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Structure and effect of sulfated fucose branches on anticoagulant activity of the fucosylated chondroitin sulfate from sea cucumber *Thelenata ananas*

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ABSTRACT

Fucosylated chondroitin sulfate (FuCS) is a glycosaminoglycan from sea cucumber, made up of alternating β-D-glucuronic acid and *N*-acetyl-β-D-galactosamine units. The β-D-glucuronic acid residues have branches of sulfated fucose, while the fucose branches may have distinguishable patterns and proportions of sulfate substitution. The structure of the sulfated fucose branches of the fucosylated chondroitin sulfate from the sea cucumber *Thelenata ananas* has been characterized by mild acid hydrolysis and NMR technology. The results showed that the fucose residues are made up of 3-,4-mono-*O*-sulfated fucose and 2,4-di-*O*-sulfated fucose with about 25:22:53, respectively. The sulfated fucose branches are essential for the anticoagulant action of FuCS, and this potent effect is possibly related to the occurrence of 2,4-di-*O*-sulfated fucose units. Furthermore, these branches could constitute the structural requirement for the binding of the glycosaminoglycan to coagulant enzymes, such as thrombin by HCII, factor X by the intrinsic tenase complex.

1. Introduction

Sea cucumber has been used as a fundamental source of tonic food and drug in traditional Chinese medicine for centuries. In the past decades, a kind of novel glycosaminoglycan had been isolated from the body wall of sea cucumber, which is also called as fucosylated chondroitin sulfate (FuCS). The glycosaminoglycan from the sea cucumber *Stichopus japonicas* (Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992) and *Ludwigothurea grisea* (Mourão et al., 1996) have been found to exhibit various biological activities, such as antiviral, antitumor, antithrombotic and anticoagulant properties (Buyue & Sheehan, 2009; Hoshino & Heiwamachi, 1990; Sheehan & Walke, 2006).

Recently, there have been several reports on the structures of the FuCS isolated from the body walls of two sea cucumbers, *S. japonicus* (Kariya, Watabe, Hashimoto, & Yoshida, 1990; Kariya, Watabe, Kyogashima, Ishihara, & Ishii, 1997) and *L. grisea* (Mourão et al., 1996; Vieira, Mulloy, & Mourão, 1991), using mild acid hydrolysis, enzymatic degradation, methylation analysis, and 1D and 2D of ^{1}H, ^{13}C NMR spectroscopy. The fucosylated chondroitin sulfate could be 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 et al., 1991; Wu, Xu, Zhao, Kang, & Ding, 2010a,b; Yoshida et al., 1992), while the fucose branches of FuCS may have distinguishable patterns and proportions of sulfate substitution.

Due to its heavily sulfated fucose side chains at position 3 of the β-D-glucuronic acid, the sea cucumber chondroitin sulfate shows potent anticoagulant activity (Mourão et al., 1996), thus the chondroitin sulfate has become one of the promising anticoagulant polysaccharides (Fonseca, Santos, & Mourão, 2009; Mourão et al., 2001; Nagase et al., 1995, 1996). The anticoagulant activities of the sulfated polysaccharide and its chemically modified analogues have been demonstrated multiple potential mechanisms of action, 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 (Fonseca & Mourão, 2006; Minamiguchi et al., 2003; Mourão et al., 2001; Nagase et al., 1995, 1996). In contrast, these activities remain after carboxyl-reduction of the glucuronic acid residues to glucose (Zancan & Mourão, 2004). The sulfated fucose branches were considered as the key factor for their anticoagulant activities, as removal of the sulfated fucose side chains by mild acid hydrolysis, as well as desulfation, reduces its anticoagulant and antithrombotic activities to the same levels as mammalian chondroitin sulfate (Mourão, Giumaraes, Mulloy, Thomas, & Gray, 1998; Mourão et al., 2001). Furthermore, 2,4-*O*-disulfation of the branches may be important for anticoagulant activity (Chen et al., 2011). Mourão et al. (1996) found that the sulfated branches could constitute the structural requirement for the binding of the glycosaminoglycan

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to heparin cofactor II and antithrombin. However, there has been no detailed investigation the effects on the constitution of sulfated fucose branches for the binding of the polysaccharide to other coagulation enzymes, such as thrombin by HCII, factor X by the intrinsic tenase complex.

Recently, we isolated a new fucosylated chondroitin sulfate from the body wall of the sea cucumber *Thelenata ananas* (Wu, Xu, Zhao, Kang, & Ding, 2010a,b), which consisted of *N*-acetylgalactosamine (GalNAc), glucuronic acid (GlcUA), fucose and ester sulfate with about 1:1:1:3.7, respectively. The chemical composition of the sulfated polysaccharide is different from that of glycosaminoglycan from *S. japonicas* (Yoshida et al., 1992) and from *L. grisea* (Mourão et al., 1996). However, there has been no detailed data concerning the patterns and proportions of sulfate substitution for the fucose residue from the sea cucumber *T. ananas* currently available.

