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Sequence determination and anticoagulant and antithrombotic activities of a novel sulfated fucan isolated from the sea cucumber *Isostichopus badionotus*

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ABSTRACT

Background: The aim is to analyze the structure, anticoagulant and antithrombotic activities of a sulfated fucan isolated from sea cucumber *Isostichopus badionotus* (fucan-*Ib*).
Methods: Fucan-*Ib* was hydrolyzed under mild acid conditions. The oligosaccharide fragments were fractionated by gel-filtration chromatography and the structures were determined by negative-ion electrospray tandem mass spectrometry with collision-induced dissociation and two-dimensional NMR. Anticoagulant activities were measured by activated partial thromboplastin, thrombin and prothrombin times, and by *in vitro* inhibition experiments with factors IIa and Xa. Antithrombotic activities were determined *in vitro* by measuring the length and weight of the thrombus generated.
Result: The linear polysaccharide sequence of fucan-*Ib* was deduced from the structures of its oligosaccharide fragments produced by acid hydrolysis. Under mild conditions, the glycosidic bonds between the non-sulfated and 2,4-*O*-disulfated fucose residues were selectively cleaved and highly ordered oligosaccharide fragments with a tetrasaccharide repeating unit $[\rightarrow 3Fuc(2S,4S)\alpha 1\rightarrow 3Fuc(2S)\alpha 1\rightarrow 3Fuc(2S)\alpha 1\rightarrow 3Fuc\alpha 1\rightarrow]_n$ were obtained. In *in vitro* assays fucan-*Ib* showed good anticoagulant and antithrombotic activities compared with heparin and the fucosylated chondroitin sulfate isolated from the same source (fCS-*Ib*). The two polysaccharides, fucan-*Ib* and fCS-*Ib*, differ in the mechanism of action; the former exhibited activity mainly by potentiation of antithrombin acted on thrombin and factor Xa whereas the latter mainly through heparin cofactor II.
Conclusion: Fucan-*Ib* has a well defined structure with tetrasaccharide tandem repeats and good anticoagulant and antithrombotic activities.
General importance: Fucan-*Ib* has a well defined structure and can be readily quality-controlled, and therefore has potential therapeutic value as an affective antithrombotic drug with low risk of bleeding.

1. Introduction

Sea cucumber has been a traditional tonic food in China and other Asian countries for thousands of years [1–3]. In traditional Chinese medicine, sea cucumbers are used to treat a variety of symptoms, *e.g.* high blood pressure in humans and joint pain in pot-bellied pigs [4]. Acidic polysaccharides are the most important bioactive polymers in sea cucumbers. It has been reported recently that the acidic polysaccharides isolated from sea cucumbers have anticoagulant and antithrombotic activities [5–7], and they can also modulate angiogenesis [8] and inhibit metastasis of tumor [9]. Therefore, sulfated polysaccharides have attracted considerable interests in recently years due to their potential therapeutic application [10,11]. Previous studies indicated that the anticoagulant activity was not merely a consequence of the charge density and the sulfate content [12–14]. The structural requirement for these polysaccharides to interact with coagulation cofactors and their target proteases are stereospecific. The site of sulfation and the position of the glycosidic linkage are important for activities [15].

Two types of polysaccharides have been found in sea cucumbers: fucosylated chondroitin sulfate (fCS) [7,16] and fucan [17,18]. Although structures and bioactivities of the fCS have been described in details [7,15,18,19], there has been limited reports on sea cucumber fucans [20,21]. The sequences of fucans from sea cucumbers are relatively simple [21]; they are linear polysaccharides consisted of regular tandem repeat, *e.g.* di-, tri- or tetrasaccharide repeating unit, with defined glycosidic linkages and distinctive sulfation patterns [21]. These features are in sharp contrast to the complex structures of algae-derived fucans, which are composed of multiple fucose

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(Fuc) branches with different glycosidic linkages and sulfation patterns. As the structure and bioactivity studies of sulfated fucans have been normally carried out with the polysaccharides, there have been some ambiguities in the structural assignment due mainly to the difficulties in detailed interpretation of NMR spectra and possible desulfation during methylation linkage analysis of the sulfated polysaccharides [20].

Mass spectrometry has become increasingly important for structural analysis of carbohydrates, including glycan profiling [22] and oligosaccharide sequencing [23], due to its high sensitivity, high accuracy, and fast processing compared with NMR and various chromatographic methods [24]. Mass spectrometry has contributed considerably to the structural analysis of sulfated oligosaccharides, *e.g.* using ionic liquid matrices in matrix-assisted laser desorption/ionization mass spectrometry for analysis of sulfated heparin oligosaccharides [24], or using sodium adducts and multiply charged ions as precursors in negative-ion electrospray ionization with collision-induced dissociation tandem mass spectrometry (ES-CID-MS/MS) [25] for analysis of carrageenan oligosaccharides. However, despite these advances, sequence assignment of highly-sulfated oligosaccharides by mass spectrometry remains a challenge area, especially for those derived from sulfated fucans.

