



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 H₂O₂ 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 ¹H/¹³C 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.

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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 japonicus* (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 μ L 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

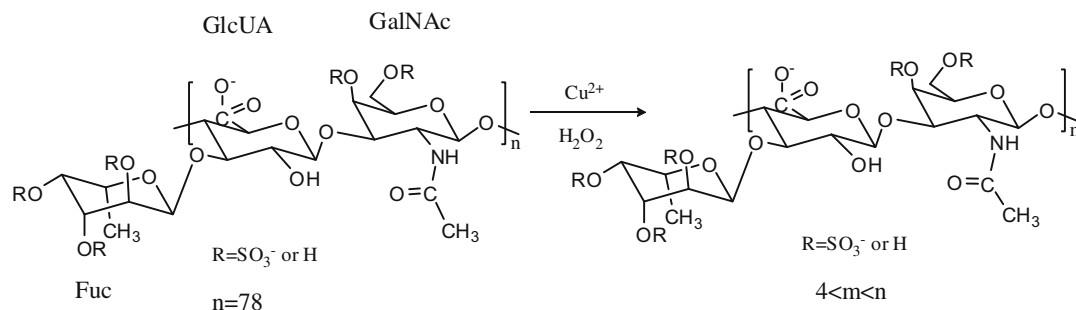
Viscosity measurements were made on dilute solutions of each sample in NaCl (0.1 M) at 25 ± 0.1 °C using an Ubbelohde viscometer (capillary length 130 mm, capillary internal diameter 0.5 mm, bulb volume 1.0 mL). The viscometer gave an average efflux time of 164.6 s when calibrated with NaCl (0.1 M) solution.

2.6. Composition of THG and DTHG samples

The aldohexuronic and acetamidohexose contents of native THG and DTHG were estimated by IR spectrophotometry (DCI/ 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 $^{13}C/^1H$ 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



Scheme 1. Free radical depolymerization of the glycosaminoglycan from the sea cucumber *T. ananas* with hydrogen peroxide in the presence of copper (II) ion.

HOD signal was presaturated by a presaturation sequence. All samples were previously dissolved in deuterium (D_2O , 99.9% D) and lyophilized thrice to replace exchangeable protons with deuterium. The lyophilized samples were then dissolved in D_2O at a 10–20 g/L concentration. All chemical shifts are relative to internal trimethylsilyl-propionic acid (TSP).

3. Results and discussion

3.1. Effect of copper ions

Iron and copper are the two metals most used to degrade polysaccharides by free-radical depolymerization. In this work, copper was preferred to catalytic, because copper has potential to achieve some selectivity of cleavage (Liu & Perlin, 1994), and copper is of interest for its biological activities in view of further applications in cosmetics (Petit et al., 2006). The influence of the amount of the copper cation and the experimental procedure on the depolymerization process was observed (Fig. 1). Carboxylic moieties from acids and hydroxyl groups can be involved as electron donors in the chelation of cations, as has been well described in the literature (Angyal, 1989; Petit et al., 2006). For instance, a recent study concerning exopolysaccharide (EPS) produced by a bacterium isolated from a deep-sea hydrothermal vent polychaete annelid (Petit et al., 2006) showed that metal capture is a chemical, equilibrated and saturable mechanism. For an $\text{H}_2\text{O}_2/\text{EPS}$ (w/w) ratio of 0.2, the system appeared saturated with metal for a concentration of about 2 g/L. The same experiments were performed with copper sulfate,

copper chloride and copper acetate, using the same molar amount of metal. For these three counter-ions, the same results were obtained. Here, for the depolymerized system of THG, increasing amounts of copper (II) acetate were added to a solution of THG, and high amounts of Cu^{2+} did not significantly improve depolymerization. When the depolymerization was performed using copper (II) as catalyst under milder chemical condition in a 5 h reaction, the reaction was highly controllable and reproducible (Table 1).

