Carbohydrate Polymers 83 (2011) 688–696

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Comparison of structures and anticoagulant activities of fucosylated chondroitin sulfates from different sea cucumbers

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ARTICLE INFO

Article history:
Received 16 March 2010
Received in revised form 4 August 2010
Accepted 17 August 2010
Available online 24 August 2010

Keywords:
Sea cucumber
Fucosylated chondroitin sulfate
Chondroitin sulfate
NMR
Anticoagulant activity

ABSTRACT

Fucosylated chondroitin sulfates (fCSs) were isolated from four sea cucumbers *Pearsonothuria graeffei* (Indo-Pacific), *Stichopus tremulus* (Western Indian Ocean), *Holothuria vagabunda* (Norwegian coast), and *Isostichopus badionotus* (Western Atlantic). The detailed sequences of fCSs, particularly their fucose branches, were characterized and compared. ^{1}H and ^{13}C NMR of the polysaccharides clearly identified three different sulfation patterns on the branched fucoses, 4-*O*-mono-, 2,3-*O*-di- and 2,4-*O*-di-sulfation, variously distributed in the four fCSs. The chondroitin sulfate backbones were established based on the monosaccharide composition of the polysaccharides and two-dimensional NMR of their oligosaccharide fragments. Anticoagulant activities of the four fCSs were assessed and compared. The results indicated that the difference in their anticoagulant activities can be attributed to the difference in sulfation pattern of the fucose branch of the chondroitin sulfate, and 2,4-*O*-disulfation is important for anticoagulant activity.

1. Introduction

Sea cucumbers have been used as a traditional tonic food in China and other Asian countries for thousands of years (Fu et al., 2005; Kalinin, 2000; Zhou et al., 2006). In the oceans, there are hundreds of varieties of sea cucumbers. Although the commercial value of species grown in different areas under different conditions can range from a few to thousands of US dollars per kilogram, no detailed comparison has been made of their chemical components and nutritional value.

The major edible parts of sea cucumbers are the body walls, which contain mainly collagen and acidic polysaccharides (Mourao et al., 1996; Trotter, Lyons-Levy, Thurmond, & Koob, 1995; Vieira & Mourao, 1988; Vieira, Mulloy, & Mourao, 1991), together with some other minor components such as triterpene glycosides, gangliosides and various lipids (Avilov et al., 2000; Kitagawa, Kobayashi, & Kyogoku, 1982; Matsuno & Tsushima, 1995; Zou et al., 2003). The acidic polysaccharides are the most important components in sea cucumbers because of their various biological activities. It has been reported that polysaccharides isolated from sea cucumbers have anticoagulant and antithrombotic activities (Fonseca & Mourao, 2006; Mourao, Giumaraes, Mulloy, Thomas, & Gray, 1998; Mourao et al., 2001; Nagase et al., 1995, 1996), and they can also modulate angiogenesis (Tapon-Bretaudiere et al., 2002) and inhibit tumor metastasis (Borsig et al., 2007). Two types of polysaccharides have been isolated from sea cucumbers: fucosylated chondroitin sulfate (fCS) and fucan (Kariya, Watabe, Hashimoto, & Yoshida, 1990; Kariya, Watabe, Kyogashima, Ishihara, & Ishii, 1997; Mourao et al., 1996; Ribeiro, Vieira, Mourao, & Mulloy, 1994; Vieira et al., 1991). There have been several studies on the structures of the fCS isolated from two sea cucumbers, *Stichopus japonicus* (Kariya et al., 1990, 1997) and *Ludwigothurea grisea* (Mourao et al., 1996; Vieira et al., 1991), using mild acid hydrolysis, methylation analysis, and ^{1}H and ^{13}C NMR spectroscopy. It was found that chondroitin sulfate from these sea cucumbers uniquely had a sulfated fucose branch at the 3-*O*-position of the glucuronic acid (GlcA) residue while the sulfation patterns of the fucose branches of fCSs were different.

Sulfated polysaccharides have attracted considerable interest in recently years due to their potential therapeutic application.

Abbreviations: fCS, fucosylated chondroitin sulfate; *Pg*, *Pearsonothuria graeffei*; *Hv*, *Holothuria vagabunda*; *St*, *Stichopus tremulus*; *Ib*, *Isostichopus badionotus*; CSE, chondroitin sulfate E; TFA, trifluoroacetic acid; PMP, 1-phenyl-3-methyl-5-pyrazolone; Fuc, fucose; Ara, arabinose; Man, mannose; Gal, galactose; GalN, galactosamine; GlcN, glucosamine; GlcA, glucuronic acid; GalA, galacturonic acid; GalNAc, N-acetylgalactosamine; APTT, activated partial thromboplastin time; TT, thrombin time; PT, prothrombin time; Fuc4S, 4-*O*-sulfated fucose; Fuc2,4S, 2,4-*O*-disulfated fucose; Fuc3,4S, 3,4-*O*-disulfated fucose.

