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Research paper

Fucosylated chondroitin sulfate oligosaccharides exert anticoagulant activity by targeting at intrinsic tenase complex with low FXII activation: Importance of sulfation pattern and molecular size

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ABSTRACT

Fucosylated chondroitin sulfates (fCSs) are structurally unusual glycosaminoglycans isolated from sea cucumbers that exhibit potent anticoagulant activity. These fCSs were isolated from sea cucumber, *Isostichopus badionotus* and *Pearsonothuria graeffei*. Fenton reaction followed by gel filtration chromatography afforded fCS oligosaccharides, with different sulfation patterns identified by mass and NMR spectroscopy, and these were used to clarify the relationship between the structures and the anticoagulant activities of fCSs. In vitro activities were measured by activated partial thromboplastin time (APTT), thrombin time (TT), thrombin and factor Xa inhibition, and activation of FXII. The results showed that free radicals preferentially acted on GlcA residues affording oligosaccharides that were purified from both fCSs. The inhibition of thrombin and factor X activities, mediated through antithrombin III and heparin cofactor II of fCSs oligosaccharides were affected by their molecular weight and fucose branches. Oligosaccharides with different sulfation patterns of the fucose branching had a similar ability to inhibit the FXa by the intrinsic factor Xase (factor IXa-VIIIa complex). Oligosaccharides with 2,4-*O*-sulfo fucose branches from fCS-*Ib* showed higher activities than ones with 3,4-*O*-disulfo branches obtained from fCS-*Pg*. Furthermore, a heptasaccharide is the minimum size oligosaccharide required for anticoagulation and FXII activation. This activity was absent for fCS oligosaccharides smaller than nonasaccharides. Molecular size and fucose branch sulfation are important for anticoagulant activity and reduction of size can reverse the activation of FXII caused by native fCSs.

1. Introduction

The leading causes of death throughout the world are now diseases of the heart and blood vessels involving thrombosis [1]. Anticoagulant therapy is demanded in the most thromboembolic conditions. Unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) have been widely used as anticoagulants for a long time, but the risk of hemorrhagic complication during therapy is still a major unsolved problem [2,3].

Sea cucumbers have been used as a traditional functional food in China and other Asian countries for thousands of years [4,5]. Fucosylated chondroitin sulfate (fCS) is a glycosaminoglycan extracted from the sea cucumber, composed of alternating β-D-glucuronic acid and *N*-acetyl-β-D-galactosamine units [6]. Unlike typical mammalian glycosaminoglycans, fCSs possess sulfated fucose branches, which represent a key factor in their anticoagulant activities [7]. The potential therapeutic application of fCSs has attracted considerable attention and these include anti-human immunodeficiency virus (anti-HIV) activity [8], attenuation of renal fibrosis through a P-selectin-mediated mechanism [9], inhibition of tumor metastasis [10], and anti-hyperlipidemia activity

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[11]. The anticoagulant and antithrombotic properties also suggest the potential use of fCS as a substitute for heparin [12,13]. The anticoagulant activity of fCSs involves multiple mechanisms, including antithrombin III (AT III)-dependent inhibition of thrombin (FIIa) and factor Xa (FXa), heparin co-factor II (HCII)-dependent inhibition of FIIa [14], inhibition of factor VIII activation by FIIa [3] and inhibition of factor Xa generation by the intrinsic tenase complex [15]. However, fCSs also cause undesirable side effects, including the activation of FXII, platelet aggregation, hypertension and spontaneous bleeding in humans and some animals [16,17]. Moreover, the absence of the fCS degradation enzymes makes its breakdown difficult in human body, and its continued circulation can cause kidney damage and other toxicity.

The selective inhibition of the intrinsic coagulation pathway is considered a promising strategy for developing safer anticoagulants without serious bleeding consequences. Depolymerized fCS fragments inhibit plasma thrombin generation primarily by reducing prothrombin activation, rather than by accelerating the inhibition of thrombin by HCII, and exert strong anticoagulant and antithrombotic activity by selectively inhibiting the intrinsic tenase, resulting in less bleeding than UFH and LMWH in dogs and rats and without such side effects as FXII activation [16,18,19]. Therefore, low molecular weight fCS fragments targeting the intrinsic coagulation pathway, may represent attractive alternatives to heparin as anticoagulant and antithrombotic drugs. Most importantly, the partial degradation of sea cucumber fCS with chondroitinase leads to oligosaccharides that can be excreted eliminating toxic effects associated with continuous circulation, making such compounds potential targets for developing anticoagulant and antithrombotic drugs [20,21]. Unfortunately, most previous research on the anticoagulant activity of depolymerized fCSs relies on mixtures and there is little understanding of their detailed structure. Thus, it has been difficult to elucidate the precise structure-anticoagulant activity relationship of these fCSs required for their safe use as anticoagulants. The anticoagulant evaluation of purified fCSs oligosaccharides is crucial for elucidating structure—activity relationships. A method relying on the partial *N*-deacetylation and deaminative cleavage [22] of fCS has been used to prepare a series of fCS oligosaccharides. However, this method is difficult to scale-up because it is time-consuming and not environmentally friendly. Acid-catalyzed hydrolysis is also an efficient method for the large-scale preparation of low molecular fCSs [23]. Unfortunately, acid-catalyzed hydrolysis can lead to the partial loss of sulfated fucose branches, significantly impacting the anticoagulant activity of the resulting low molecular weight fucosylated chondroitin sulfate [24,25]. Thus, a new method for the controlled depolymerization of fCS is required for the preparation of fCS oligosaccharides for purification and evaluation.

Two fCSs with well-defined repeating oligosaccharide units have been identified by our laboratory from 2D NMR (Fig. 1): fCS from *Pearsonothuria graeffei* (fCS-*Pg*) that mainly contains 3,4-*O*-sulfo fucose (Fuc3,4S) branches, and fCS from *Isostichopus badionotus* (fCS-*Ib*) that mainly contains 2,4-*O*-sulfo fucose (Fuc2,4S) branches [24]. Evaluation of their anticoagulant and antithrombotic activities indicated 2,4-*O*-sulfo fucose branching was most important for anticoagulation, while the antithrombotic activity was determined by the overall structure of the polysaccharide. However, more recent research indicates there is no difference in the activity of four fCSs obtained from different sources.

In the present study, we prepared a series of pure oligosaccharides from the two fCSs using the modified Fenton-system that we previously reported [26]. The structure of the pure oligosaccharides was determined by electrospray-tandem mass spectrometry (ES-MS-MS) together with nuclear magnetic resonance (NMR) spectroscopy and a degradation mechanism for fCS consistent with the Fenton-system was proposed [26]. The anticoagulant activity was determined using activated partial thromboplastin time (APTT) and thrombin time (TT), inhibition of thrombin by HC II and AT, and factor X activation by the intrinsic factor Xase (factor IXa-VIIIa complex) and the resulting data were used to obtain a structure-anticoagulant activity relationship. The capability of these oligosaccharides to activate factor XII (FXII) was investigated to reduce the potential side effects of fCSs, facilitating their further development as drugs for the treatment of thromboembolic diseases.