3.2. Effect of pH

Another parameter that could influence the reaction is pH. Therefore, the influence of pH and the experimental procedure on the depolymerization process was observed. During the addition of metallic cation to polysaccharide solutions, a sudden decrease in pH immediately occurred due to the chelation process (Petit et al., 2006). The pH of the solution then remained stable with values ranging from 6.3 to 6.8 at 35 °C depending on the initial concentration of the added cation. Without pH control, the reproducibility was not very good at $\text{RSD} > 10\%$ (Table 1) and the polydispersity was about 1.87. For pH 6.5 or 8.0, the reproducibility was good, but the final molecular weight was not different, and when the reaction took place at acidic pH, the depolymerization was much drastic. The molecular weight of the final products was approximately 12,000 Da when the reaction was run at pH 6.5 for 5 h. The observation is consistent with that of Rota, Liverani, and Spelta (2005) according to the hydroxyl radical attack of heparin by the electron spin resonance (ESR) spin trapping technique. Every reaction mixture included a considerable amount of hydroxyl radical species at the earliest time points. Carbon-centered free radical, arising from hydrogen abstraction on fragments, appeared at later time points when the reaction was performed at neutral pH. So, a neutral pH is required for an optimized depolymerization of the polysaccharide backbone by hydroxyl to avoid acidic or basic hydrolysis of the polysaccharide. This was also observed by Petit et al. (2006) in the depolymerization of an exopolysaccharide produced by a bacterium isolated from a deep-sea hydrothermal vent polychaete annelid.

3.3. Kinetics during molecular weight reduction

3.3.1. Effects of reaction temperature

Temperature is critical to the reaction rate, the product yield, and distribution. Without the addition of H_2O_2 , the sample viscosity decreased gradually. Raising the temperature from 35 to 65 °C increased slightly the rate of viscosity reduction. The decrease in the molecular weight of THG was much faster when temperature was increased (Fig. 2A) to 65 °C.

The degree of chain scission (α) is defined as $n_{\text{red}}/n_{\text{tot}}$, where n_{red} is the number of reducing ends and n_{tot} is the total number of glucose residues in the main chain. At the beginning of reaction, the

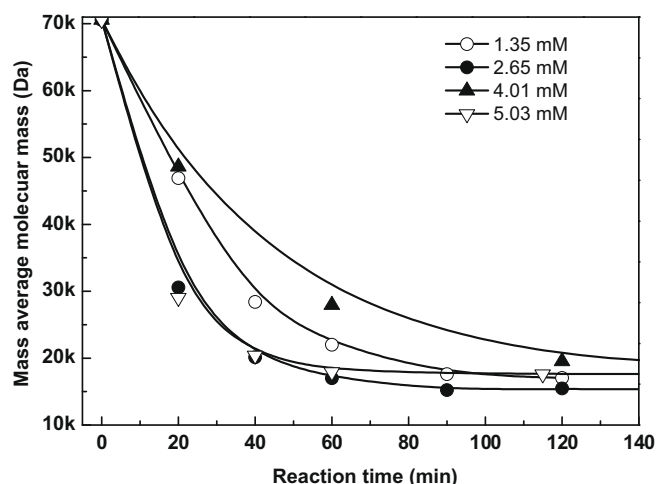


Fig. 1. Effect of the concentration of Cu^{2+} on the depolymerization reaction. In the process, 25 g/L of THG was degraded by 0.41 M H_2O_2 in the presence of different concentration of $\text{Cu}(\text{CH}_3\text{COO})_2$ at 35 °C.

Table 1Molecular weight, polydispersity and reproducibility according to different pH and reaction time during the depolymerization reaction.^a

pH ^b	Reaction time (min)	Average of molecular weight ($n = 4$)		Polydispersity (M_w/M_n)	Reproducibility ^c ($n = 4$; RSD, %)
		Weight average (M_w)	Number-average (M_n)		
6.5	60	19,729 ± 515	12,492 ± 414	1.58 ± 0.01	2.6
	180	12,510 ± 391	7007 ± 279	1.79 ± 0.12	3.1
	300	12,230 ± 372	6830 ± 270	1.79 ± 0.19	3.0
7.3	60	19,545 ± 619	12,344 ± 212	1.58 ± 0.02	3.2
	180	16,993 ± 140	9569 ± 105	1.77 ± 0.08	0.8
	300	16,348 ± 114	9840 ± 87	1.66 ± 0.11	0.7
8.0	60	18,221 ± 1728	11,311 ± 1402	1.61 ± 0.04	9.5
	180	16,752 ± 484	10,151 ± 372	1.65 ± 0.11	2.9
	300	16,520 ± 477	9972 ± 365	1.66 ± 0.12	2.9
No control	60	21,406 ± 3782	13,929 ± 3104	1.54 ± 0.26	17.7
	180	11,308 ± 1445	6164 ± 1307	1.83 ± 0.15	12.8
	300	10,630 ± 1233	5697 ± 1138	1.87 ± 0.11	11.6

^a In the process of depolymerization, 25 g/L of THG was degraded by 0.41 M H₂O₂ in the presence of 4 mM Cu(CH₃COO)₂ at 35 °C.^b The pH value is the value during the depolymerization reaction.^c n is the number of experiments; RSD is the relative standard deviation of the weight average molecular mass.