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doi:10.1016/j.carbpol.2010.08.040

An example of this is the glycosaminoglycan heparin and its low molecular weight fragments which has been used as anticoagulants for prophylaxis and treatment of thrombosis (Mulloy & Johnson, 1987). Dermatan sulfate (Tollefsen, Majerus, & Blank, 1982; Tollefsen, Pestka, & Monafo, 1983) and sulfated fucans (Colliec et al., 1991; Mauray et al., 1995; Millet et al., 1999) from brown algae also exhibit anticoagulant properties although are of lower potencies than heparin. Recently, there have been several reports on the anticoagulant activities of sea cucumber fCSs and their chemically modified analogues (Fonseca, Santos, & Mouro, 2009; Mourao et al., 2001; Nagase et al., 1995, 1996). Their anticoagulant activities have been demonstrated at various stages of coagulation reactions by inhibiting the initial reaction of the extrinsic pathway, and by inhibiting tenase and thrombin (HC II-dependent) (Fonseca et al., 2009; Minamiguchi et al., 2003; Mourao et al., 2001; Nagase et al., 1995, 1996). The sulfated fucose branches were considered as the key factor for their anticoagulant activities, as partial release or desulfation of the fucose branches greatly reduced the effect on prolonged clotting time (Mourao et al., 1998, 2001). However, there has been no detailed investigation on the effect of different sulfation patterns of fucose on anticoagulant activities.

Structural characterization of these complex carbohydrates has become possible with the development of modern spectroscopic techniques, of which the most powerful is NMR spectroscopy. Both ^{1}H and ^{13}C NMR spectroscopies have been used for sequence analyses of heparin oligosaccharides and characterization of subtle structural differences between different preparations of heparin (Casu et al., 1996; Colliec et al., 1991). Recently NMR techniques have been used for direct analysis of heparin polysaccharide materials to identify other sulfated sugar contaminants associated with adverse clinical events (Guerrini, Beccati, & Shriver, 2008).

In the present study, we isolated fCSs from four sea cucumbers with different commercial values from seawaters of three different geographical zones: *Pearsonothuria graeffei* from Indo-Pacific, *Holothuria vagabunda* from Western Indian Ocean, *Stichopus tremulus* from Norwegian coast, and *Isostichopus badionotus* from Western Atlantic. The fCS components of these four sea cucumbers have not been investigated previously. Here we examine and compare their structures and anticoagulant activities. Previously, detailed structural study of other fCSs was based on oligosaccharide fragments obtained from partial depolymerization of fCS by acid hydrolysis (Kariya, Sakai, Kaneko, Suzuki, & Kyogashima, 2002; Mourao et al., 1996; Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992). However, as sulfated fucose is highly sensitive to acid treatment, partial loss of sulfate and fucose are inevitable even under very mild conditions (Kariya et al., 2002; Mourao et al., 1996; Yoshida et al., 1992), and therefore, it has been difficult to characterise the fine detail of the fCS fucose branches. Our comparison of the fucose branches is based on polysaccharides with NMR spectroscopy as the primary analytical method. We attribute the difference in anticoaulant activities to the subtle differences in sulfate content and sulfation pattern of the fucose branch.

2. Materials and methods

2.1. Materials

Sea cucumber *P. graeffei* (from Indo-Pacific), *S. tremulus* (from Western Indian Ocean), *H. vagabunda* (from Norwegian coast) and *I. badionotus* (from Western Atlantic Ocean) were purchased from a local market in Qingdao (China). TSK-G4000 and -G3000 PWXL columns were from TOSOH Biosep (Tokyo, Japan) and DEAE–cellulose anion-exchange resin from Whatman (Brentford, England). Papain and cystein were purchased from Fluka (Seelze, Germany). The carbohydrate standards D-mannose, L-fucose, L-arabinose, D-galactose, D-galactosamine, D-glucosamine, D-glucuronic acid, D-galacturonic acid, lactose, and chondroitin sulfate A (bovine trachea) were from Sigma (St. Louis, MO, USA). The derivatization reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) was from Sinopharm Chemical Reagent (Shanghai, China).