In the present study, the structure of the sulfated fucose branches of the fucosylated chondroitin sulfate from the sea cucumber *T. ananas* was investigated. The results, where the extent and position of sulfate substitution have been fully characterized by mild acid hydrolysis and NMR technology, are a valuable tool to trace the relationship between structures *versus* anticoagulant activity of this novel glycosaminoglycan. Furthermore, anticoagulant activities of the defucosylated sample (together with that of native fucosylated chondroitin sulfate), including activated partial thromboplastin time (APTT), inhibition of thrombin by heparin cofactor II, and factor X activation by the intrinsic tenase complex, were examined to investigate the effect of the branches on anticoagulant activities.

2. Experimental

2.1. Materials

Heparin (204 IU/mg), dermatan sulfate (DS) and thrombin (123 NIH U/mg) were purchased from Sigma (USA). Low molecular weight heparin (LMWH, 3500–5500 Da, 0.4 ml × 4000 ml AXaIU) were purchased from Sanofi-Aventis (France). Activated partial thromboplastin time (APTT) reagents were from Teco Medical (Germany). Human heparin cofactor II (HCII) and antithrombin were from Hyphen Biomed (France). Human coagulation factor VIII was from Green Cross China, Inc. (China). Chromogenic assay for measuring factor VIII: C in concentrates was purchased from Hyphen Biomed (France), containing reagent 1 (R1: human factor X and fibrin polymerization inhibitor), reagent 2 (R2: factor IXa with thrombin, phospholipids and calcium), reagent 3 (R3: factor Xa specific chromogenic substrate S-2765) and reagent 4 (R4: Tris–BSA buffer). Chromogenic substrate S-2238 (*H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline-dihydrochloride) was also from Hyphen Biomed (France). All other chemicals were of reagent grade and were obtained commercially.

2.2. Preparation of the fucosylated chondroitin sulfate from sea cucumber

The fucosylated chondroitin sulfate was extracted from the body wall of the sea cucumber *T. ananas*, collected from Sanya of Hainan province of China. The extraction, preparation and purification of the native fucosylated chondroitin sulfate were performed as previously described (Wu et al., 2010a,b). The yield of the fucosylated chondroitin sulfate was about 0.7% from the dried body wall of sea cucumber.

2.3. Mild acid hydrolysis of the fucosylated chondroitin sulfate from sea cucumber

Removal of sulfated fucose branches from the fucosylated chondroitin sulfate was performed by partial acid hydrolysis according to modification of the method described previously (Vieira & Mourão, 1988; Vieira et al., 1991). About 50 mg of the fucosylated chondroitin sulfate from sea cucumber *T. ananas* was subjected to partial acid hydrolysis in 10 ml of 100 mM H_2SO_4 at 100 °C for 30 min. A saturated solution of $Ba(OH)_2$ was added to give a pH of 7 and the $BaSO_4$ was removed by centrifugation (4000 × *g* for 20 min at 25 °C). The supernatant was purified by gel filtration with a Sephadex G-25. The separated fractions were assayed by Dubois, Gilles, Hamilton, Rebers, and Smith (1956) and carbazole (Kosakai & Yosizawa, 1979) reactions. Finally, the fractions were pooled, concentrated and lyophilized. The acid-resistant fragment (de-Fuc), structure of which is similar to that of chondroitin sulfate, and the sulfated fucoses were obtained.

2.4. NMR methods

NMR analyses were performed at 35 °C with a Bruker Avance 400 spectrometer of 400 MHz equipped with $^{13}C/^{1}H$ dual probe in the FT mode as described previously (Vilela-Silva, Castro, Valente, Biermann, & Mourão, 2002). 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. 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 HOD signal was presaturated by a presaturation sequence. COSY, TOCSY, and $^{1}H/^{13}C$ heteronuclear correlation (HMQC) spectra were recorded using states-time proportion phase incrementation for quadrature detection in the indirect dimension. TOCSY spectra were run with 4096 × 400 points with a spin-lock field of about 10 kHz and a mixing time of 80 ms. HMQC were run with 1024 × 256 points and globally optimized alternating phase rectangular pulses for decoupling. NOESY spectra were run with a mixing time of 100 ms. All chemical shifts are relative to internal trimethylsilyl-propionic acid (TSP).