plot of $-\ln(1 - \alpha)$ vs. time is linear, indicating that the degradation obeys first-order kinetics for the initial time (Fig. 2A). The degradation rate constant for 0.41 M H₂O₂ on about 2.5% THG solution was

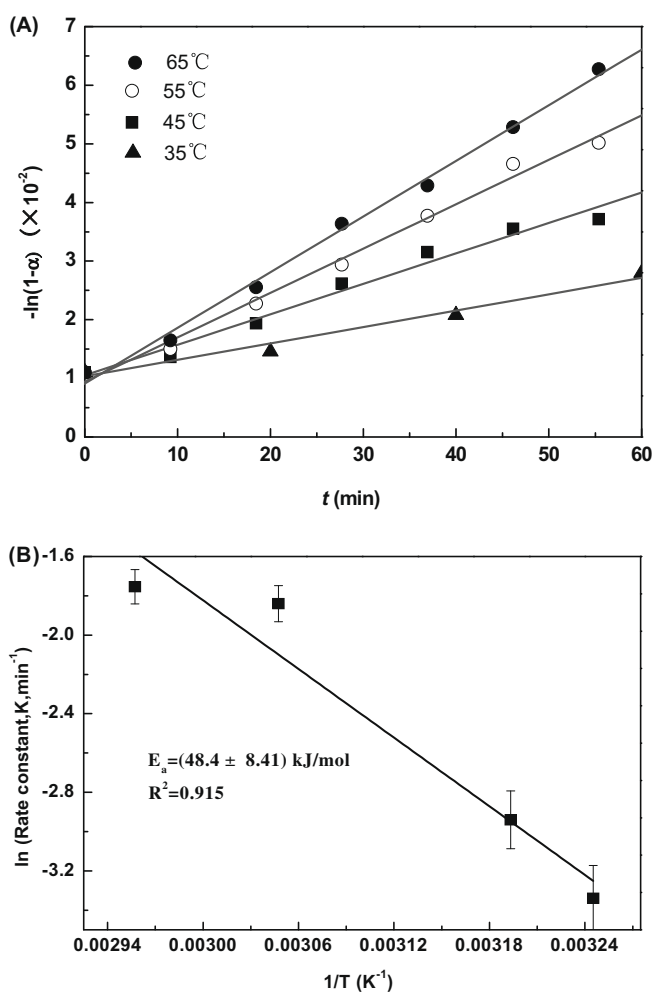


Fig. 2. (A) Changes in the average number of chain scission, α , as a function of reaction time. The degree of chain scission (α) is defined as $n_{\text{red}}/n_{\text{tot}}$, where n_{red} is the number of reducing ends and n_{tot} is the total number of glucose residues in the main chain. (B) Arrhenius plot of the polymerization initial rate constants from the first exponential kinetic model. In the process, 25 g/L of THG was degraded at different temperatures in the presence of Cu(CH₃COO)₂ for different durations.

plotted in the Arrhenius plot (Fig. 2B). The apparent activation energy (E_a) was found to be 48.4 ± 8.4 kJ/mol. This value of E_a is close to that determined for ultrasonic degradation ($E_a = 52.13$ kJ/mol) of polysaccharide from *Porphyra yezoensis* (Ma, Zhou, Zheng, Luo, & Wang, 2007) and arabinan degradation ($E_a = 62.06$ kJ/mol) (Shatalov & Pereira, 2005), but is much lower than the value reported for the acidic hydrolysis of dextran sulfate ($E_a = 103$ kJ/mol) and κ -, ι - or λ -carrageenan ($E_a = 105, 162, 116$ kJ/mol) (Karlsson & Singh, 1999).

3.3.2. Effect of H₂O₂ concentration

Amount of H₂O₂ is also critical to the reaction rate and distribution of the products. Fig. 3 shows the effect of H₂O₂ concentration on average number of chain scission, N . The average number of chain scission (N) is defined as $(M_{w0} - M_{wt})/M_{wt}$, where M_{w0} is weight average molecular weight of native THG at time $t = 0$, and M_{wt} is weight average molecular weight of the fragment at the end of reaction time t . The curve was close to linear ($R^2 = 0.9898$) in the range of 0.22–1.30 M, suggesting that main chain scission was of pseudo-first order. The rate constants were first obtained in the range of $3.5\text{--}6.0 \times 10^{-2} \text{ min}^{-1}$.