2. Materials and methods

2.1. Materials

Dry sea cucumbers *P. graeffei* and *I. badionotus* were purchased from a local market in Qingdao (China). Gel-filtration column Ultrahydrogel 250 was from Waters (Milford, USA) and DEAE ion-exchange resin was from Whatman (Brentford, England). The colorimetric substrates for thrombin (FIIa) and Factor Xa, AT, FIIa, FXa and heparin were purchased from Adhoc (Beijing, China). Factor VIII (FVIIIa), Factor IX (FIXa) and Factor X were achieved from Boatman Biotech CO (Shanghai, China). Factor XII (FXII) was from AssayPro (St. Charles, USA). Prekallikrein Pool and kallikrein substrate S-2302 were from Hyphen BioMed (Strasbourg, France). Phosphatidylserine (PS), phosphatidylcholine (PC) and cholesterol were purchased from Aladdin (Shanghai, China). Phosphatidylcholine: phosphatidlylserine: cholesterol (molar ratio 75:25:1) phospholipid vesicles (PC:PS vesicles) were prepared by extrusion through a 100 nm polycarbonate filter as previously described [7].

2.2. Preparation and purification of fCSs oligosaccharides by a modified Fenton system

The fCSs were extracted and purified from the body wall of the sea cucumber *P. graeffei* and *I. badionotus* as previously described [27]. The depolymerized fCS-*Ib* and fCS-*Pg* fragments were prepared by our modified free-radical depolymerization induced by Cu^{2+} catalyzed Fenton system [26]. In brief, fCS (200 mg) was dissolved in 100 mL 0.1 M sodium acetate-acetic acid solution containing 0.2 mM copper (II) acetate and adjusted to pH 6.0. Hydrogen peroxide was added with mixing and maintained 55 °C for 5 h. The reaction was stopped by removal of Cu^{2+} ions by Chelex 100 resin. The resulting products were analyzed by HPLC with a Waters Ultrahydrogel 250 column for determination of the molecular weight distribution. The oligosaccharide mixture was fractionated by gel filtration on a Superdex 30 column (1.6 × 100 cm) eluted with 0.3 M NH_4HCO_3 at a flow rate of 0.3 mL/min, and monitored by RI detection (Agilent, Wilmington, DE, USA). The pooled oligosaccharide fractions were purified again using a Superdex peptide column (10 × 300 mm).

2.3. Negative-ion ES-MS

Negative-ion ES-MS was carried out on a Waters Ultima mass spectrometer (Manchester, UK) with a Q-TOF configuration. Nitrogen was used for desolvation and nebulizer gas at flow rates of 250 L/h and 150 L/h, respectively. Source temperature was 80 °C, and the desolvation temperature was 150 °C. A cone voltage of 60–150 V was used for negative-ion detection, and the capillary voltage was maintained at 3 kV. A scan rate of 1.0 s/scan was used for ES-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 5 μL was loop-injected. Solvent (acetonitrile/1 mM ammonium bicarbonate, 1:1, v/v) was delivered by a Harvard

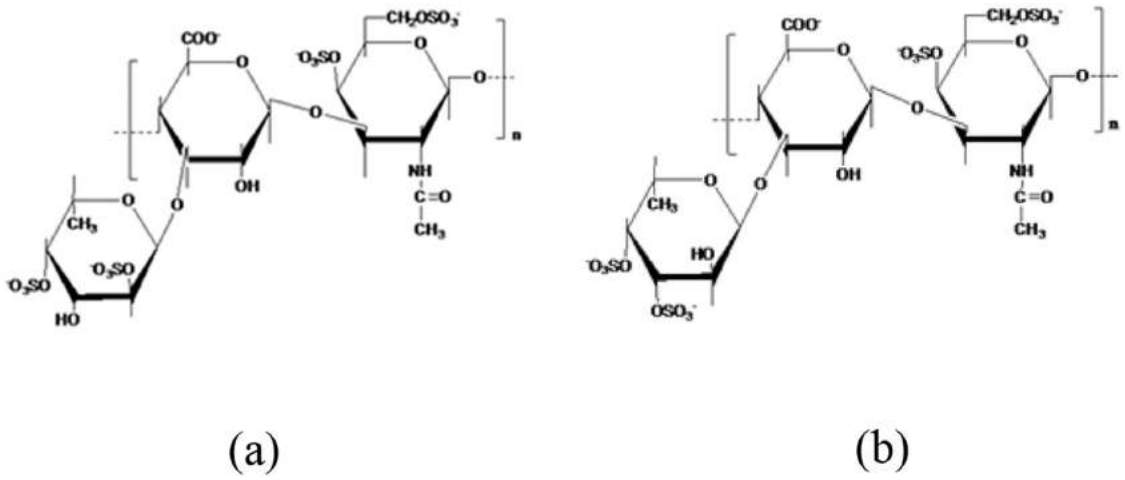

Fig. 1. The main structures of the sulfated polysaccharides from sea cucumbers. (a) fCS-*Ib*; (b) fCS-*Pg*.

syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 10 μL/min.

2.4. NMR spectroscopy

Nuclear magnetic resonance (NMR) experiments were carried out on Bruker AVANCE III 600 (Bruker, Germany) or Hudson—-Bruker SB 800 MHz Spectrometer (Madison, WI, USA). All samples were dissolved in 500 μL D_2O (99.9%, Sigma-Aldrich, St. Louis, MO) and lyophilized three-times to remove the exchangeable protons. The spectra of ^1H, ^{13}C, homonuclear $^1H/^1H$ total correlation spectroscopy (TOCSY), correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC) and Heteronuclear multiple bond correlation (HMBC) experiments were conducted at room temperature. The number of scans (ns) in each experiment was dependent on the sample concentrations.

2.5. Anticoagulant assays

Healthy human blood was donated by a 24-year old man and was collected into a solution of 3.8% sodium citrate. The plasma was separated by centrifugation at 3000 rpm for 10 min. The anticoagulant assays, including the activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT) were performed according to the manufacturer's recommended procedures with a coagulometer (RAC-120, China). The results were expressed as units/mg using a parallel standard curve based on a standard heparin (150 units/mg, Jiangsu Pharmacia, Jiangshu, China).

