COs are antiangiogenic and that the effect observed was not only due to decreased number of EAT cells or ascites fluid in the peritoneal cavity of treated mice, a CAM assay was carried out, which showed inhibition especially towards sprouted small capillaries (Fig. 8). The involvement of vascular endothelial growth factor (VEGF) (most potent inducer of angiogenesis) in the formation of malignant ascites has been well documented [27]. Since there is inhibition of angiogenesis by LMWC and COs, it supports that these chitosan-derived products may repress the expression of VEGF or inhibit the secretion of such factors, thereby
inhibiting the formation of new blood vessels. It is well documented [38] that chitosan and its depolymerized products diminish glycolysis in EAT cells, by decreasing glucose uptake and ATP level, probably due to inhibition of tumor-specific variant of pyruvate kinase. On the other hand, they do not exert a similar inhibitory effect on glycolytic activity of mouse normal liver and muscle supernatants. In another study, mice fed diet containing LMWC showed significant reduction in the number of aberrant crypt foci and cell proliferation in the colonic epithelium [39].

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Fig. 7. Suppression of in vivo angiogenesis by LMWC and COs (a) normal mouse peritoneum, (b) peritoneal lining of mouse that bear tumor cells, showing extensive vasculature, (c,d) inhibition of angiogenesis in LMWC- and COs-treated mice, respectively.

Fig. 8. Chorioallantoic membrane (CAM) assay showing inhibition by LMWC and COs towards sprouted small capillaries.
References


