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Free-radical depolymerization of glycosaminoglycan from sea cucumber *Thelenata ananas* by hydrogen peroxide and copper ions

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ABSTRACT

A process of depolymerization of a new fucosylated chondroitin sulfate from *Thelenata ananas* by free radicals was developed. The fractions with different molecular weights and narrow molecular weight distribution were obtained. The parameters of the process were investigated by high-performance gel permeation chromatography. The kinetics of the depolymerization of THG by H_2O_2 was studied, and a possible mechanism was proposed. The results indicated that the levels of final products fragmentation and reproducibility were different depending on the conditions of depolymerization used. The fragmentation of the main chain of THG occurred randomly and obeyed pseudo-first-order kinetics, and produced species with rather narrow and unimodal distribution of molar mass. Chemical compositions of partially depolymerized samples by $^{1}H/^{13}C$ nuclear magnetic resonance spectroscopy and by elementary analyzer suggested that there was no preferential cleavage of sulfated α-L-fucopyranose side-chain, and chemical composition of products was kept almost unchanged from that of native THG.

1. Introduction

In the past decades, a kind of novel glycosaminoglycan isolated from the body wall of sea cucumber have appeared as a potentially useful therapeutic for antithrombotic applications. They have a chondroitin sulfate-like structure, containing large numbers of sulfated α-L-fucopyranose branch linked to position 3 of the β-D-glucuronic acid residues (Mourão et al., 1996; Vieira, Mulloy, & Mourão, 1991; Yoshida et al., 1992; Zhao et al., 2009). The complex biosynthesis of glycosaminoglycan from the body wall sea cucumber leads to different chemical composition of sequences containing different ratio of *N*-acetylgalactosamine (GalNAc), glucuronic acid (GlcUA), fucose (Fuc) and ester sulfate. Recently, we isolated a new glycosaminoglycan with molecular weight of around 70 kDa from the body wall of sea cucumber *Thelenata ananas* (holothurian glycosaminoglycan from *T. ananas*, THG), which consisted of GalNAc, GlcUA, fucose and ester sulfate with about 1:1:1:3.7, respectively (Zhao et al., 2009). The chemical composition and molecular weight of THG are different from those of glycosaminoglycan from *Stichopus japonicas* (Yoshida et al., 1992) and from *Ludwigothurea grisea* (Mourão et al., 1996).

The glycosaminoglycan containing the branch of sulfated α-L-fucopyranose from the body wall of sea cucumber has been known to show a heparin-like anticoagulant activity (Mourão et al., 1996), together with an undesirable effect of platelet aggregation (Li & Lian, 1988). In order to minimize the side effect, its low molecular weight derivative, depolymerized holothurian glycosaminoglycan (DHG) had been prepared (Suzuki, Kitazato, Takamatsu, & Saito, 1991). Whereas the pharmacological properties of glycosaminoglycan from the body wall of sea cucumber have been widely studied, it is surprising that the methods of preparation of its low molecular weight derivative have showed comparatively little interest in the literature. In order to extend its area of interest, we have carried out investigations to obtain low molecular weight fractions from this high molecular weight THG (60–70 kDa).

In the literature, several methods are described for depolymerizing polysaccharides, such as irradiation (Bertolini, Mestres, Colonna, & Raffi, 2001), ultrasonic degradation (Portenlanger & Heusinger, 1997), free-radical depolymerization with or without metallic catalysts (Nardella et al., 1996; Petit et al., 2006; Volpi, Mascellani, & Bianchini, 1992; Yang, Li, & Guan, 2004), acid hydrolysis (Hjerde, Smidsrød, Stokke, & Christensen, 1998; Karlsson & Singh, 1999) and enzymatic depolymerization (Cheng & Prud'homme, 2000). These preparation processes produce different products with different structures and distributions of molecular weight, quite different anticoagulant activities, and different pharmacological properties (Linhardt et al., 1990).

For the degradation of glycosaminoglycan, free-radical depolymerization is an interesting route because it enables, with

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reproducibility and constant composition, the extent of the depolymerization to be controlled (Petit et al., 2006). Moreover, compared with acid hydrolysis, there is no preferential cleavage of side-chains (Hjerde, Stokke, Smidsrød, & Christensen, 1998) and the primary structure after chemical depolymerization is retained (Zhang et al., 2008). The reaction also has potential to achieve some selectivity of cleavage by the appropriate choice of metal ion catalyst (Liu & Perlin, 1994). The conditions of the reaction are mild and it can be quenched at any point by removal of the metal ions by a chelating agent. The reagents are inexpensive and suitable for use on a large scale (Bianchini & Mascellani, 1988). Thus, free-radical depolymerization has considerable promise for the industrial production of low molecular weight THG.

A controlled free-radical depolymerization process of THG from sea cucumber has been developed. Here, we present new results on free-radical depolymerization of glycosaminoglycan from sea cucumber *T. ananas* by hydrogen peroxide in the presence of cupric ion, report a kinetic behavior during the free radical reaction, and discuss a possible mechanism of depolymerization of the novel glycosaminoglycan. Furthermore, the effect of variables such as pH, temperature and the concentration of hydrogen peroxide were investigated.