2.6. Inhibition of thrombin (FIIa) or FXa by AT III and HCII in the presence of fCSs and their oligosaccharide fractions

The inhibition experiments were carried out in a 96-well microtiter plate as described [24]. The reactant solutions used included AT (0.5 IU/mL) or HCII (0.5 μmol/L) and samples or the standard heparin at different concentrations in 40 μL of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl and 1.0 mg/mL PEG 8000, pH 7.4). FIIa (40 μL of 5 IU/mL) or FXa (40 μL of 0.4 IU/mL) was added to initiate the reaction. After incubation at 37 °C for 60 s, 40 μL of TS/PEG buffer containing 0.625 mM colorimetric substrate of FIIa or 1 mM chromogenic substrate SXa-11 of FXa was added and the absorbance at 405 nm was measured at intervals of 15 s within a period of 300 s in a micro-plate reader [28]. The plasma sample (30 μL) and a glycosaminoglycan solution (30 μL) were incubated at 37 °C for 2 min. Then, 30 μL of 6 mM S-2302 was added and the absorbance at 405 nm was recorded for 450 s. The absorbance change rate was proportional to the FIIa and FXa activity remaining in the incubation mixtures. Heparin was used as a control and the experimental results were expressed as the percent of control (n = 3).

2.7. Inhibition of FXa of by the intrinsic tenase complex

Inhibition of FXa by the FIXa-FVIIIa—phospholipids complex was evaluated according to the previous report with small modifications [3]. Different concentrations of glycosaminoglycan were incubated with 60 nM human FIXa, 5.0 nM thrombin-activated FVIIIa, and 50 mM PC/PS phospholipids vesicles in the "tenase buffer" containing 0.15 M NaCl, 2 mM $CaCl_2$, and 0.1% polyethylene glycol with an average of 8000 (PEG-8000) in a total volume of 200 μL, for 2 min at 37 °C. The reaction was initiated by addition of 50 nM FX (20 μL) immediately for 1 min at 37 °C. Then 50 nM factor X (30 μL) was added and the absorbance at 405 nm was measured at 37 °C at intervals of 15 s within a period of 300 s in a micro-plate reader. The absorbance change rate was proportional to the FXa activity remaining in the incubation mixtures.

2.8. Activation of human FXII

The activation of human FXII in the presence of glycosaminoglycans was determined according to a previously described method with modifications. Standard human plasma was diluted with three times the volume of TS buffer (0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4). The plasma sample (30 μL) and a glycosaminoglycan solution (30 μL) were incubated at 37 °C for 2 min. Then, 30 μL of 6 mM S-2302 was added and the absorbance at 405 nm was recorded for 450 s. S-2302 is a chromogenic substrate for plasma kallikrein, which is activated from its precursor, prekallikrein by the action of FXIIa.

3. Results and discussion

3.1. Preparation of oligosaccharide fractions by free-radical depolymerization

The development of a glycosidic bond-selective depolymer-

ization method was critical to obtain pure oligosaccharide fractions of fCSs. Our previous study has demonstrated that free radical depolymerization is very efficient for preparing sulfated oligosaccharides within several hours [26]. In addition, the degradation method is selective, causing no obvious sulfo group loss or Fuc debranching [26]. This selectivity is not observed in photochemical depolymerization [29] or acid hydrolysis [30]. Thus, free-radical depolymerization was employed for preparation of pure oligosaccharides from fCSs. The depolymerized DfCS-*Ib* and DfCS-*Pg* (Fig. 2a) contained a mixture of fragments of average molecular weight of 4.3 kDa as developed using a Waters Ultrahydrogel 250 column. The depolymerized fCS mixtures were size-fractionated by gel filtration chromatography on a Superdex 30 column (1.6 × 100 cm, Fig. 2b) and both fCSs generated six major fractions, labeled fCS-D1-D6. The six fractions were further purified on super peptide column (1.0 × 30 cm) and all these fractions showed only

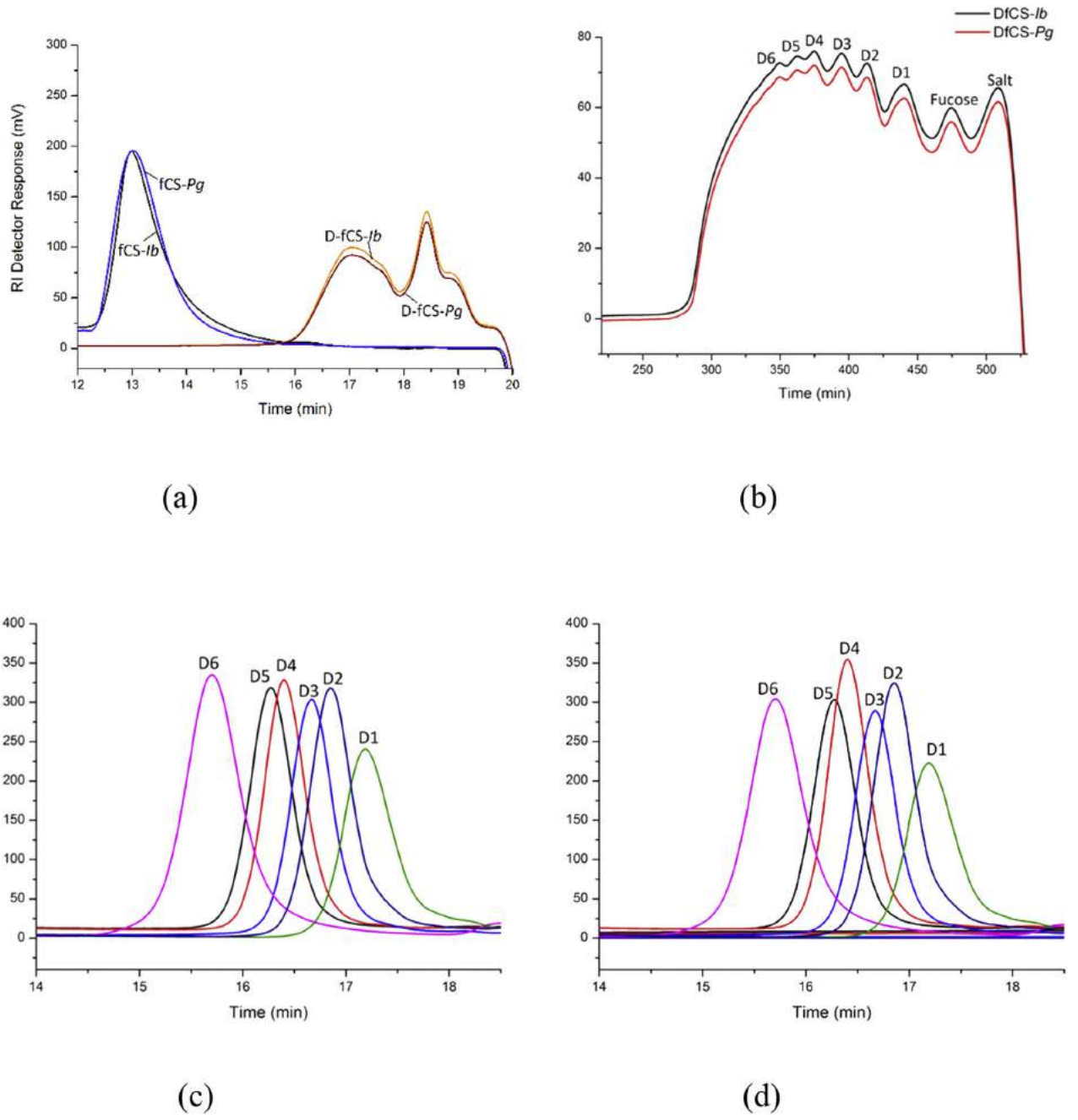

Fig. 2. Preparation of pure fCS oligosaccharide fragments. (a) Free radical degradation of fCS-*Ib* and fCS-*Pg* with a modified Fenton system [26]; (b) Fractionation of fCS oligosaccharides by gel filtration chromatography on a Superdex 30 column (1.6 × 100 cm) with elution by 0.3 M NH_4HCO_3 at a flow rate of 18 mL/h and detection by RI; HPLC profile of D1-D6 from fCS-*Ib* (c) and fCS-*Pg* (d), respectively, analyzed using Waters Ultrahydrogel 250 column and detected by RI.