2.5. Anticoagulant action of the fucosylated chondroitin sulfate measured by activated partial thromboplastin time (APTT)

APTT clotting assays were carried out as described previously (Wu et al., 2010a,b). The biological activities of native FuCS, de-Fuc, heparin, and low molecular weight heparin were obtained using the APTT Kit (Organon Technika, France) of either buffer Owen Koller (controls, 100 μl), heparin solution, low molecular weight heparin solution, or dFuCS-I dilution, 100 μl of platelet poor plasma (PPP) and 100 μl of APTT test reagent were incubated for 3 min at 37 °C. The clotting time was measured after the addition of 100 μl of 25 mM $CaCl_2$ solution.

2.6. Inhibition of thrombin by heparin cofactor II and factor X activation by the intrinsic tenase complex

Antithrombin activity in the presence of HCII was measured with chromogenic substrate S-2238 by a slight modification of the method described previously (Nagase et al., 1996). Factor X activation by the factor IXa–factor VIIIa–phospholipid complex was determined by a modification of the method described previously (Glauser, Pereira, Monteriro, & Mourão, 2008; Sheehan & Walke, 2006) according to the package leaflet of chromogenic assay for measuring factor VIII: C in plasma or in concentrates.

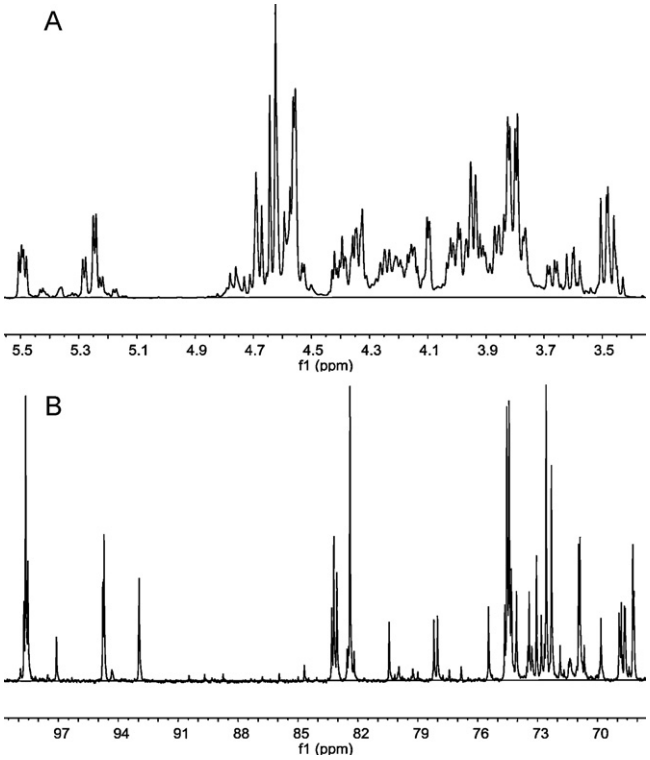

Fig. 1. ^{1}H (A) and ^{13}C (B) NMR spectrum of the sulfated fucose released by partial acid hydrolysis of the fucosylated chondroitin sulfate.

3. Results and discussion

3.1. NMR spectroscopy of sulfated fucose residues obtained by partial acid hydrolysis of the fucosylated chondroitin sulfate from sea cucumber

The ^{1}H and ^{13}C one-dimensional spectrum of the sulfated fucose residues obtained by partial acid hydrolysis of the fucosylated chondroitin sulfate from sea cucumber *T. ananas* were shown in Fig. 1A and B. The chemical shifts in Tables 1 and 2 were based on the interpretations of TOCSY, COSY and HMQC spectra (Fig. 2).

In the ^{1}H NMR spectrum (Fig. 1A), the signals between 5.2 and 5.6 ppm were the five α anomeric protons of sulfated fucose residues obtained by partial acid hydrolysis of the fucosylated chondroitin sulfate (Mourão et al., 1996), since their *J* values (*d* 3.6–4.6 Hz) were in agreement with that of α anomeric proton of fucose. Comparison of the chemical shifts for each of these fucose residues with shifts for standard unsulfated fucose (literature values in Table 1) showed strong downfield shifts of some signals consistent with sulfation at those positions, and less strong downfield shifts attributable to sulfation at the adjoining position. The four sulfated fucose systems correspond to those for 2,4-*O*-disulfated fucose (Fuc2S4S), 2-,3- and 4-*O*-monosulfated fucose (Fuc2S, Fuc3S and Fuc4S). But the chemical shifts for each β anomeric protons of these fucose residues were between 4.5 and 4.8 ppm, where the signals were overlapped with those for H-4 of Fuc2S4S and Fuc4S, so it was difficult to assign them in the ^{1}H NMR spectrum. The signals at 1.2–1.3 ppm can be readily assigned to the methyl protons of variously sulfated fucose residues (not shown).

