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Purification, structural characterization and anticoagulant properties of fucosylated chondroitin sulfate isolated from *Holothuria mexicana*

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ABSTRACT

A novel fucosylated chondroitin sulfate (HmG) was isolated from sea cucumber *Holothuria mexicana*, the structure of which was characterized by monosaccharide composition, disaccharide composition, IR, ^{1}H and ^{13}C NMR spectrum, additionally with two dimensional NMR spectrum of degraded HmG (DHmG). The backbone of HmG was identified as chondroitin 6-O sulfate, while the major O-4 sulfated fucose branches linked to O-3 position of glucuronic acid in almost every disaccharide unit. The anticoagulant activities of HmG and DHmG were assessed and compared with heparin and low molecular weight heparin. The results indicated that HmG and DHmG both could significantly prolong the activated partial thrombo-plastin time, and the properties were well related to its molecular weight. DHmG showed similar anticoagulant properties to low molecular weight heparin with less bleeding risks, making it a safer anticoagulant drug.

1. Introduction

Sea cucumber has been used as a prized food sources and traditional Chinese medicine for centuries [1]. There were hundreds species of sea cucumbers, which distributed in all oceans the world over, generally living near corals, rocks or sea weeds in warm shallow waters [2]. The edible parts of sea cucumbers are mainly the body walls, which were primarily composed of collagen and polysaccharides with some micro substances, like triterpene glycosides, gangliosides and lipids [3–5]. There were two kinds of sulfated polysaccharides obtained from sea cucumbers, involving fucosylated chondroitin sulfate (fCS) and fucan [6–9]. The fCS was a highly sulfated polysaccharide, possessing a backbone of chondroitin sulfate and large amount of sulfated fucose branches attaching to the O-3 position of β-D-glucuronic [10], the sulfate patterns of which made the mainly distinctions among fCS isolated from different species of sea cucumbers [11].

Due to its unique structures, fCS had attracted considerable interests in recent years owing to their potential therapeutic application, such as antitumor [12], antiviral [13] and antithrombotic properties [6,14,15]. Moreover, the fCS showed a heparin-like anticoagulant activity with less bleeding risks [16]. The fCS exerted its anticoagulant activities in different ways, including acceleration of thrombin inhibition by heparin cofactor II (HCII), inhibition of factor VIII activation by thrombin, and inhibition of factor X activation by the intrinsic tenase complex [7]. Owing to the lack of antithrombin-dependent activities, the fCS decreased the bleeding risk compared with heparin [16], which made it a promising candidate of anticoagulation drug.

The traditional technique methods for characterization of fCS were monosaccharide composition, disaccharide composition analysis and methylation analysis [10,17]. However, during the process of disaccharide composition and methylation analysis, it was inevitable for degradation of the sulfate esters and fucose branches, which made it difficult to obtain the accurate information of the sulfation substituent and glycosidic linkage patterns. With the development of 1/2D NMR spectroscopy, the detailed structure of fCS could be defined, which had been achieved for different holothurian glycosaminoglycans [8,18–20]. Besides, low molecular

Abbreviations: fCS, fucosylated chondroitin sulfate; CSE, chondroitin sulfate E; TFA, trifluoroacetic acid; PMP, 1-phenyl-3-methyl-5-pyrazolone; Man, mannose; GlcN, glucosamine; GlcA, glucuronic acid; GalA, galacturonic acid; GalNAc, N-acetylgalactosamine; Glc, glucose; Gal, galactose; Xyl, xylose; Fuc, fucose; Tris, tris(hydroxymethyl)aminomethane; ΔDi-0S, ΔUA-GalNAc; ΔDi-4S, ΔUA-GalNAc4S; ΔDi-6S, ΔUA-GalNAc6S; ΔDi-diS_E, ΔUA-GalNAc4S6S; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; LMWH, low molecular weight heparin; APTT, activated partial thromboplastin time; TT, thrombin time; PT, prothrombin time; Fuc2,4S, 2,4-O-disulfated fucose; Fuc4S, 4-O-sulfated fucose.

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fCS prepared by free radical degradation showed good resolution in 2D NMR, which would benefit the structure characterization of intact fCS [11].

In the present studies, we obtained a novel fCS from sea cucumber *H. mexicana* (named HmG), established a convenient and effective method to isolate fCS from sea cucumber and characterized its detailed structures using 1/2D NMR as the primary analytical method. We prepared low molecular weight HmG by free radical degradation and investigated its 2D NMR spectrum with good resolution to get the precise structure information of HmG. Furthermore, the comparison of anticoagulant properties for intact HmG and its depolymerized products were examined to elucidate the structure-activities relationships.