single peak on the TSK 3000 column (7.8 mm × 30 cm) (Fig. 2c–d). The major fractions fCS-D1, fCS-D2, fCS-D3, fCS-D4, containing a relatively single size-pure component, were identified as tri-, tetra-, hexa-, hepta-saccharides, respectively, by negative-ion ES-MS (Table 1), with the formula of (GlcA-44), Fuc, GalNAc, S2 or $GlcA_n$, Fuc_n, $GalNAc_{(m-1)}$, GalNAcOOH, S2(n + m), where n = 1–2, m = n or n + 1 and "S" represents sulfo groups. The results unambiguously indicate that the basic structure of fCS polysaccharides was retained and no loss of sulfo groups took place during depolymerization reaction. Moreover, the free radical preferentially acted on the GlcA in the backbone, similar to our previous report [26]. In addition, analysis also suggested that the trisaccharide fraction (fCS-D1) had undergone decarboxylation and the GalNAc at the oligosaccharide terminus were easily oxidized, generating a carboxylic acid.

3.2. Structural analysis of the fCS oligosaccharide fractions

3.2.1. ES-CID-MS of fCSs oligosaccharides

The product-ion spectra were acquired for the tri- and tetrasaccharide fractions to determine their sequences. In addition, the overall sequence of fucosylated chondroitin sulfate could be deduced from the unequivocal sequences obtained for the individual oligosaccharides.

Trisaccharide fCS-*Ib*-D1: Based on the ES-MS result (Supplementary Fig. 1a), we selected the partially desulfation and doubly charged ion of sodiated molecule of fCS-D1-*Ib*, $[M\text{-}6H\text{-}4Na\text{-}2S]^{2-}$ (*m*/*z* 328.5) as the precursor for negative-ion ES-CID-MS/MS experiment, to deduce the sequence of the trisaccharide. As shown in Supplementary Fig. 1b, a set of glycosidic fragment ions were observed: Y1 at *m*/*z* 225, B1 at *m*/*z* 300, B2 at *m*/*z* 432, an indicative of the presence of fucose residue, GalNAc residue and GlcA-CO_2 residue. The occurrence of B2 (*m*/*z* 432) confirmed the presence of the GlcA-GalNAc backbone. In contrast, the GalNAc branch in a non-sulfated polysaccharide from the ink sac of squid *Ommastrephes bartrami* [31] is hard to establish due to the lack of ion information, for example the Y1 (*m*/*z* 225) was generated from the sulfated fucose branch observed in fCS fragmentation. The mass difference of 132 Da between B2 and B1 clearly indicates that GlcA was non-sulfated and decarboxylation had occurred at the GlcA. The ions at *m*/*z* 288.5, *m*/*z* 560 and *m*/*z* 325 were generated from desulfation of quasi-molecular ion peaks and B2. Taken together, the trisaccharide fragment could be established as a GlcA-GalNAcS disaccharide backbone with FucS branch on the GlcA residue, a typical trisaccharide repeating-unit of fCS.

Tetrasaccharide fCS-*Ib*-D2: This tetrasaccharide gave a negative product-ion spectrum (Supplementary Fig. 1c) with some doubly charged ions. Here, we used a partially desulfated doubly charged ion $[M\text{-}8H\text{-}6Na\text{-}4S]^{2-}$ at *m*/*z* 460 of the tetrasaccharide as precursor to determine its sequence. As shown in Supplementary Fig. 1d, the B1 ion at *m*/*z* 300/310 suggested that the amino galacturonic acid (B1) was so unstable that it was easily oxidized. The presence of deoxygenation in $[M\text{-}7H\text{-}6Na\text{-}4S]^-$ further identified the instability of the amino galacturonic acid (B1). The doubly charged ion B2 at *m*/*z* 341.6 indicates the existence of GlcA(FucS)-GalNAcS, whereas a gap of 384 Da (GlcA + FucS-H_2O) between B2 and B1 clearly suggests a FucS branch at the GlcA position. Therefore, the sequence of the tetrasaccharide was GalNAcOOH-S GlcA(FucS)-GalNAc. The fCSs-*Pg* oligosaccharides, which showed spectra similar to fCS-*Ib*, could be deduced to contain the same repeating units. However, the sulfation pattern information could not be obtained using mass spectrometry.

3.2.2. NMR of fCSs oligosaccharides

Although the repeating sequence had been identified by ES–CID–MS/MS, additional structure information especially the sulfation pattern was still unclear. The 1D and 2D NMR spectra of oligosaccharide fragments were investigated to obtain this information.

The ^1H NMR of native fCS-*Ib* and its oligosaccharide fractions are presented in Supplementary Fig. 2. All of the polysaccharide and oligosaccharide fractions showed the similar spectra with characteristic signals except for D1. The main signals were easily assigned based on previous literature [27]. The signals at 1.17 and 1.86 ppm could be assigned to the methyl protons of Fuc (CH_3) and GalNAc (CH_3CO), respectively, indicating that free radical depolymerization had no impact on the Fuc and GalNAc residues. The signals between 5.10 and 5.62 ppm were the anomeric protons of various sulfated fucose residues [6]. Signals from the anomeric proton of fucose branches of oligosaccharides remained almost identical to native fCS, suggesting that no obvious loss of sulfation pattern of fucose branches and fucose branches had occurred during the oxidation. Newly appearing signals at 3.4–3.55 ppm in the spectra of the oligosaccharides could be assigned to H-2 and H-3 of glucuronic acid. These results from reduced chain size and the degradation of GlcA at reducing terminus, indicate that free radical depolymerization is selective and preferentially acts on the GlcA residues.