2.2. Isolation and purification of sea cucumber chondroitin sulfates

Preparation of crude sea cucumber polysaccharide was based on the method reported previously (Zou et al., 2003). Briefly, sea cucumber body wall (ca. 100 g) was dried, minced and homogenized. The homogenate was treated with $CHCl_3$/MeOH (4:1, v/v) to remove lipids before autoclaving at 50 °C for 4 h. The resulting residue was digested with papain (in 5 mM EDTA and 5 mM cysteine) at 60 °C for 10 h. The digested mixture was centrifuged (2000 × *g* for 15 min at 10 °C) and the polysaccharide in the clear supernatant was precipitated with 1.6 mL of 10% aqueous cetylpyridinium chloride solution. After standing at room temperature for 24 h, the mixture was centrifuged (2000 × *g* for 15 min) and the precipitated polysaccharide was collected and re-dissolved in 1000 mL of a 2 M NaCl:ethanol (100:15, v/v) solution before further precipitation with 2000 mL of 95% ethanol. After standing at 4 °C for 24 h, the precipitate formed was collected after centrifugation (2000 × *g* for 15 min). The precipitate was dissolved in water and dialyzed against distilled water (with two changes). The polysaccharide solution was lyophilized before analysis.

The crude polysaccharide (~1 g) was fractionated by anion-exchange chromatography on a DEAE–cellulose column (2.6 cm × 40 cm) with elution by a linear gradient of NaCl, 0–1.2 M NaCl (in 0.1 M sodium acetate, pH 5.0) in 1000 min at a flow rate of 1.0 mL/min. Carbohydrate fractions were detected by phenol/sulfuric assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and further purified by HPLC with a TSK-G4000 column.

The purity of the polysaccharide fractions was confirmed by cellulose–acetate membrane electrophoresis which was carried out in 0.1 M HCl at 3 mA for 30 min as described (Wessler, 1971). Membrane was stained with 0.2% Alcian blue in 0.1% acetic acid solution. The molecular weights of the polysaccharides were determined by liquid chromatography on an Agilent 1100 system (Palo Alto, CA, USA) with a TSK-G4000 PWXL column by elution with 0.2 M NaCl and with detection by refractive index.

2.3. Determination of chemical compositions

Monosaccharide composition was determination by HPLC as described (Strydom, 1994). In brief, sea cucumber polysaccharides (typically 1 mg) were hydrolyzed with 2 M TFA at 110 °C for 4 h. The hydrolysate was dried under vacuum and conjugated to PMP. The derivatization was carried out with 450 μL PMP solution (0.5 M, in methanol) and 450 μL of 0.3 M NaOH at 70 °C for 30 min. The reaction was stopped by neutralization with 450 μL of 0.3 M HCl and extraction with chloroform (1 mL 3×). HPLC analyses were performed on an Agilent ZORBAX Eclipse XDB-C18 column (5 μm, 4.6 mm × 150 mm) at 25 °C with detection at UV 250 nm. The mobile phase was aqueous 0.05 M KH_2PO_4 (pH 6.9) with 15% (solvent A) and 40% acetonitrile (solvent B), respectively. A gradient of B from 8% to 19% in 25 min was used.

Sulfate content was determined by ion chromatography as described (Ohira & Toda, 2006). Briefly, ~1 mg of sea cucumber polysaccharide was hydrolyzed with 2 M TFA at 110 °C under nitrogen for 8 h. The hydrolysate was dried under vacuum before dissolved in water prior to ion chromatography.

