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An anticoagulant fucan sulfate with hexasaccharide repeating units from the sea cucumber *Holothuria albiventer*

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ABSTRACT

A fucan sulfate was isolated and purified from the sea cucumber *Holothuria albiventer* by papain enzymolysis, alkaline hydrolysis and ion-exchange chromatography. The water-soluble polysaccharide had high molecular weight and contained fucose and sulfate in a molar ratio of about 1:0.83. Methylation analysis of the native polysaccharide indicated that its glycosidic linkages and sulfate substituents might be at *O*-3 or *O*-3, 4 or *O*-2, 3, or *O*-2, 3, 4 positions. FT-IR and 2D NMR spectroscopies further revealed that the fucan sulfate is characteristically composed of a regular α (1 → 3) linked hexasaccharide repeating unit which is substituted with sulfate esters in a distinctive pattern. Anticoagulant properties of the fucan sulfate and its depolymerized product were assessed *in vitro* in comparison with a low-molecular-weight heparin. The fucan sulfate exhibits strong APTT and TT prolonging activities and intrinsic factor Xase inhibitory activity, and its molecular size seemed to be required for these activities.

1. Introduction

Unfractionated heparin (UFH) and low-molecular-weight heparins (LMWHs) have been the cornerstones of antithrombotic treatment and prophylaxis for the last 70 years, which are the only type of sulfated polysaccharide currently used as anticoagulant drugs [1,2]. The commercial sources of heparins are mainly porcine intestinal mucosa and bovine lung, where the heparin content is low [3]. The possibility that concomitants such as prions and viruses may be carried by the biological products combined with the increasing demand for antithrombotic therapies indicates the necessity to research for alternative sources of anticoagulant agents [4].

Marine echinoderm and brown alga are abundant sources of anticoagulant polysaccharides, such as fucoidans and fucan sulfates [4–17]. Brown algal polysaccharides, called fucoidans, usually are mixtures of several sulfated polymers with complex monosaccharide composition, thus they are structurally non-regular and heterogeneous [4–10], which hinders the clarification of their structure-activity relationship [10,11]. Distinguishing from fucoidans, the fucans from echinoderm such as sea cucumber and sea urchin, often known as fucan sulfates (FSs), are structurally more regular for they comprise only one kind of monosaccharide [12–18]. The chemical structures of these fucan sulfates were found to be species-specific [12,17]. Thus, each new sulfated polysaccharide purified from a certain sea cucumber or sea urchin would be a new compound with unique structures and, consequently, with potential novel biological activities.

Recently, when searching for new anticoagulant sulfated polysaccharides, we obtained two fucan sulfates from two species of sea cucumbers *Holothuria edulis* and *Ludwigothurea grisea* [16,17]. Both fucan sulfates have a unique structure composed of a central core of regular (1 → 2) and (1 → 3)-linked tetrasaccharide repeating units. Approximately 50% units of the FS from *L. grisea* (100% for *H. edulis* FS) contain side chains that are formed by nonsulfated fucose residues and linked to the *O*-4 positions of the central core. Anticoagulant activity assays indicated that the sea cucumber FSs can strongly inhibit human blood clotting through the intrinsic pathway of the coagulation cascade. Their distinctive structure with the tetrasaccharide repeating units contributes to the anticoagulant action [17].

Further exploration of sulfated polysaccharides from other sea cucumber species would provide a wider insight into the studies on their chemical structures and functional activities. In the present study, we discovered a new fucan sulfate from the sea cucumber *Holothuria albiventer*. The chemical structure was analyzed by chemical and instrumental methods such as Fourier transform infrared spectroscopy

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Table 1
GC–MS of alditol acetate derivatives from the methylated product of the fucan sulfate from the sea cucumber *H. albiventer*.

Methylated Derivatives (as alditol acetates)[a]	Positions of substitution	Relative retention time[b]	Molar ratio	Primary mass fragments (m/z)
2, 4-Me_2-Fuc	3	1.00	1.00	89, 101, 117, 131,159, 173, 233
2-Me-Fuc	3, 4	1.14	0.51	87, 99, 117, 129, 173, 275
4-Me-Fuc	2, 3	1.22	0.79	89, 99, 131, 159, 201, 261
Fuc	2, 3, 4	1.35	5.60	115, 128, 145, 170, 217, 231, 289

[a] 2, 4-Me_2-Fuc: 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-*L*-fucitol; 2-Me-Fuc: 1, 3, 4, 5-tetra-*O*-acetyl-2-*O*-methyl-*L*-fucitol; 4-Me-Fuc: 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl-*L*-fucitol; Fuc: 1,2,3,4,5-penta-*O*-acetyl-*L*-fucitol.

[b] Relative retention times of the corresponding alditol acetate derivatives compared to 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-*L*-fucitol.

(FT − IR), high performance liquid chromatography (HPLC), monosaccharide composition analysis, and nuclear magnetic resonance (NMR) spectroscopies (1D ^{1}H, ^{13}C, 2D ^{1}H/^{1}H COSY, TOCSY, ROESY, 2D^{13}C/^{1}H HSQC and HMBC). Moreover, its effect on the clotting time of human plasma and intrinsic coagulation factor Xase complex were investigated. Our results may provide valuable information for understanding the structure-function relationships of the well-defined polysaccharides from invertebrate.