The assignment can be corroborated by their COSY (Fig. 2A) and TOCSY spectra (Fig. 2B). The COSY spectrum showed the correlation signals between anomeric protons H-1/H-2 (1–2), H-2/H-3 (2–3), H-3/H-4 (3–4), H-5/H-6 (5–6) of α and β fucoses (I–V and I′–V′). The cross-peak of H-4 and H-5 was weaker, due to equatorial bond of H-4 from fucoses. But the signals at 4.1–4.4 ppm can be readily

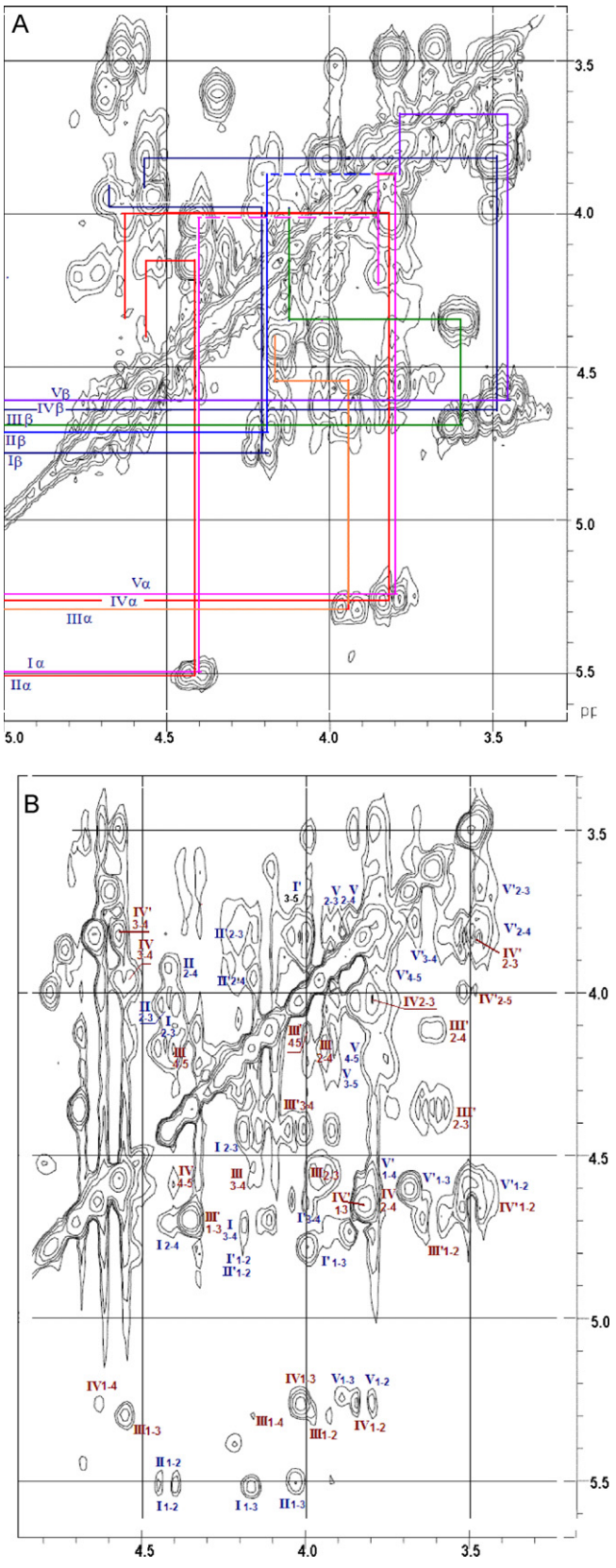

Fig. 2. Parts of the ^{1}H–^{1}H COSY (A) and ^{1}H–^{1}H TOCSY (B) spectra of the sulfated fucose released by partial acid hydrolysis of the fucosylated chondroitin sulfate.

Table 1
Proton chemical shifts of sulfated fucose residues obtained by partial acid hydrolysis of the fucosylated chondroitin sulfates from sea cucumber.