The kinetics of the depolymerization of THG by H₂O₂ in the presence of copper (II) was studied. The result indicates that the depolymerization obeys first-order kinetics (Figs. 2 and 3). An important observation is that the rate constant was independent of the degree of chain scission or consequently, independent of M_w . The apparent activation energy (E_a) of the depolymerization by copper (II) catalysis was much lower than that of the degradation without catalyst (E_a is about 105 kJ/mol), indicating that energy barrier of the depolymerization was great reduced so that conditions of the reaction was milder. At just 35 °C, the rate of depolymerization by copper (II) catalysis was much faster than that of the reaction at 55 °C without a catalyst. The molecular weight of the final products was approximately 14,000 Da when the reaction was run at pH 7.5 for just 3 h. The reaction time was sharply shorter than the value reported for the depolymerization of glycosaminoglycan from sea cucumber *S. japonicas* by H₂O₂ without a catalyst (Suzuki et al., 1991).

3.4. Molecular weight distribution during the free-radical depolymerization

The molecular weight distribution of native THG and depolymerized samples during the free-radical depolymerization was determined by HPGPC. The results are given for selected samples

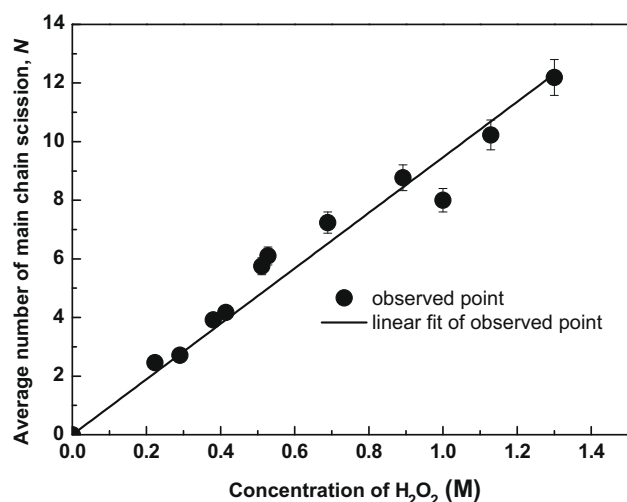


Fig. 3. Average number of main chain scission, N , as a function of concentration of H_2O_2 . The average number of chain scission (N) is defined as $(M_{w0} - M_{wt})/M_{wt}$, where M_{w0} is weight average molecular weight of native THG at time $t = 0$, and M_{wt} is weight average molecular weight of the fragment at the end of reaction time t . In the process, 25 g/L of THG was degraded by different concentration of H_2O_2 in the presence of $Cu(CH_3COO)_2$ at 35 °C.

with different degree of depolymerization in Table 1. The results show that the polydispersity index stays close to two during the depolymerization process, which indicate a random depolymerization (Yoon, Chin, Kim, & Kim, 1996).

Fig. 4 illustrates differential and cumulative molecular weight distributions of DTHG at different depolymerization time using the HPGPC method. The fact that final products fragmentation revealed strictly unimodal and symmetrical MWD implies that character/kinetics of the depolymerization of the THG sample can be classified as a random process. The distribution did not significantly broaden as the molecular weight decreased. This suggests that the larger macromolecules were preferentially fragmented and smaller macromolecules were not, and fragmentation did not occur near the extremities of macromolecules. The kind of process of stochastic chain cleavage has been modeled earlier by Singh, Shen, Chou, and Fan (1994) according to acid hydrolysis of κ -carrageenan in a batch reactor.

3.5. ^{13}C NMR spectroscopy of THG and DTHG samples

The structures of native THG and its low molecular weight fragment DTHG-2 by free-radical depolymerization as a representative of the fragments were further studied by ^{13}C NMR spectroscopy. The comparison of the ^{13}C NMR spectrum of fraction DTHG-2 with that of the native THG shows the coincidence among almost all signals of both polysaccharides. The anomeric region of the ^{13}C spectrum of DTHG-2 (Fig. 5C) shows peaks coincident with carbon 1 of the β -D-glucuronic acid (U-1) and the N -acetyl- β -D-galactosamine (A-1) units of native THG from sea cucumber (Fig. 5A), together with several other signals of α - and β -glycosidically linked carbons (Mourão et al., 1996; Vieira & Mourão, 1988; Vieira et al., 1991). In addition, the ^{13}C spectrum of show several signals in the vicinity of $\delta = 68$ –80 ppm, which have chemical shifts similar to the nuclei of native THG from sea cucumber. The spectra of both compounds also show signals attributable to carbonyl (CO), acetamidomethyl (COCH₃) and ring carbon-2 (A-2) of the N -acetyl-galactosamine moiety, which resonate at approximately $\delta = 177.7$, 25.4 and 55.3 ppm, respectively ($\delta = 177.7$, 25.4 ppm not shown). Due to the fucose residues linked to the chondroitin sulfate core (Vieira & Mourão, 1988; Vieira et al., 1991), the ^{13}C spectrum of both com-