Assignment of ^1H and ^{13}C chemical shifts of fucose branch and CSE backbone in fCS-*Ib*-D1 (Table 2) were based on the interpretations of the COSY (Supplementary Fig. 3a), HMBC (Supplementary Fig. 3b), and HSQC (Supplementary Fig. 3c) spectra. Compared with native fCS-*Ib*, the anomeric proton signals of sulfated fucose residue of fCS-*Ib*-D1 were shifted upfield from 5.56 ppm to 4.95 ppm, which might be affected by the decarboxylation. Some signals assigned to GlcA were also shifted downfield due to decarboxylation (Table 2). The sulfate substitution could be deduced from the apparent downfield shift of proton on

Table 1
Negative-ion ES-CID-MS of fCS oligosaccharide fractions.

Fraction	Purity (%)	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$	Mw	Compositions
fCS-*Ib*-D1	98	907.1	452.5		908.1	Fuc•GalNAc•4S+(GlcA-44)+4Na
fCS-*Ib*-D2	98	1357.0	678.0		1358.0	$Fuc•GalNAc_2•GlcA•6S+6Na$
fCS-*Ib*-D3	86		919.0		1840.0	$Fuc_2•GalNAc_2•GlcA_2•8S+6Na$
fCS-*Ib*-D4	90		1138.5	751.3	2279.0	$Fuc_2•GalNAc_2•GlcA_2•GalNAcOOH•10S + Na$
fCS-*Ib*-D5	75		1284.7	856.1	2571.4	$Fuc_3•GalNAc_3•GlcA_3•12S + Na$
fCS-*Ib*-D6	65			1332.5	4000.5	$Fuc_5•GalNAc_5•GlcA_5•16S+3Na$
fCS-*Pg*-D1	96	907.1	452.5		908.1	Fuc•GalNAc•4S+(GlcA-44)+4Na
fCS-*Pg*-D2	98	1357.0	678.0		1358.0	$Fuc•GalNAc_2•GlcA•6S+6Na$
fCS-*Pg*-D3	85		919.0		1840.0	$Fuc_2•GalNAc_2•GlcA_2•8S+6Na$
fCS-*Pg*-D4	92		1138.5	751.3	2279	$Fuc_2•GalNAc_2•GlcA_2•GalNAcCOOH_3•10S + Na$
fCS-*Pg*-D5	69		1284.7	856.1	2571.4	$Fuc_3•GalNAc_3•GlcA_3•12S + Na$
fCS-*Pg*-D6	68			1332.5	4000.5	$Fuc_5•GalNAc_5•GlcA_5•16S+3Na$

"S" represents a sulfo group.

Table 2
1H/13C NMR chemical shift assignments of fCS-*Ib* oligosaccharides.

Sample (Formula)		H1 (C1)	H2 (C2)	H3 (C3)	H4 (C4)	H5 (C5)	H6 (C6)
fCS-D1	GlcA	4.43 (101.32)	3.84	4.34	4.43	3.65	–
[(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]			(73.84)	(78.84)	(79.33)	(70.12)	
	GalNAc	4.45	3.79	3.80	4.57	3.84	–
		(100.70)	(52.84)	(75.32)	(76.43)	(73.96)	
	Fuc2S,4S	4.95	4.30	3.97	4.51	4.04	–
		(95.62)	(75.73)	(64.51)	(80.81)	(67.65)	
fCS-D2	GlcA	4.36	3.51	3.59	3.74	3.74	–
GalNAc-β1,4- [(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]		(101.62)	(74.72)	(77.24)	(77.03)	(77.96)	
	GalNAcI	4.43	3.79	3.80	4.57	3.84	–
		(101.3)	(50.84)	(77.63)	(76.87)	(73.92)	
	GalNAcII	4.43	4.32	3.84	4.57	4.04	–
		(101.2)	(54.43)	(72.35)	(76.85)	(67.82)	
	Fuc2S,4S	5.56	4.30	3.97	4.51	4.04	–
		(95.6)	(75.73)	(67.38)	(82.43)	(67.65)	
fCS-D3	GlcA	4.48	3.56	3.78	3.96	3.75	–
[(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]$_2$		(103.88)	(73.35)	(77.32)	(75.46)	(69.98)	
	GalNAc	4.53	3.85	3.87	4.64	4.10	–
		(100.08)	(51.82)	(75.02)	(81.04)	(72.66)	
	Fuc2S,4S	5.62	4.38	4.07	4.76	4.72	–
		(96.64)	(76.62)	(67.32)	(81.03)	(66.46)	
fCS-D4	GlcA	4.48	3.56	3.78	3.96	3.75	–
GalNAc-β1,4- [(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]$_2$		(103.88)	(73.35)	(77.32)	(75.46)	(69.98)	
	GalNAc	4.53	3.92	3.90	4.64	4.09	–
		(100.08)	(51.82)	(75.02)	(81.04)	(72.66)	
	GalNAc'	4.53	4.38	3.94	4.64	4.12	–
		(100.08)	(54.48)	(76.45)	(81.04)	(67.82)	
	Fuc2S,4S	5.62	4.38	4.07	4.76	4.72	–
		(96.64)	(76.62)	(67.32)	(81.03)	(66.46)	
fCS-D5	GlcA	4.48	3.56	3.78	3.96	3.75	–
[(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]$_3$		(103.88)	(73.35)	(77.32)	(75.46)	(69.98)	
	GalNAc	4.53	3.85	3.87	4.64	4.10	–
		(100.08)	(51.82)	(75.02)	(81.04)	(72.66)	
	Fuc2S,4S	5.62	4.38	4.07	4.76	4.72	–
		(96.64)	(76.62)	(67.32)	(81.03)	(66.46)	
fCS-D6	GlcA	4.48	3.56	3.78	3.96	3.75	–
[(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]$_5$		(103.88)	(73.35)	(77.32)	(75.46)	(69.98)	
	GalNAc	4.53	3.85	3.87	4.64	4.10	–
		(100.08)	(51.82)	(75.02)	(81.04)	(72.66)	
	Fuc2S,4S	5.62	4.38	4.07	4.76	4.72	–
		(96.64)	(76.62)	(67.32)	(81.03)	(66.46)	

attached carbons by careful comparison with that of corresponding unsubstituted monosaccharide, which showed that the Fuc is sulfated at C-2 and C-4 and that GalNAc is sulfated at both C-4 and C-6 positions. Further analysis using HMBC spectrum confirmed that the C-1 position of Fuc was linked to the C-3 position of GlcA and GalNAc was connected to the C-4 positions of the GlcA. In combination with the results from mass spectrometry, the complete structure of fCS-*Ib*-D1 was identified as:

GalNAcβ(4,6S)1-4GlcAβ(Fucα(2,4S)1−3) with loss of CO_2

The chemical shift assignments of heptasaccharide fraction were also made from COSY (Supplementary Fig. 4a), HMBC (Supplementary Fig. 4b) and HSQC (Supplementary Fig. 4c) spectrum. As shown in Table 2, in contrast to trisaccharide, heptasaccharide showed two different GalNAc signals (GalNAc I and GalNAc II), suggesting that GalNAc II was likely oxidized to a carboxylic acid and the C-3 position of GalNAc I was substituted. The sulfation positions were deduced from the downfield shifts of their proton signals compared with corresponding monosaccharides. The linkage and anomeric configuration could be deduced from correlation signals in HMBC spectra and H1/H2 coupling constants. Together with the mass spectrum, the structure of fCS-*Ib*-D4 was assigned as:

GalNAcOOHβ(4,6S)1-4GlcAβ(Fucα(2,4S)1−3)1-3GalNAcβ(4,6S)
4GlcAβ(Fucα(2,4S)1−3)1-3GalNAcβ(4,6S)

Further analysis of NMR spectra of another oligosaccharide fraction, fCS-*Ib*-D2 (Supplementary Fig. 5a–5c and Table 2), showed that these oligosaccharides match the formula: [(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]$_k$ or GalNAc-β1,4- [(Fuc-α1,3-) GlcA-β1,3-GalNAc-β1,4-]$_k$, where the Fuc side chains and the GalNAc residues are primarily Fuc_{2S4S} and $GalNAc_{4S6S}$, respectively. This result again confirms that oxidative depolymerization with Fenton system was selective and free radical preferentially acts on GlcA in the backbone.

The structures of fCS-*Pg* oligosaccharides were similarly determined, and the chemical shits are listed in Supplementary Table 1. Some typical 2D NMR spectra of fCS-*Pg* oligosaccharide fractions (Supplementary Figs. 6 and 7) are shown. A previous study showed that the two fCSs had identical chondroitin sulfate E backbones and similar fucose branches, but different sulfation patterns of the fucose branches [24]. Therefore, the structures of fCS-*Ib* oligosaccharide fractions were similar to those of fCS-*Pg* except for differences in the sulfation patterns of the fucose branches and this is

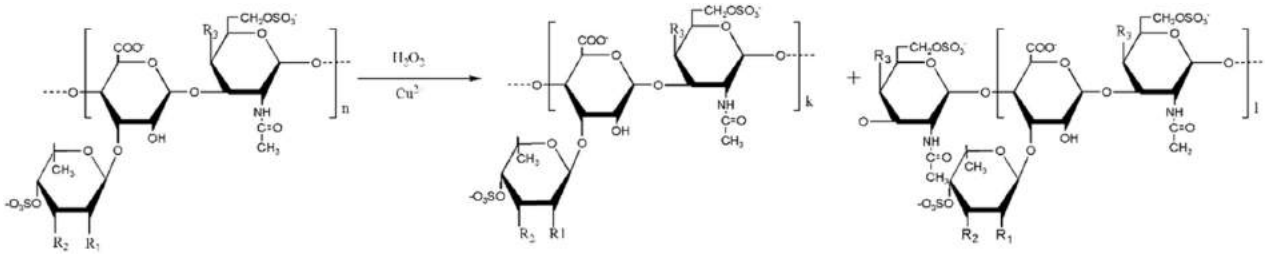

Fig. 3. Free radical depolymerization scheme of fCS from sea cucumber with hydrogen peroxide in the presence of copper (II) ion.

reflected in the similar GPC profile of their Fenton degradation products.

3.2.3. The proposed mechanism of fCS depolymerization by Fenton system

Based on the results from our data and related published literature [32], a mechanism for the depolymerization of fCS by Fenton system can be proposed. The radical degradation process occurs through the formation of free hydroxyl radical OH· from the hydrogen peroxide-metal redox system. This very reactive species primarily attacks at the glycosidic bond and GlcA residues of glycosaminoglycans are more reactive with free radicals, leading to the chain scission by free radical through action on the GlcA residues (Fig. 3). Wu et al. [33] has reported that the Fenton-type depolymerization of holothurian glycosaminoglycan from *T. ananas* (THG) is random and nonselective by investigating chemical compositions of partially depolymerized samples. These conclusions were based on fragment mixtures and it is difficult to draw the same conclusions for oxidative depolymerization mechanism of fCS. Cristina Rota et al. [34] investigated free radical-mediated heparin depolymerization under different chemical conditions. Their result suggests that when free radical depolymerization was performed using copper (II) as catalyst under mild chemical conditions, the reaction was highly controllable and reproducible and the parent heparin structure did not undergo substantial structural changes in the LMWHs obtained, consistent with our results on fCS. Moreover, changes of reaction conditions (pH, temperature, reaction time, catalyst types) [32,35,36] can lead to great differences in free radical species during the depolymerization process, thus affecting the final products. This explains why some research suggests there is no preferential cleavage of side-chains and that Fenton depolymerization is a totally random process [37,38]. These results further indicate that free radical degradation by Fenton-system in a mild process was useful in unraveling the structure of unknown marine GAGs [39], as well as helping with the identification of functionally important patterns in their sequences [40].

3.3. Anticoagulant and anticoagulant factor activities

Following structural clarification, we next characterized the anticoagulant properties of the fCS oligosaccharides. The anticoagulant activities of all samples were evaluated by APTT, TT and PT of plasma clotting assays (Table 3), which are used to describe the ability of compounds to inhibit blood clotting through the intrinsic, extrinsic, and common pathways of the coagulation cascade, respectively.

The results of the APTT assays indicate that molecular weight significantly influences anticoagulant activity and a reduction in molecular weight decreases APTT activity (Table 3). Notably, native fCS-*Ib* showed an APTT of 183 IU/mg, a little higher than the standard heparin (150 IU/mg) used. In comparison, the APTT-prolonging activity of fCS-*Ib*-D6, fCS-*Ib*-D5, fCS-*Ib*-D4 and fCS-*Ib*-D3 were reduced by 70.1%, 78.6%, 84.1%, 91.3%, respectively. Similarly, compared with native fCS-*Pg*, the depolymerized fCS-*Pg* fragments were also markedly reduced (Table 3). It is worth noting that both fCS-D1 (<1 IU/mg) and fCS-D2 (<10 IU/mg) showed weak or negligible effect on APTT. In addition, the APTT of fCS-*Pg* and its oligosaccharides is much lower than fCS-*Ib* and its corresponding oligosaccharides, further indicating the importance of sulfation pattern of fucose branch to the anticoagulant activity. In terms of TT and PT, no observable effects were found for any of the fCS oligosaccharides, suggesting that in contrast to the fCS polysaccharides, which can act on the intrinsic, extrinsic and common pathways, the fCS oligosaccharides exhibit anticoagulant activity mainly by inhibition of intrinsic coagulation pathway and have little or no effect on the extrinsic and common coagulation pathways. The variation between native polysaccharides and their oligosaccharide fragments on anticoagulant properties clearly suggest a completely

Table 3
Anticoagulant properties of native fCSs and their oligosaccharides.