2. Results and discussion

2.1. Purification and physicochemical characterization

The body wall of sea cucumber usually contains two types of sulfated polysaccharides, fucosylated chondroitin sulfate and fucan sulfate [16–19]. The crude polysaccharides, with the yield of about 8.0% by dry weight, were extracted from the sea cucumber *H. albiventer* by the papain enzymolysis and alkaline hydrolysis [16]. The fucosylated chondroitin sulfate was partially removed by ethanol precipitation and KOAc salting out [19,20]. Then the crude fucan sulfate was further purified into different fractions by anion exchange chromatography using a FPA98 column. The fucan sulfate fraction obtained from the sea cucumber seemed to display high purity as determined by the high-performance gel permeation chromatography (HPGPC) (Figure S1). Ultraviolet absorption at around 260 or 280 nm was not observed as detected by an UV-detector, indicating the absence of contaminants of protein or peptides.

Additionally, the HPGPC profile of the fucan sulfate displayed a single wide peak, indicating that the polysaccharide might be homogenous with a wide distribution. And its average molecular weight was over 2000 kDa as calculated by GPC. The monosaccharide composition of the sea cucumber polysaccharide was qualitatively identified by reverse-phase HPLC after PMP derivatization procedures [21,22]. The result showed that the fucan sulfate contained the only monosaccharide fucose, which consists with those from other sea cucumber species [15,16,18] (Figure S2). The specific rotations of the polysaccharide and its depolymerized product were −168° and −172°, respectively, similar to those from other sea cucumber species [16,17]. This result is in conformity with *L*-configuration of fucose residues [17,23].

Among other diagnostic information, the content of possible charged groups, such as sulfate groups, is essential to evaluate the charge distribution along the polyelectrolyte chain. Thus, the charge of the native fucan sulfate and its depolymerized product was measured by conductometric titration [24]. Both of conductimetric titration curves showed only one inflection point (Figure S3), indicating that the fucan sulfate contains only a negatively charged functional group. Further calculation indicated that the sulfate (SO_4) content of the native fucan sulfate was 34.4% (37.9% for the depolymerized fucan sulfate), and the molar ratio of sulfate ester to monosaccharide was 0.83 (0.93 for the depolymerized fucan sulfate).

To analyze the positions of its glycosidic linkages and sulfate ester substituents in the fucan sulfate, methylation and GC-MS analysis was carried out by the previous method [17,21,25]. Consequently, the four monosaccharide derivatives identified by MS analysis were 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-*L*-fucitol (2, 4-Me_2-Fuc), 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl-*L*-fucitol (2-Me-Fuc), 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl- *L*-fucitol (4-Me-Fuc), and 1,2,3,4,5-penta-*O*-acetyl-*L*-fucitol (Fuc) with a molar ratio of 1.00: 0.51: 0.79: 5.60 as calculated by their peak areas, respectively (Table 1). The result suggested that its glycosidic linkages and sulfate substituents might be at *O*-3 or *O*-3, 4 or *O*-2, 3, or *O*-2, 3, 4 positions of fucose residues in the polymer chains. Since the four substituents were all at *O*-3 positions, we inferred that the fucan sulfate might consist of (1 → 3) linked fucose residues sequences. Other techniques such as NMR analysis of its desulfated product would further confirm the $[(1 \rightarrow 3)\text{-Fuc}]_n$ backbone in a structure of the fucan sulfate (see the following results).

2.2. IR and NMR analysis

The organic functional groups of the *H. albiventer* fucan sulfate were further characterized by IR spectroscopy (Figure S4A). The bands in the region of 4000–1800 cm^{-1} showed the characteristic O–H and C–H stretching vibrations of this polysaccharide at 3442 cm^{-1} and 2942 cm^{-1}, respectively [26]. Three signal groups were assigned to the sulfate groups, particularly, those appeared at 1262 and 1231 cm^{-1} were caused by S = O asymmetric stretching vibration, that at 854 cm^{-1} were assigned to the symmetric C–O–S stretching vibration and that at 583 cm^{-1} were caused by S–O stretching vibration [26–28]. These data confirmed that the sea cucumber polysaccharide was substituted by sulfate esters. Carbohydrate signals in the 1500–1200 cm^{-1} region indicated the deformation vibrations of H–C–H, C–O–H, C–H and C–O–C groups. Bands in this region at 1453 cm^{-1} and 1384 cm^{-1} may be assigned to the asymmetric and symmetric deformation vibrations of CH_3, respectively. These bands were also observed in the second-derivative spectrum of fucoidan [29]. In the finger print region, band at 962 cm^{-1} was assigned to the asymmetric and symmetric deformation vibrations of the methenyl groups in fucose residues [27]. The FT-IR spectrum of the depolymerized fucan sulfate (Figure S4B) was similar to that of the native fucan sulfate, implying their structural group similarity.

The 1D ^{1}H NMR spectrum of the fucan sulfate displayed overlapping and broad signals with line widths of several Hz (Figure S5), owing to its highly polymeric nature as high-molecular-weight compound, which hampered the resolution. It would be useful to study the desulfated product of the native sulfated polysaccharide to clarify the glycosidic linkages of its backbone. The sulfate content (Figure S3C) and the molar rate of sulfate ester to fucose of the desulfated product were 15.6% and 0.28, respectively, and the desulfation rate was about 70% compared to the native fucan sulfate. Minor signals at about 5.3 ppm in the ^{1}H NMR spectrum of the soluble desulfated product might indicate incomplete desulfation (Figure S6). The molecular weight of the desulfated product was 1638 Da. The result indicated that the fucan sulfate may be excessively degraded under desulfation conditions. Degradation of other fucan sulfates was also observed previously in the desulfation procedure [30,31]. Nevertheless, the 1D NMR spectra of the desulfated product (Figure S6) indeed became simpler than those of the native sulfated polysaccharide. The chemical shifts of individual residues in the desulfated product were fully assigned (Table S1) according to 1D (^{1}H, ^{13}C) and 2D ^{1}H/^{1}H COSY, TOCSY, ROESY, 2D^{13}C/^{1}H HSQC, and