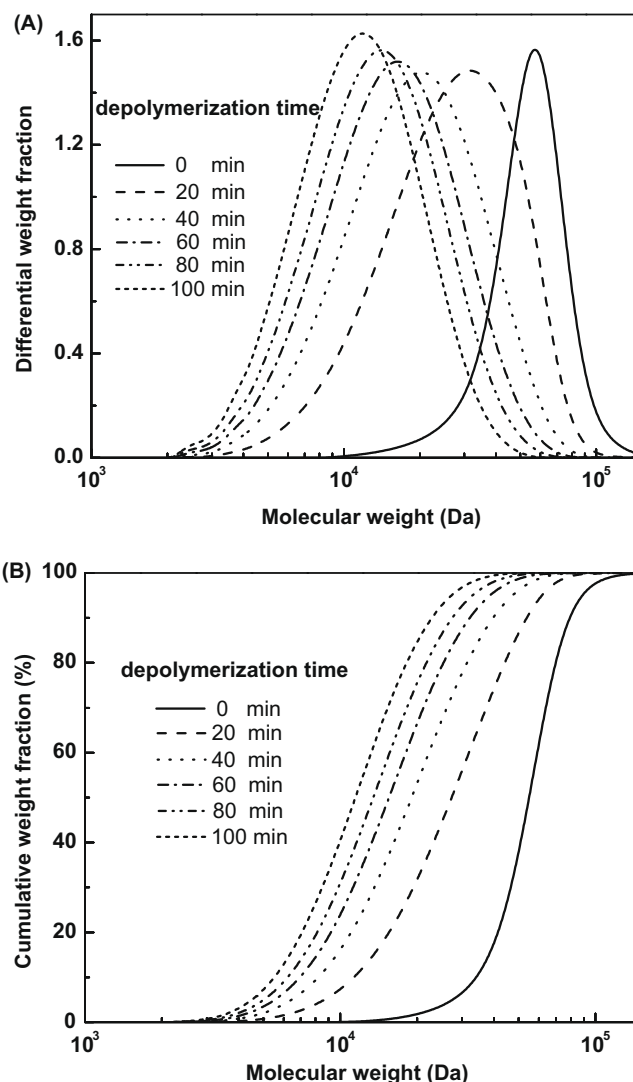


Fig. 4. Molecular weight distribution curves of THG depolymerization during the free-radical depolymerization for different time. (A) Differential molecular weight distribution curves of THG. (B) Cumulative molecular weight distribution. In the process, 25 g/L of THG was degraded at 35 °C with 2.0% H_2O_2 in the presence of $Cu(CH_3COO)_2$ for different durations.

pounds also show additional complexity attributable to anomeric carbons of α -linked fucose, ring carbons of fucose and deoxy CH₃, which resonate in the vicinity of $\delta = 101.3$ –99.2, 83.8–69.0 and 18.6 ppm, respectively. The signal at $\delta = 64.1$ ppm is attributed to non-substituted carbon-6 (A-6) of the N -acetyl- β -D-glactosamine (A-1) units of both compounds. Furthermore, the signal at approximately $\delta = 69.3$ ppm, which is obscured by the overall complexity of the spectrum in the region of fucose signals, is attributed to sulfated carbon-6 of the N -acetylgalactosamine (Fig. 5B). These data indicate that the basic structure of DTHG is similar to native THG from sea cucumber.

3.6. Mechanism of free-radical depolymerization of THG

The mechanisms of free-radical attack leading to depolymerization of polysaccharides are complex and largely unknown, but it is assumed that the number of reducing ends (n_{red}) is at least proportional to the number of chain breaks (Hjerde, Smidsrød et al., 1998; Hjerde, Stokke et al., 1998). Uchiyama, Dobashi, Ohkouchi, and Nagasawa (1990) have shown that reducing sugars correspond to