Samples	Mw (kDa)	APTT[a] (IU/mg)		TT[a](IU/mg)		Anti-FXa/AT[a] (IU/mg)		Anti-Xase[b] (μg/ml)	
		Ib	*pg*	*Ib*	*pg*	*Ib*	*pg*	*Ib*	*pg*
D1	0.9	<1	<1	<1	<1	>500	>500	>500	>500
D2	1.4	6.8	3.06	<1	<1	52.5	>500	>500	>500
D3	1.8	16	7.03	<1	<1	16.6	56.5	>500	>500
D4	2.3	29.1	13.0	<1	<1	16.4	16.6	33.18 ± 4.87	31.77 ± 5.03
D5	2.6	39.1	19.7	<1	<1	12.4	16.4	8.15 ± 1.93	12.4 ± 2.32
D6	4.0	54.8	21.5	<1	<1	9.97	12.4	1.21 ± 0.21	1.41 ± 0.29
fCS	10.8/7.3	183	35	33.5	4.57	0.22	8.97	0.72 ± 0.12	0.77 ± 0.09
LMWH	4.5	69		64		1.16		26.04 ± 4.26	
Heparin	18.0	150		150		0.1		0.77 ± 0.14	

[a] The activity is expressed as international units/mg using a parallel standard curve based on the International Heparin Standard (150 IU/mg).
[b] EC50 value, the concentration required to inhibit 50% of protease activity. ($n \geq 3$).

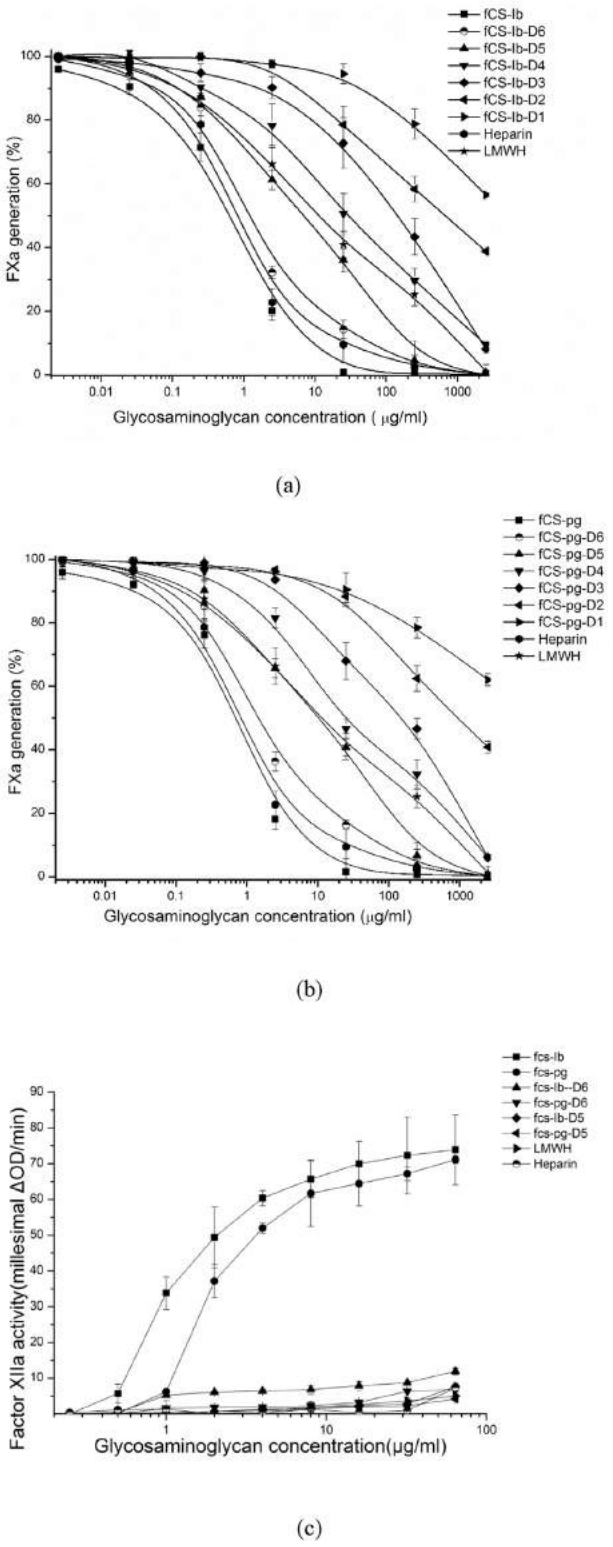

Fig. 4. Effect of fCSs and oligosaccharide fractions on inhibition of FXa by the intrinsic tenase complex. (a) fCS-*Ib* and its oligosaccharide fractions; (b) fCS-*Pg* and its oligosaccharide fractions. Human FIXa (60 nM), thrombin-activated FVIIIa (5.0 nM), and phospholipids vesicles (50 mM PC/PS) in "tenase buffer" were incubated with FX (50 nM) in the presence of sulfated polysaccharides or oligosaccharides at various concentrations. After 60 s of incubation at room temperature, the remaining FXa was determined with a chromogenic substrate (A405 nm/min). Results were expressed as mean ± SD (n = 3/group). (c) Activation of FXII in the presence of native fCSs and their oligosaccharides.

different anticoagulant mechanism.

Based on the results of coagulation-based assays, anticoagulant factor activities were next investigated and compared with those of unfractionated heparin and low molecular weight heparin (LMWH) to clarify the anticoagulant properties and mechanism of action of fCS and fCS oligosaccharides. The results show that antithrombin-dependent activities against both thrombin and factor Xa of all fCS oligosaccharides were concentration-dependent with activity less than 1 IU/mg (1.68 IU/mg for fCS-*Ib*-D6) (Table 3 and Supplementary Fig. 8), suggesting that the required chain size for fCS to accelerate the inhibition of coagulation proteases by conformational activation of serine protease inhibitor (serpin) is longer than heparin (≥18 sugar units and including the AT-binding pentasaccharide). When the molecular weight of fCS was reduced to 4.0 kDa (corresponding to a pentadecasaccharide), the binding affinity of oligosaccharides appears to be lost. In contrast, native fCS-*Ib* and fCS-*Pg*, heparin and LMWH exhibit strong AT-dependent anti-FIIa and anti-FXa activity. These results are consistent with our previous study that showed the intensity of ATIII-mediated anti-FIIa and anti-FXa activities of fCS and its depolymerized products decreased dramatically with decreasing molecular weight [26]. Additionally, fCS-*Ib* oligosaccharides, with Fuc2,4S branches, have greater activity than fCS-*Pg* oligosaccharides, with Fuc3,4S branches, on the inactivation of thrombin (FIIa) mediated by ATIII. The anti-FIIa activity, mediated by HCII, was also concentration-dependent. However, the inhibitory effect of all of the fCS oligosaccharides was much weaker than the native fCS polysaccharides. At a concentration of 2500 μg/mL, both native fCS polysaccharides and fCS-D6 afforded nearly complete inhibition of thrombin, but fCS nonasaccharides showed only 56% inhibition. The decreased inhibitory effect of the fCS nonasaccharide on thrombin mediated by ATIII and HCII may be associated to its negligible risk of bleeding [16], decreasing toxicity and mild anti-inflammatory effects *in vivo* [41].