FucCS	Sugar	Anomeric form	Chemical shift (ppm)					
			H-1	H-2	H-3	H-4	H-5	H-6
This work[a]								
T. ananas	Fucose 2,4-*O*-disulfate (I)	α	5.50	4.40	4.15	4.57	4.35	1.26
	Fucose 2-*O*-sulfate (II)	α	5.49	4.39	4.02	3.87	4.23	1.23
	Fucose 3-*O*-sulfate (III)	α	5.24	3.94	4.52	4.17	4.25	1.26
	Fucose 4-*O*-sulfate (IV)	α	5.24	3.80	3.99	4.62	4.33	1.26
	Fucose non-sulfate (V)	α	5.22	3.79	3.91	3.86	4.23	1.20
	Fucose 2,4-*O*-disulfate (I′)	β	4.77	4.21	3.97	4.62	3.84	1.29
	Fucose 2-*O*-sulfate (II′)	β	4.70	4.19	3.87	3.84	3.85-3.40	1.29
	Fucose 3-*O*-sulfate (III′)	β	4.67	3.60	4.36	4.09	3.86-3.92	1.29
	Fucose 4-*O*-sulfate (IV′)	β	4.64	3.50	3.82	4.55	3.92-3.96	1.29
	Fucose non-sulfate (V′)	β	4.59	3.48	3.68	3.79	3.84	1.27
Literature values								
S. japonicus[b]	Fucose 2,4-*O*-disulfate	α	5.66	4.49	4.14	4.81	4.87	1.34
	Fucose 3,4-*O*-disulfate	α	5.31	3.96	4.53	4.94	4.73	1.37
	Fucose 4-*O*-sulfate	α	5.38	3.80	4.02	4.73	4.80	1.34
L. grisea[c,d,e]	Standard unsulfated fucose	α	5.20	3.76	3.86	3.81	4.20	1.21
	Fucose 4-*O*-sulfate (major)	α	5.22	3.81	3.97	4.61	4.31-4.33	1.26
	Fucose 2,4-*O*-disulfate	α	5.49	4.41	4.13	4.67	4.34-4.36	1.29
	Fucose 3,4-*O*-disulfate	α	5.28	3.94	4.60	4.89	NR[f]	NR
	Fucose 4-*O*-sulfate (minor)	α	5.26	3.92	4.13	4.54	NR	NR
	Standard unsulfated fucose	β	4.55	3.46	3.63	3.75	3.80	1.25
	Fucose 4-*O*-sulfate	β	4.60	3.48	3.75	4.54	3.95	1.31
	Fucose 2,4-*O*-disulfate	β	4.73	3.92	4.21	4.61	3.93	1.28
	Fucose 3,4-*O*-disulfate	β	4.68	3.60	4.37	4.82	3.92	1.28
	Fucose 3-*O*-sulfate	β	4.65	3.64	4.31	4.09	3.84	1.26

[a] ^{1}H spectra were recorded at 400 MHz, 45 °C in D_2O. Chemical shifts are referenced to internal TSP at 0 ppm in the fucosylated chondroitin sulfate sample.
[b] Data from Yoshida et al. (1992). Chemical shifts relative to external TMS; 70 °C in D_2O.
[c] Data of ^{1}H spectra from Vieira et al. (1991). Measured at 500 MHz in D_2O at 70 °C. Chemical shifts relative to external TSP.
[d] Data from Borsig et al. (2007).
[e] Data from Mourão et al. (1996). Measured at 500 MHz in D_2O at 60 °C. Chemical shifts relative to external TSP.
[f] NR, ^{1}H spectra were shown, but data in detail were not reported.

assigned to the H-5 of α sulfated fucoses, while 3.8–4.0 ppm to that of α sulfated fucoses, according to the correlation signals H-5/H-6. The TOCSY spectrum (Fig. 2B) extended the assignment to the remaining proton signals.

In ^{13}C NMR (Fig. 1B) and ^{1}H–^{13}C HSQC (not shown), the ^{13}C signals at 97–99 ppm were correlated with β anomeric protons, while the signals at 95–92 ppm with α anomeric protons, consistent with the distribution rule for the ^{13}C signal from monosaccharide. The signals between 68 and 84 ppm were the sulfate substituted C-4 from α- and β-Fuc2S4S, α- and β-Fuc2S, α- and β-Fuc3S, and α-and β-Fuc4S, respectively. The signals at 18.1–18.3 ppm and 18.6–18.9 ppm can be readily assigned to the methyl protons (C-6) of α- and β-variously fucose residues, respectively.

Table 2
Carbon chemical shifts of sulfated fucose residues obtained by partial acid hydrolysis of the fucosylated chondroitin sulfates from sea cucumber.

FucCS	Sugar	Anomeric form	Chemical shift (ppm)					
			C-1	C-2	C-3	C-4	C-5	C-6
This work[a]								
T. ananas	Fucose 2,4-*O*-disulfate (I)	α	92.96	78.12	68.86	82.39	68.17	18.15
	Fucose 2-*O*-sulfate (II)	α	92.96	74.01	70.87	73.48	68.86	18.29
	Fucose 3-*O*-sulfate (III)	α	94.78	72.31	80.43	68.91	68.23	18.26
	Fucose 4-*O*-sulfate (IV)	α	94.72	74.31	70.94	83.20	68.22	18.21
	Fucose non-sulfate (V)	α	94.72	74.05	74.64	73.05	68.87	18.29
	Fucose 2,4-*O*-disulfate (I′)	β	97.10	82.47	72.31	83.31	72.57	18.70
	Fucose 2-*O*-sulfate (II′)	β	97.10	82.19	74.64	74.05	72.81	18.82
	Fucose 3-*O*-sulfate (III′)	β	98.53	71.87	83.05	69.83	72.65	18.75
	Fucose 4-*O*-sulfate (IV′)	β	98.65	74.43	74.64	82.39	72.57	18.66
	Fucose non-sulfate (V′)	β	98.73	74.43	75.46	74.64	72.81	18.82
Literature values								
S. japonicus[b]	Fucose 2,4-*O*-disulfate	α	97.5	76.1	67.5	82.1	67.0	16.6
	Fucose 3,4-*O*-disulfate	α	100.1	67.4	76.4	80.2	67.4	17.0
	Fucose 4-*O*-sulfate	α	ND[c]	ND	ND	ND	ND	ND
L. grisea[d,e]		NR[f]	NR	NR	NR	NR	NR	