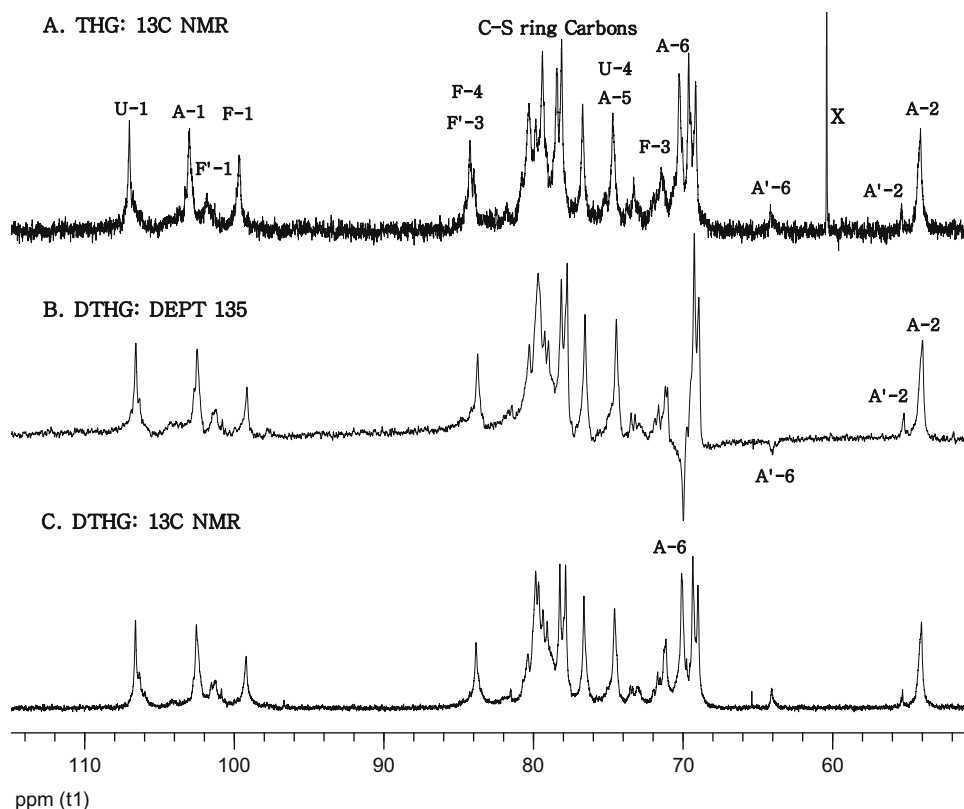


Fig. 5. ^{13}C NMR spectrum (100.62 MHz) in D_2O of (A) native fucosylated chondroitin sulfate (THG), (B) DEPT 135° spectrum of fraction DTHG-2, and (C) ^{13}C NMR spectrum of fraction DTHG-2 obtained by free-radical depolymerization. Signals designate by "A" or "A'" refer to those produced by acetamidodeoxyhexose residues, whereas those of uronosyl residues are labeled "U", and those of fucose residues are labeled "F" or "F'". Signals marked "X" arise from contaminants.

about 75% of the reducing terminal residues in hyaluronic acid after depolymerization with free radical.

The observed linear decrease in $-\ln(1 - \alpha)$ with time (Fig. 3A) and the polydispersity index stayed close to two (Table 1) suggest that the cleavage of the main chain of THG occurred randomly. A corresponding behavior upon depolymerization with Fenton's reagent has previously been observed in several other polysaccharides (Chang, Tai, & Cheng, 2001; Christensen, Myhr, & Smidsrød, 1996; Vismara et al., 2007).

Analysis of the composition of native and partially depolymerized samples may give additional information on the random depolymerization process. The change in the content of branching was evaluated by sulfated α -L-fucopyranose (Fuc)/N-acetylgalactosamine (GalNAc) (molar ratio) according to integration of ^1H NMR signals of their methyl. The ^1H NMR spectra of the native THG and two partially depolymerized THG samples are shown in Fig. 6. The spectra reveal peaks corresponding to proton atoms in the side-chain sulfated α -L-fucopyranose (Fuc; see figure legend).

