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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.

Keywords: Low molecular weight chitosan; Chitooligosaccharide; Anti-angiogenesis; Apoptosis; DNA

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 their 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

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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 characterization of the products, especially with respect to their biological (antitumour) activities. The main objective of the present study was to elucidate the structural features of LMWC/COs and their effect in inhibiting angiogenesis and inducing apoptosis in Ehrlich ascites tumor (EAT) bearing mice.

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]. Dextrans (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_2O 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 θ–θ goniometer and EG-7G solid state germanium liquid nitrogen cooled detector, under following conditions; 30 kV and 25 mA with $CuK\alpha_1$ radiation at λ1.54184 Å. The relative intensity was recorded in the scattering range (2θ) 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 integrater (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.