2. Experimental

2.1. Materials

The sea cucumber *T. ananas* was collected in Sanya of Hainan province of China. Diastase vera (EC 3.3.21.4) was obtained from Aolipharm, Inc. (Chongqing, China). Hydrogen peroxide (30% wt. solution in water), copper (II) acetate monohydrate, sodium acetate and disodium ethylenediamine tetra-acetate dihydrate (EDTA) were purchased from DamaoChem., Ltd. (Tianjin, China). All other chemicals were of reagent grade and were obtained commercially.

2.2. Analysis of molecular weight

The molecular mass, weight average molecular mass (M_w), number-average molecular mass (M_n) and molecular weight distribution (MWD or M_w/M_n) of THG and DTHG were examined by high-performance gel permeation chromatography and low-angle laser light scattering (HPGPC–LALLS) using a Agilent technologies1200 series (Agilent Co., USA) apparatus, equipped with a Shodex OH-pak SB-804 HQ column (8 mm × 300 mm). The elution solvent was 0.1 M NaCl solution. The flow rate was 0.5 mL/min, and the temperature of the column was 35 °C. Wyatt Dawn E and Shodex RI-71 systems were used as a low-angle laser light-scattering detector and a differential refractive index detector, respectively. Data acquisition and molecular weight calculations were performed using the GPC soft, version B01.01 (Agilent Co., USA). The specific refractive index increment (dn/dc) of DTHG was measured in 0.1 M NaCl solution, by a Wyatt OPTILAB DSP interferometric refract meter at 633 nm, and $(dn/dc)_{NaCl-H_2O} = 0.1190$ mL/g was obtained. Detailed sample preparation and recording conditions were performed according to Tsukamoto, Hattori, Sakabe and Haginaka (2001) previously.

2.3. Extraction and purification of THG

The origin and extraction of the THG from the body wall of the sea cucumber *T. ananas* were performed by modification of the method described previously (Vieira et al., 1991). The preparation was purified by gel filtration with a Sephadex G-100 and ion-exchange chromatography with a DEAE-cellulose column. This preparation was purified by gel filtration with a Sephadex G-100 and ion-exchange chromatography with a DEAE-cellulose column. The separated fractions were assayed by the Dubois (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and carbazole (Kosakai & Yosizawa, 1979) reactions. Finally, THG fractions were pooled, dialyzed (cutoff 3000 Da) against distilled water, concentrated and lyophilized. The yield of THG from the dried body wall of sea cucumber was about 0.7%.

2.4. Conditions of copper (II)-catalyzed depolymerization of THG

The well-established method of dermatan sulfate depolymerization, obtained in the presence of hydrogen peroxide and cupric acetate (Volpi, 1994), was analyzed. Scheme 1 gives a reaction equation of free-radical depolymerization of the glycosaminoglycan from *T. ananas*. Here, free-radical depolymerization copper (II)-catalyzed of THG was investigated while varying different parameters (pH, concentration of H_2O_2 and temperature). The following protocol is representative of the different experiments.

Native THG (5.00 g) and 160 mg of copper (II) acetate monohydrate were dissolved in 180 mL of 6% sodium acetate and sodium chloride solution at 35 °C. A 10% H_2O_2 solution was added by using a peristaltic pump at the rate of 0.20 mL/min. The pH of the solution was maintained at 7.0–7.5 by addition of 1 M NaOH solution. After 100 min, the pump was stopped, but the reaction was continued for 80 min. The reaction mass was then cooled down to room temperature, and added 0.5 g of disodium ethylenediamine tetra-acetate dihydrate (EDTA); the pH was adjusted at a 6.0 value with acetic acid. During the course of the reaction, about 100 μL samples were taken, quenched with an equivalent volume of EDTA and analyzed by HPGPC-LALLS.

Depolymerized THG (DTHG) was precipitated with ethanol (1:2.5 [v/v] reaction mixture/ethanol). The crude product was collected by centrifugation (4000g for 20 min at 20 °C), washed with ethanol. Then the precipitate was dissolved in water desalinated by ultra filtration with a 3000 Da cutoff membrane (Millipore), concentrated and subsequently lyophilized.

The same experiment was carried out four times with the same batch of production of the THG to check the reproducibility of the manipulation, and R statistical software (version 2.7.2, University of Auckland, USA) was used for analysis of variance (ANOVA).

2.5. Viscometry measurement

Viscosity measurements were made on dilute solutions of each sample in NaCl (0.1 M) at 25 ± 0.1 °C using an Ubbelohde viscometer (capillary length 130 mm, capillary internal diameter 0.5 mm, bulb volume 1.0 mL). The viscometer gave an average efflux time of 164.6 s when calibrated with NaCl (0.1 M) solution.

2.6. Composition of THG and DTHG samples

The aldohexuronic and acetamidoxyhexose contents of native THG and DTHG were estimated by IR spectrophotometry (DCl/D_2O) (Casu, Scovenna, Cifonelli, & Perlin, 1978). The contents of sulfate and nitrogen (sulfur/nitrogen ratio) of native THG and DTHG were determined by Elementar Vario EL. The sulfate/carboxyl groups in the native THG and DTHG were determined by a conductimetric method (Casu & Gennaro, 1975).

2.7. NMR spectrometry methods

NMR analyses were performed at 35 °C with a Bruker Avance 400 spectrometer of 400 MHz equipped with $^{13}C/^{1}H$ dual probe in the FT mode. The NMR experiments were recorded with a spectral width of 3000 Hz, an acquisition time of 1.36 s, a pulse width of 7 s, a relaxation time of 1 s and a number of 256 scans. The