Factor IXa (FIXa), a serine protease, and factor VIIIa (FVIIIa), a protein cofactor, form a Ca^{2+} and phospholipid surface-dependent complex referred to as the intrinsic tenase complex, which efficiently converts zymogen factor X (FX) to FXa [2]. Factor Xa promotes platelet activation and factor IXa promotes rapid thrombin generation on the activated platelet surface by activating factor Xa. Activation of factors IXa and Xa in proximity to platelet surfaces are required for effective initiation of coagulation and optimal thrombin generation is achieved only when both factor X and factor IX are activated [42]. Factor X activation by factor Xase (factor IX and VIIIa complex) is the rate-limiting step in thrombin generation [43,44]. Selective targeting of the intrinsic tenase complex in animal models of thrombosis is associated with significantly less bleeding risk compared with heparin and LMWH at equivalent therapeutic doses [45]. Therefore, inhibitors that selectively act on intrinsic tenase might be ideal alternatives for heparin as anticoagulant and antithrombotic drugs. Increasing concentrations of fCS-D6 and D5 results in essentially complete inhibition of factor Xa generation as is observed for fCS polysaccharides and heparin in Fig. 4. In terms of their mass concentrations, the EC_{50} values for direct factor Xase-inhibition activity for both fCS-*Ib*-D6/fCS-*Pg*-D6 (1.21 μg/mL/1.41 μg/mL) and fCS-*Ib*-D5/fCS-*Pg*-D5 (8.15 μg/mL/12.4 μg/mL) are stronger than that observed for LMWH (26.04 μg/mL). Moreover, the EC_{50} of fCS-*Ib*-D4/fCS-*Pg*-D4 (33.18 μg/mL/31.77 μg/mL) was only slightly lower than that of LMWH. However, further reduction in fCS polymer size markedly reduces the Xase-

inhibiting activities. Therefore, we hypothesize that the fCS heptasaccharide might be the threshold chain length required to inhibit the factor Xase. Remarkably, the EC_{50} for the fCS polysaccharides and fCS oligosaccharides were similar, suggesting that the sulfation pattern of the fucose branch had no effect on the binding activity of fCS to the intrinsic tenase complex for inhibition of FXa and that the anticoagulant mechanisms of fCS oligosaccharides were significantly different from heparin products and fCS polysaccharides.

Because the HCII-dependent FIIa inhibition and ATIII-dependent anti-IIa activities of fCS oligosaccharides of Mw lower than 4.0 kDa were too weak to significantly affect APTT activity, the decrease observed in APTT was most likely the result of a reduction in anti-factor Xase activity, indicating that fCS oligosaccharides exert highly selective anticoagulant activity mainly inhibiting the intrinsic coagulation pathway [3] and that the anticoagulant mechanisms of fCS oligosaccharides were significantly different from heparin products and fCS polysaccharides [46].

3.4. Activation of FXII

FXII is a component of the coagulation system that may be activated by polyanionic materials, such as oversulfated chondroitin sulfates (OSCS) [47], dextran sulfate [48] and heparin [49]. The fCS from *L. grisea* reportedly also activates FXII and causes hypotension when injected *intravenously* into rats [12,50]. Our previous study also demonstrated that fCS-*Ib* and fCS-*Pg* exhibit a potent activity to activate FXII [27] and suggested that a reduction in its molecular size might reverse this activity. However, at the beginning of this study, the structure and size requirements for reduction of FXII inhibition was still unclear. Therefore, the effect of the fCS-*Ib* and fCS-*Pg* oligosaccharides on the activation of FXII was determined.

FXII activation activity of fCS-*Ib* oligosaccharides and fCS-*Pg* oligosaccharides were markedly lower than those of fCS polysaccharides within the studied concentrations (Fig. 4c). With the reduction in chain length, the FXII activation activity of oligosaccharides drastically decreased, and both fCS-*Ib*-D5 and fCS-*Pg*-D5 showed negligible effect on FXII activation activity over the concentrations studied and were lower than the effects of heparin and LMWH. When the molecular weight was decreased further, no FXII activation was observed (data not shown). Additionally, oligosaccharides prepared from fCS-*Ib* and fCS-*Pg* showed no obvious differences in FXII activation, further demonstrating that sulfation pattern of the fucose branches may not the crucial for the activation of FXII [27]. In addition, structural similarities between the fCSs polysaccharides and OSCS (i.e., the presence of a CS-E containing repeating disaccharide sequence), suggest a reason for fCS having the same pro-inflammatory effects as OSCS through the contact system/kinin-kallikrein pathway [41]. Shorter chain fCS oligosaccharides do not carry sufficiently high net negative charge or are of insufficient size to enable complex (PK, high molecular weight kininogen, and FXII) formation, and thus no or less activation of prekallikrein (PK) and pro-inflammatory effect takes place.

4. Conclusion

In the present study, a series of pure fCS oligosaccharides from two sea cucumbers, *I. badionotus* (fCS-*Ib*) and *P. graeffei* (fCS-*Pg*), were prepared through a controllable Fenton depolymerization reaction. NMR and ES-MS analysis of fCS oligosaccharide fractions, with different sulfation patterns, further indicated that unlike other chemical methods [29] and acid hydrolysis [30], free-radical depolymerization was highly selective and preferentially cleaved the GlcA in the backbone without influencing on Fuc branching or sulfation.

Anticoagulant assay on native fCS and fCS oligosaccharides indicated that fCS oligosaccharides exerted anticoagulant activity only through the intrinsic pathway and had no or little effect on extrinsic and common coagulation pathways. APTT activity was found to be proportional to the Mw of fCS. Furthermore, anticoagulant factor assays suggested that inhibitory effects of thrombin mediated through ATIII/HCII of the two fCSs were sharply reduced when depolymerized and fCS–4-fCS-D6 retained considerably stronger anti-Xase activity than anti-FXa activity and anti-FIIa activity mediated by AT and HCII. The results suggest that in contrast to native fCSs, which acted on both serpin-related and serpin-independent pathways, fCS oligosaccharides exert their main activity on coagulation through the serpin-independent pathways, especially targeting the intrinsic tenase complex rather than the anticoagulant serpin-related system. Furthermore, the sulfation pattern of the fucose branch plays an important role in the anticoagulant activity, but its impact on anticoagulant activity is less than molecular weight.

Targeting of the intrinsic tenase complex may improve the benefit/risk ratio of antithrombotic therapy. Our data revealed that depolymerization of fCS could markedly decrease adverse effect of activation FXII and a heptasaccharide was the minimum fragment that possesses potent anticoagulant activity by selective inhibition of the intrinsic tenase with negligible FXII activation activity. Hence, the therapeutic potential of fCS oligosaccharides deserves further investigation for its clinical application and for its development as a drug to better treat thromboembolic diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.07.065.

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