Detailed knowledge of the polysaccharide structure is important for better understanding of their biological roles. However, the high sulfate content poses a major challenge in sequence analysis and assignment of structure–function specificities of sulfated fucans. Traditionally, sequences of sulfated fucans were deduced from NMR spectroscopy of the polysaccharide and methylation linkage analysis of the monosaccharide components. For unambiguous sequence assignment and for testing the activities in order to derive the structure-function specificities it is important to prepare oligosaccharide fragments. Unfortunately, specific fucan-degrading enzymes have not been generally available. Acid hydrolysis has been widely used for partial depolymerization of fucan polysaccharides. However, acid hydrolysis is generally non-specific and it can also destroy some of the sulfates. Oligosaccharides thus obtained are complex and extremely difficult to purify. Recently, in the structural characterization of the linear sulfated fucan from the sea urchin *Lytechinus variegates,* Mourao and colleagues [26] were able to show, unexpectedly, that under mild conditions the apparently non-specific acid hydrolysis selectively removed a 2-*O*-sulfate from a mono-sulfated Fuc residue followed by preferential cleavage of the glycosidic bond between this newly created non-sulfated and the adjacent 2,4-*O*-disulfated Fuc residues. The hydrolysate containing the oligosaccharide mixture was analyzed directly by NMR to derive the sequence of the polysaccharide and the mechanism of the hydrolytic cleavage.

In the present study, prompted by the interesting work of acid hydrolysis [26], we employed the mild conditions to cleave a sulfated fucan isolated from the sea cucumber *Isostichopus badionotus* (fucan-*Ib*). The highly sulfated oligosaccharide fragments were isolated and their sequences were characterized by a unique ES-CID-MS/MS strategy together with 2D NMR. The structure of the polysaccharide was unambiguously assigned and the mechanism of cleavage was proposed which was somewhat different from the previously described [26]. We further assessed the antithrombotic and anticoagulant activities of fucan-*Ib*, including activated partial thromboplastin time (APTT) and thrombin time (TT), inhibition of thrombin (F.IIa) by heparin cofactor II (HC II) and antithrombin (AT), factor X (F.Xa) activation by AT, were examined to investigate the mechanism of action.

2. Experimental Section

2.1. Materials

Dry sea cucumber *I. badionotus* was purchased from a local market in Qingdao (China). Gel-filtration columns TSK G4000PWXL and G3000 PWXL were from TOSOH BIOSEP (Tokyo, Japan). DEAE ion-exchange resin was from Whatman (Brentford, England). Monosaccharides standards and disaccharide lactose were purchased from Sigma (St. Louis, Missouri, USA). The derivatization reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) was from Sinopharm Chemical Reagent (Shanghai, China). Low molecular weight heparin (LMWH) with an average MW of 4,000 Da was prepared in our laboratory and heparin was from Jiangsu Pharmacia (Jiangshu, China). The colorimetric substrates for F.IIa, F.Xa, AT, HC II and F.IIa were purchased from Calbiochem (Darmstadt, Germany) and that for F.Xa was from Sigma (St. Louis, MO, USA).

2.2. Isolation, purification and chemical composition analysis of fucan-Ib

Sulfated fucan was extracted from *I. badionotus* after papain digestion as previously described with some revision [12]. The crude polysaccharide participated by cetylpyridinium chloride was dissolved in a solution of 2 M NaC1: ethanol (100:15, v/v). Additional ethanol was added to a final concentration of 40% to precipitate fCS, which was purified using anion-exchange chromatography as described previously [15]. After centrifugation (2000×*g*, 15 min) and removal of the precipitate, ethanol was added to the supernatant to a final concentration of 60%. The precipitate formed was collected by centrifugation (2000×g, 15 min) and dissolved in water before dialysis against water for 24 h. The retained solution was lyophilized and crude fucan was obtained.

The crude fucan was purified by anion-exchange chromatography as previously described [15] (Supplementary Fig. 1). The fractions which showed single peaks on HPLC were pooled for further investigation. The polysaccharide collected, named as fucan-*Ib*, was with high viscosity, and the average molecular mass of fucan was determined to be 450 kDa by gel filtration chromatography on a TSK G4000PWXL column.

Monosaccharide composition of fucan-*Ib* was determined by HPLC after derivatization with PMP [28], and sulfate content was determined by ion-chromatography [29]. Fucose was the only monosaccharide detected. The sulfate content was determined to be about 32.9%, and the molar ratio of Fuc to sulfates was 1.00 to 0.92, the protein content was determined to be 7.2%.

2.3. Mild acid hydrolysis of fucan-Ib

For partial depolymerization of the polysaccharide fucan-*Ib*, mild acid hydrolysis was carried out with 0.05 M H_2SO_4 at 60 °C for 4–9 h. The hydrolytic products were analyzed by polyacrylamide gel electrophoresis (22%), and by HPLC with a TSK 3000 PWXL column for determinnation of the molecular weight distribution. The oligosaccharide mixture was fractionated by gel filtration chromatography on a Bio-Gel P-4 column (2.6×120 cm) eluted with 0.3 M NH_4HCO_3 at a flow rate of 0.3 mL/min, and monitored by refractive index. The pooled oligosaccharide fractions were lyophilized.

2.4. Negative-ion ES-MS and ES-CID-MS/MS

Negative-ion ES-MS and CID-MS/MS were carried out on a Waters Ultima mass spectrometer (Manchester, UK) with a Q-TOF configuration. Nitrogen was used as desolvation and nebulizer gas at a flow rate of 250 L/h and 150 L/h, respectively. Source temperature was 80 °C, and the desolvation temperature 150 °C. A cone voltage of 60–150 V was used for negative-ion detection, and the capillary voltage was maintained at 3 kV. Product-ion spectra were obtained from CID using argon as the collision gas at a pressure of 0.17 mPa. The collision energy was adjusted between 20 and 36 V for optimal fragmentation. A scan rate of 1.0 s/scan was used for both ES-MS and CID-MS/MS experiments, and the acquired spectra were summed for presentation. For analysis, oligosaccharides were dissolved in acetonitrile/water (1:1, v/v), typically at a concentration of 20 pmol/μL, of which