[a] ^{13}C spectra were recorded at 100.6 MHz, 45 °C in D_2O. Chemical shifts relative to internal TSP in the fucosylated chondroitin sulfate sample.
[b] Yoshida et al. (1992). Chemical shifts relative to external TMS; 70 °C in D_2O.
[c] ND, not determined.
[d] Vieira et al. (1991). Measured at 500 MHz in D_2O at 70 °C. Chemical shifts relative to external TSP.
[e] Mourão et al. (1996). Measured at 500 MHz in D_2O at 60 °C. Chemical shifts relative to external TSP.
[f] NR, ^{13}C spectra were shown, but data in detail were not reported.

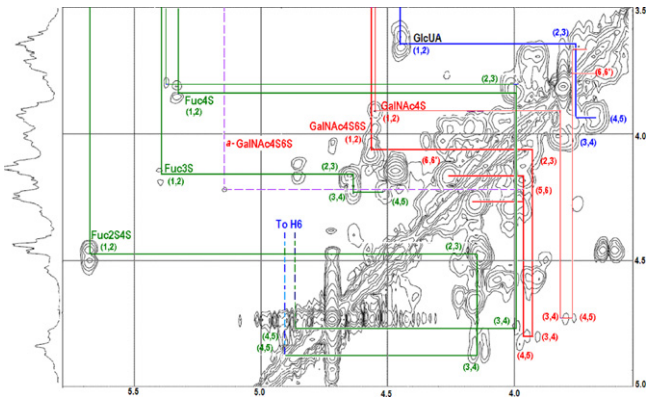

Fig. 3. Part of the COSY spectrum of the fucosylated chondroitin sulfate from the sea cucumber *T. ananas*, at 400 MHz, 45 °C, in D_2O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. Signals designated by "Fuc2S4S", "Fuc3S" and "Fuc4S" refer to those produced by 2,4-di-sulfated-fucose, 3-sulfated-fucose and 4-sulfated-fucose residues, respectively, whereas those of 4,6-di-sulfated-acetyl-galactosamine and 4-sulfated-acetyl-galactosamine residues are labeled "GalNAc4S6S" and "GalNAc4S", respectively.

Overall, NMR analysis of sulfated fucose residues obtained by partial acid hydrolysis of the fucosylated chondroitin sulfate from sea cucumber indicated that there could be five fucose residues in the fucose branches of the fucosylated chondroitin sulfate.

However, in our previous study (Wu et al., 2010a,b), we found that there are mostly three fucose residues in the fucose branches of the fucosylated chondroitin sulfate by ^{1}H NMR technology. In order to clearly illuminate it, COSY spectrum of the fucosylated chondroitin sulfate from *T. ananas* was shown in Fig. 3. The signals at 5.64, 5.36, and 5.28 ppm can be readily assigned to the H-1 from α-Fuc2S4S, α-Fuc3S and α-Fuc4S according to the correlation signals H-1/H-2, respectively. Thus, approximate integration of the three anomeric signals gives the proportions of these three as 53, 25, and 22% of the sample, respectively (Table 3). Comparison with patterns and proportions of sulfate substitution of the fucose residues by partial acid hydrolysis of the fucosylated chondroitin sulfate suggested that the mild acid hydrolysis can result in the partial desulfation of sulfated fucose residues.

Comparison with other published results on the patterns and proportions of sulfate substitution of the fucose branches of the FuCSs from sea cucumber indicated that the structures of the branches vary from species to species (Table 3). Further work could help to understand if there is a close relationship between the geographical area of the habitat of sea cucumber and the sulfation patterns of the fucose branches (Chen et al., 2011), since these FuCSs from different sea cucumbers were obtained from various geographical zones. However, it is difficult to elucidate this, due to other various factors such as harvest season and the dimensions of the samples.