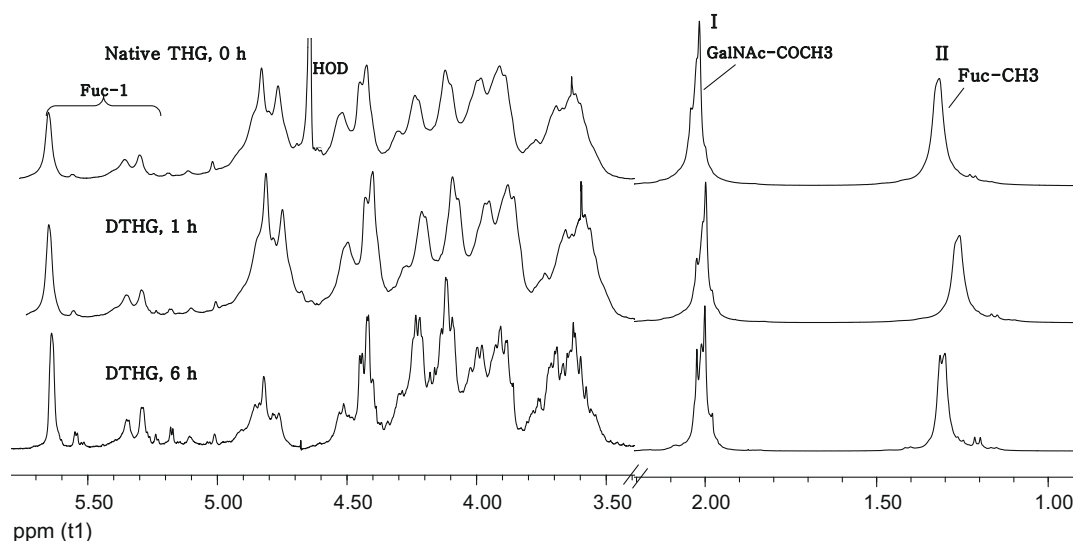


Fig. 6. Section of proton-decoupled ^1H NMR spectra (400 MHz at 35 °C) of the native and three partially depolymerized THG samples. The assignment of the peak is explained in the figure and the references (Mourão et al., 1996; Yoshida et al., 1992).

Table 2

Physico-chemical properties of native THG and DTHG.

Samples	Average molecular weight (Da)	Chemical composition (molar ratios)				–SO ₃ [−] /COO [−] (molar ratio)	[η] ²⁵ °C (mL/g)
		GalNAc	GlcUA	Fuc	Sulfate		
Native THG	69,250	1.00	0.98	0.96	3.71	3.81	48.7
DTHG-1	23,380	1.00	0.99	0.99	3.45	3.53	14.3
DTHG-2	13,950	1.00	0.92	0.97	3.52	3.51	8.0
DTHG-3	8549	1.00	0.97	0.96	3.50	3.32	4.1

The fraction of side-chains per repeating unit can be found by dividing the area of peak II, corresponding to Fuc–CH₃ in the Fuc unit, and the area of peak I, which corresponds to CO–CH₃ of GalNAc in the main chain. As shown in Table 2, the chemical compositions of DTHG, thus obtained, were kept almost unchanged from that of native THG during the depolymerization. These results are consistent with those obtained from depolymerized glycosaminoglycan from sea cucumber *S. japonicus* by Yoshida et al. (1992). The results thus show that there is no preferential cleavage of side-chains, and supports other researchers' observation of a totally random depolymerization mechanism by Fenton reagent (Hjerde, Kristiansen, Stokke, Smidsrød, & Christensen, 1994; Hjerde, Smidsrød et al., 1998; Hjerde, Stokke et al., 1998). The content of sulfate and sulfate/carboxyl group in the native and DTHG before and after depolymerization was very slightly decreased (Table 2). This could be due to the preferential attack of the free radicals on oversulfated residues (sulfated *N*-acetylgalactosamine) leading to fewer oversulfated disaccharides or to simple removal of sulfate groups (Ofman, Slim, Watt, & Yorke, 1997).

Combining results from our data and related literature reports (Chang et al., 2001; Vismara et al., 2007), the following possible

mechanism for the depolymerization of THG by H₂O₂ in the presence of copper (II) was proposed.

The Fenton reaction induces random degradation at the beginning. Due to the high numbers of involved species and intermediate reactions, the process is actually very complicated, thus at present there is a debate about the real mechanism (Vismara et al., 2007). Nevertheless, our discussions are based on the assumption that hydroxyl radicals are generated in some manner as shown in the following equations:



Metal ions are mostly bound to *N*-acetyl group on its C-2 positions in the GalNAc. The Fenton reaction produces hydrogen free radicals that abstracted hydrogen atoms from C-1 of *N*-acetylgalactosamine or uronic residues of THG. The generated primary radical from C-2 and C-3, both on an *N*-acetylgalactosamine or uronic ring,