2. Materials and methods

2.1. Materials

Sea cucumber *H. mexicana* was purchased from and identified by the Yantai Guangxiang aquatic products company, Ltd. (China). Monosaccharide standards and 1-phenyl-3-methyl-5-pyrazolone (PMP) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Diastase Vera (EC 3.3.21.4) was purchased from Xuemei Zymin Technology Ltd. (Wuxi, China). Chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* (Seikagaku American Inc., Rockville, MD) was purchased from Sigma. Unsaturated disaccharides of the chondroitin sulfate family, including ΔUA-GalNAc (ΔDi-0S), ΔUA-GalNAc4S (ΔDi-4S), ΔUA-GalNAc6S (ΔDi-6S) and ΔUA-GalNAc4S6S (ΔDi-diS_E), were from Iduron (Manchester, England). Dextran T-series standards, were from National Institutes for Drugs and Biological Products (Beijing, China). Heparin (150 IU/mg) and low molecular weight heparin (LMWH, average molecular weight 3500 Da) were from Huixing Biochemistry Reagents Company Ltd., Shanghai, China. Q Sepharose Fast Flow and Sephadex 10 were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other chemical reagents were of analytic grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Isolation and purification of native fCS

The isolation of fCS from *H. mexicana* was performed according to our previous method [21] with minor modification. Briefly, the body wall of dry sea cucumber *H. mexicana* (ca. 100 g) was minced, homogenized and immersed in acetone to remove the lipids. The resulting residue was diluted to 1 L with distilled waters. 56 g of KOH was added under continuous stirring at 60 °C for 1 h. The pH was adjusted to 8.5 with cold HCl. The mixture was added with 5 g of Diastase vera (EC 3.3.21.4) and stirred at 50 °C for 3 h. The resulting solution was cooled down and centrifuged to remove the precipitate. One fold 95% (v/v) ethanol was added to the supernatant to precipitate the polysaccharides. The formed precipitate was collected and dissolved in distilled water at the ratio of 1:20 (g/mL). 1.5 M KAc was added into the supernatant and kept at 4 °C overnight. The crude polysaccharide was collected by centrifugation, dissolved in distilled water, dialyzed against distilled water for 36 h and lyophilized.

The crude glycosaminoglycans was further purified by ion exchange chromatography on a Q Sepharose Fast Flow column (300 mm × 30 mm) coupled with a peristaltic pump, eluted with 1.5 M NaCl and detected by the phenol–sulfuric acid method [22]. The major polysaccharides fractions were collected, dialyzed and further purified on a Sephadex G10 column (100 cm × 2.6 cm) with deionized water. The polysaccharide fractions were pooled and lyophilized.

The purity and molecular weight distribution of obtained fCS were determined by high performance gel permeation chromatography (HPGPC) on a Waters Ultrahydrogel Linear column (7.8 mm × 300 mm, Japan) with a Waters 2410 refractive index detector, eluted by 0.2 M Na_2SO_4 at a flow rate of 0.5 mL/min. The molecular weight was calculated by a calibration curve by determining the retention time of a series of dextran T-series standards (Mw: 133.8, 84.4, 41.1, 21.4, 10.0, 7.1 kDa) [23].

2.3. General analysis

Total sugar content was investigated by the phenol–sulfuric acid method using fucose as the standard [22]. The protein content was measured according to the previous method described by Lowry [24]. Sulfate ester content was tested according to the $BaCl_2$-gelatin colorimetric method described by Therho and Hartiala [25].

2.4. Monosaccharide composition analysis

Monosaccharide composition was determined by reversed phase high performance liquid chromatography (HPLC). 5 mg of polysaccharide was hydrolyzed by 2 M TFA at 110 °C for 8 h. The excess acid was removed by co-evaporating with methanol. The residue was dissolved in 1.0 mL. 100 μL samples was mixed with 100 μL 0.1 M NaOH and 100 μL of 0.5 M PMP (dissolved in methanol) and maintained at 70 °C for 30 min. The solution was neutralized with 0.1 M HCl and the excess PMP was extracted by chloroform. The supernatant was subject to HPLC and UV detection as described [26]. The analysis was performed on a Zonran Bondysil AQ-C18 column (5 μm, 4.6 mm × 150 mm, Zonran technologies company Ltd., Shanghai, China) at 30 °C with detection at a UV wavelength of 245 nm.

2.5. Analysis of repeatable disaccharides composition

The repeatable disaccharides composition was determined by mild acid hydrolysis and subsequent enzymatic degradation [10,20]. Briefly, 50 mg of HmG was dissolved in 1.0 mL of 150 mM H_2SO_4, kept at 100 °C for 30 min. The excess SO_4^{2-} was removed by adding aqueous $Ba(OH)_2$. The mixture was centrifuged to remove the precipitate and the supernatant was dialyzed against distilled water for 24 h. The defucosylated HmG was lyophilized, dissolved in 100 μL distilled water, and incubated at 37 °C for 6 h by addition of 900 μL 50 mM Tris/HCl buffer (pH 8.0) containing 0.1 unit of chondroitin ABC lyase (EC 4.2.2.4). The compositions of produced unsaturated disaccharides were determined by an anion exchange HPLC method [27]. The analysis was performed on a Waters Spherisorb S5 NH_2 column (2.0 mm × 150 mm) at 30 °C with UV detection at 232 nm.

2.6. Preparation of low molecular weight HmG

The depolymerized HmG fragments were prepared by controlled free-radical depolymerization induced by Cu^{2+} [28]. Briefly, the intact HmG (100 mg) was dissolved in 10 mL 50 mM H_2O_2 (diluted in 100 mM K_2HPO_4 solution, pH 7.5) containing 1 mM $CuAc_2$ and kept at 45 °C for 1.5 h. The reaction was stopped by addition of appropriate amount of EDTA·2Na. The mixture was purified on a Sephadex G-10 column (100 cm × 1.6 cm). The depolymerized HmG (named DHmG) fractions were pooled and subsequently lyophilized. The yield of the DHmG was 85.5% by weight.

2.7. IR and NMR spectroscopic analysis

The glycosaminoglycan was mixed with dried KBr, ground and punched into 1 mm pellets for Fourier-transform infrared (FT-IR)