3.2. Effect of sulfated fucose branches of fucosylated chondroitin sulfate on the anticoagulant action

Anticoagulant activity of the partially defucosylated sample (de-Fuc) obtained by partial acid hydrolysis of the fucosylated chondroitin sulfate from the sea cucumber *T. ananas* were assessed by measuring the activated partial thromboplastin time (APTT), and compared with the same activities of native polysaccharide, unfractionated heparin, low molecular weight heparin (LMWH) and dermatan sulfate (DS) from mammalian. The APTT assays (summarized in Table 4) indicated that native polysaccharide have a high degree of anticoagulant activity (a in Table 4), comparable with that of unfractionated heparin, LMWH and DS. Partially defucosylation of the FuCS resulted in significantly decreased anticoagulant potency (b in Table 4), which suggested that sulfated fucose branches are responsible for the high anticoagulant activity of the fucosylated chondroitin sulfate. The result was high consistent with that from a previous study on the other fucosylated chondroitin sulfate from the sea cucumber *L. grisea* (Mourão et al., 1996).

Comparison with the fucosylated chondroitin sulfate from *L. grisea* in the literature (Mourão et al., 1996) showed that the sulfated polysaccharide from *T. ananas* has a higher significant anticoagulant action (a in Table 4). Their sulfation patterns may result in the difference in anticoagulant activities of the FuCSs. Comparison with the FuCSs from six sea cucumbers in the literature (Chen et al., 2011; Mourão et al., 1996; Yoshida et al., 1992; also see Table 3) indicated that this potent effect is possibly related to the occurrence of 2,4-di-*O*-sulfated fucose units.

3.3. Effect of sulfated fucose branches of fucosylated chondroitin sulfate on thrombin activity by heparin cofactor II

Thrombin inhibitions by heparin cofactor II (anti-IIa activity) of native and defucosylated polysaccharides were examined to assess the effect of sulfated fucose branches of the polysaccharide on thrombin activity, in comparison with unfractionated heparin, LMWH and DS. Increasing concentrations of the FuCS resulted in essentially complete inhibition of thrombin activation by heparin cofactor II (Fig. 4A), corresponding with a noncompetitive inhibition pattern (Sheehan & Walke, 2006). Like DS, in the presence of heparin cofactor II, the native glycosaminoglycan showed strong inhibition of thrombin (EC_{50} = 0.289 μg/ml, ~4.4 nM) as previous studies on that of the FuCSs from the sea cucumber *S. japonicas* (Nagase et al., 1995) and from *L. grisea* (Mourão et al., 1996). However, the defucosylated polysaccharide had no inhibitory effect on thrombin by heparin cofactor II. These results and APTT assays (Table 4) indicated the requirement of sulfated fucose branches for the anticoagulant activity of the fucosylated chondroitin sulfate as a previous study (Mourão et al., 1996).

3.4. Effect of sulfated fucose branches of fucosylated chondroitin sulfate on generation of factor Xa by the intrinsic tenase complex

The fucosylated chondroitin sulfate from sea cucumber has multiple potential mechanisms of action, including acceleration of thrombin inhibition by HCII, inhibition of factor VIII activation by thrombin, and inhibition of factor X activation by the intrinsic tenase complex (Nagase et al., 1995, 1996, 1997; Sheehan & Walke, 2006).

In order to directly assess the role of sulfated fucose branches in the anticoagulant activity of the fucosylated chondroitin sulfate, the effect of native FuCS and de-Fuc on factor X activation by the intrinsic tenase complex (anti-f.Xase activity) was examined, and the result was shown in Fig. 4B. Fitting the data to a noncompetitive inhibition model yielded EC_{50} values for the glycosaminoglycans according to Sheehan and Walke (2006), which were listed in Table 4. The EC_{50} for the native fucosylated chondroitin sulfate inhibition of factor X activation by the intrinsic tenase complex are ~10 times greater when compared with EC_{50} for heparin inhibition on a molar basis, respectively (0.84 nM for FuCS *vs* 7.8 nM for Heparin, in Table 4). However, the EC_{50} for the defucosylated polysaccharide (de-Fuc) is ~6620-fold higher than the native glycosaminoglycan on a molar basis (0.84 nM for FuCS *vs* 5561 nM for de-Fuc, Table 4), which indicated that the sulfated fucose branches are essentially essential for the anticoagulant action of FuCS and thus these branches could constitute the structural requirement for the binding of the glycosaminoglycan to the intrinsic tenase complex.

Table 3
Sulfation patterns and proportions of the sulfated fucose residues of the fucosylated chondroitin sulfates from six sea cucumbers.