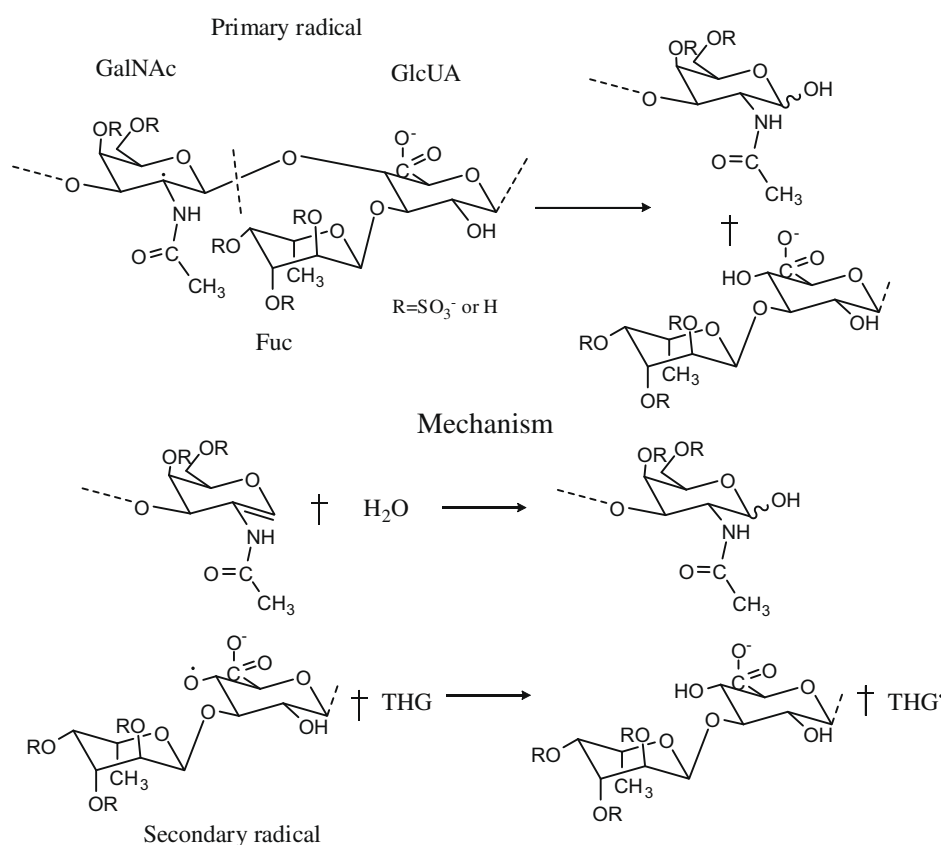


Fig. 7. Cleavage of β-D-GalNAc-(1 → 4)-β-D-GlcUA glycosidic bond of glycosaminoglycan from sea cucumber *Thelassia ananas* by hydrogen peroxide and copper ions (the cleavage of β-D-GlcUA-(1 → 3)-β-D-GalNAc glycosidic bond, not shown).

can undergo a β scission leading to a glycosidic bond breaking accomplished by a double-bond formation (Fig. 7). The secondary oxygen-centered radical abstracts one hydrogen atom, and generates a new THG carbon-centered radical, indicating that the overall depolymerization mechanism can be interpreted as a radical chain. In the presence of the catalytic acidic medium, the double bond can add water, thus regenerating a sugar ring. Vismara et al. (2007) have first proposed the kind of mechanistic hypothesis according to investigating structural modification induced in heparin by a Fenton-type depolymerization process. However, the structure of THG and DTHG is heterogeneous and more complex than heparin; therefore, it may be more difficult to make an elucidation on depolymerization mechanism of THG by H_2O_2 in the presence of copper (II).

4. Conclusion

We isolated a new glycosaminoglycan from the body wall of the sea cucumber *T. ananas*, and prepared with high reproducibility, low molecular weight and size-defined DTHG derivatives in the molecular weight range from 8500 to 30,000 Da, depending on experimental conditions by the free-radical depolymerization with hydrogen peroxide. Copper (II) was found to be a high efficient catalyst which reduced the apparent activation energy (E_a) of the depolymerization. To control the free radical reaction, and then, the efficiency and reproducibility of the process, the most parameters were pH control, the concentration of hydrogen peroxide and the temperature. Under the conditions of a low concentration of hydrogen peroxide, a neutral pH and a low temperature, DTHG whose chemical composition was kept almost unchanged from that of native THG, were obtained with good reproducibility. Moreover, the depolymerization of the main chain of THG was random, and obeyed pseudo-first-order kinetics. Now, studies are underway to elucidate the mechanism of the depolymerization, and to explore the potential of depolymerized fractions (DTHG), especially their pharmacological properties.

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