Species	Fuc0S	Fuc3S	Fuc4S	Fuc2S4S	Fuc3S4S	References
Thelenata ananas	0	~25	~22	~53	0	This work
Stichopus japonicas	0	–[a]	11.1	55.6	33.3	Yoshida et al. (1992)
Ludwigothurea grisea	0	–	~49	~20	~17	Mourão et al. (1996)
Pearsonothuria graeffei	–	–	81.6	18.4	–	Chen et al. (2011)
Holothuria vagabuda	25.6	–	50.2	15.8	8.4	Chen et al. (2011)
Stichopus tremulus	–	–	24.8	22.4	52.8	Chen et al. (2011)
Isostichopus badionotus	–	–	4.1	95.9	–	Chen et al. (2011)

[a]Not detected.

Table 4
Anticoagulant properties of different sulfated polysaccharides.

Samples	APTT[a]		Chemical modification	EC_{50} (Anti-f.Xase)		EC_{50} (Anti-IIa)	
	μg/ml	IU/mg		μg/ml	nM	μg/ml	nM
(a) FuCS	1.36	348	Native	0.055	0.84	0.289	4.4
(b) de-Fuc	>450	<1	Partially defucosylated	25.5	5561	×[b]	×
(c) LMWH	6.90	69	Depolymerized	1.84	409	1.404	312
(d) Heparin	2.32	204	Unfractionated	0.141	7.8	0.448	24.9
(e) DS	47.7	10	Native	×	×	0.464	11.2

[a]The activity of fucosylated chondroitin sulfates to prolong APTT is expressed by the concentration of each agent that is required to double the APTT (doubling APTT, μg/ml), and also is expressed as NIH units/mg (IU/mg) using a parallel standard curve based on the International Heparin Standard 204 units/mg from Sigma (USA).
[b]No activity.

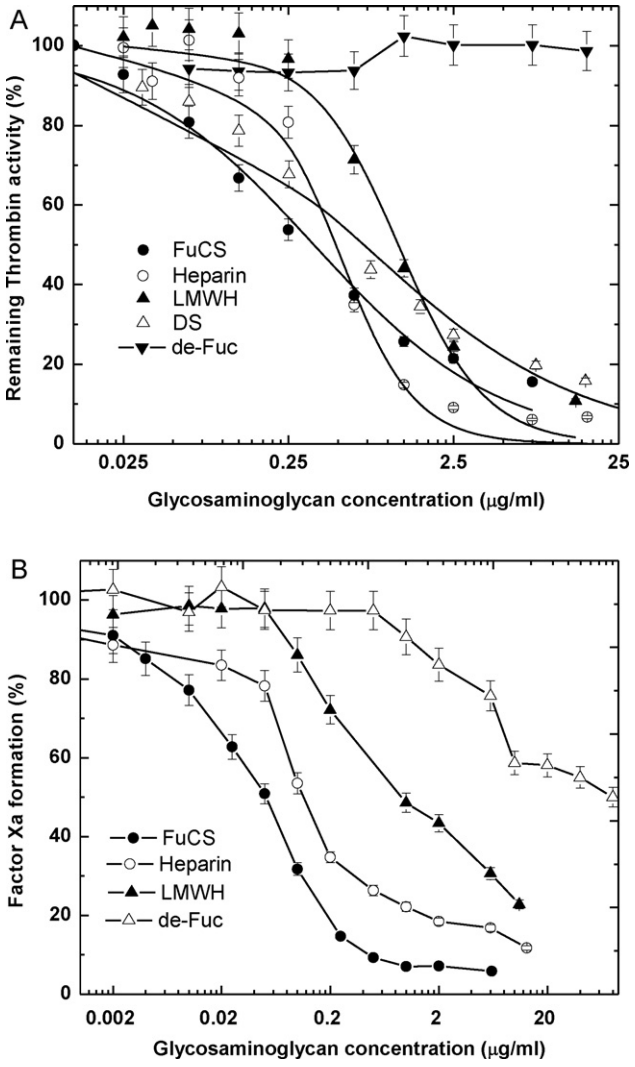

Fig. 4. Effect of sulfated fucose branches of the fucosylated chondroitin sulfate on thrombin activity by heparin cofactor II (A) and generation of factor Xa by the intrinsic tenase complex (B).

4. Conclusion

In summary, the structure of the sulfated fucose branches of the fucosylated chondroitin sulfate from the sea cucumber *T. ananas* have been fully characterized by mild acid hydrolysis and NMR technology, comparison with the native sulfated polysaccharide. The results showed that the fucose residues by the mild acid hydrolysis are significantly different from those of the native polysaccharide, which the mild acid hydrolysis can result in the partial desulfation of sulfated fucose residues.

Comparison between native and partially defucosylated polysaccharides suggested that the sulfated fucose branches are essential for the anticoagulant action of FuCS, and this potent effect is possibly related to the occurrence of 2,4-di-*O*-sulfated fucose units (Chen et al., 2011). Furthermore, these branches could constitute the structural requirement for the binding of the glycosaminoglycan to coagulant enzymes, such as thrombin by HCII, and factor X by the intrinsic tenase complex.

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