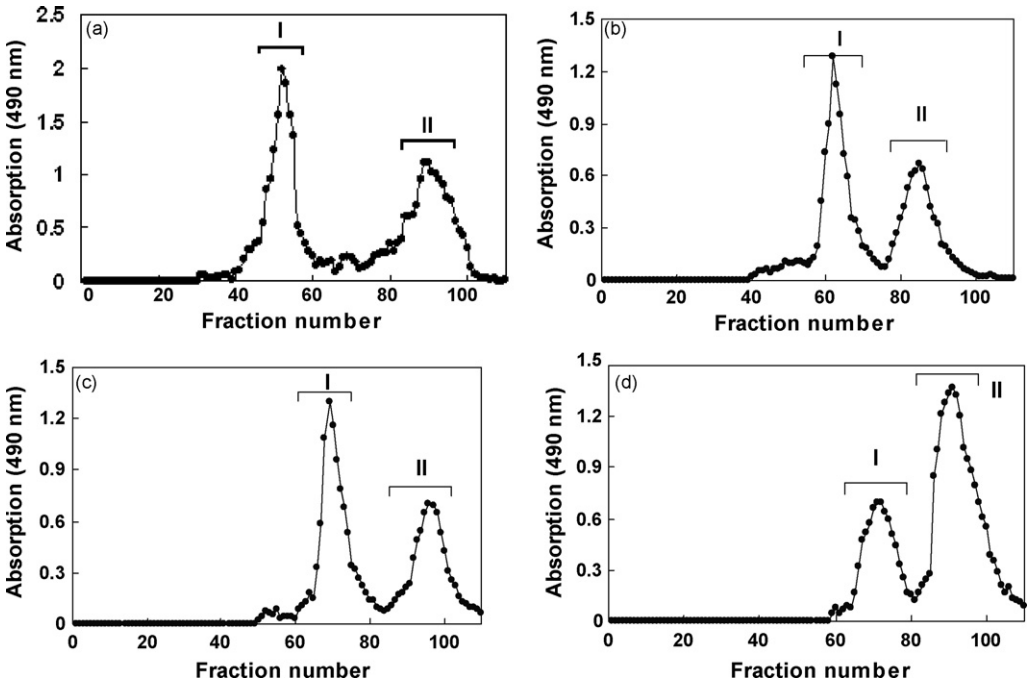

Fig. 1. Isolation of fucosylated chondroitin sulfates from polysaccharides extracted from sea cucumbers by anion-exchange chromatography: (a) *P. graeffei*, (b) *H. vagabunda*, (c) *S. tremulus*, and (d) *I. badionotus*.

2.4. Periodate oxidation

Periodate oxidation was carried out with 0.05 M aqueous $NaIO_4$ solution at 4 °C for 4 days. The reaction was stopped by dialysis (MWCO 10K) against distilled water (2×), and the retained solution was lyophilized. The lyophilized material was hydrolyzed and monosaccharides were determined by the HPLC method described above.

2.5. Preparation of oligosaccharide fragments by peroxide oxidative degradation

The polysaccharide fCS from *I. badionotus* was partially depolymerized by peroxide oxidation essentially as described (Petit et al., 2006). In brief, polysaccharide (2 g) and copper(II) acetate monohydrate (160 mg) were dissolved in 50 mL of distilled water and aqueous H_2O_2 solution was then added at a speed of 12 mL/h during a period of 4 h. The pH of the solution was maintained at 7.5 by addition of 2 M NaOH solution. The reaction was stopped by cooling down to room temperature and removal of the Cu^{2+} ions by Chelex 100 resins. The reaction mixture was desalted on a short column of Sephadex G-10 (1.6 cm × 36 cm), and fractionated on a Bio-Gel P4 column (2.6 cm × 120 cm) with elution by 0.2 M NH_4HCO_3. The fraction with a molecular mass of ~3,000 Da was pooled for NMR analysis after removal of the volatile buffer by repeated co-evaporation with water by lyophilization.

2.6. NMR and IR spectroscopy

For NMR analysis, polysaccharides (50 mg) and oligosaccharide fragments (30 mg) were co-evaporated with 500 μL D_2O (99.8%) twice by lyophilization before final dissolution in 500 μL high quality D_2O (99.96%) containing 0.1 μL acetone. ^1H NMR experiments were carried out at 600 MHz and ^{13}C NMR at 150 MHz. Spectra were recorded at 25 °C for ^{13}C NMR and 60 °C for ^1H NMR. The observed ^1H and ^{13}C chemical shifts were reported relative to internal acetone standard (2.03 and 33.1 ppm, respectively). Two-dimensional COSY and TOCSY spectra of fCSs were recorded at 20 °C.

IR spectra of the polysaccharides (in KBr pellets, 0.5 mg sample/150 mg KBr mg) were taken on a Perkin-Elmer instrument.

2.7. Anticoagulant assay.

The anticoagulant assays, including activated partial thromboplastin time (APTT) (assay kit from Organon-Tecknica, Fresnes, France), thrombin time (TT) (5 NIH U/mL human thrombin, from Diagnostica Stago, AsnieAres, France) and prothrombin time (PT) with thromboplastin (from Diagnostica Stago, AsnieAres, France), were performed according to the manufacturer's specifications as described (Mauray et al., 1995). The healthy human blood was donated from a local young man aged 24 years. The results were expressed as international units/mg using a parallel standard curve based on the International Heparin Standard (150 units/mg).

3. Results and discussion

3.1. Isolation and purification

The yields of polysaccharides isolated from the four sea cucumbers, *P. graeffei* (*Pg*), *H. vagabunda* (*Hv*), *S. tremulus* (*St*), and *I. badionotus* (*Ib*), were 11.0%, 6.3%, 7.0%, and 9.9% by weight, respectively. Ion exchange chromatography of the extracted polysaccharides on a DEAE–cellulose column showed two peaks for each of the four sea cucumbers (Fig. 1), which were identified as fCSs (fractions I) and fucans (fractions II), based on their chemical compositions (Table 1, and see below for discussion). The contents of the two polysaccharides were different: fCS was more abundant in *S. tremulus*, *P. graeffei* and *H. vagabunda* (Fig. 1a–c); whereas fucan was more abundant in *I. badionotus* (Fig. 1d). The retention times of the four fCSs in anion-exchange chromatography were also different, with an order of fCS-*Pg* < fCS-*Hv* < fCS-*Ib* ~ fCS-*St* (Fig. 1), indicating difference in sulfate content.

The purity of the four fCSs was confirmed by cellulose–acetate membrane electrophoresis as each polysaccharide gave a single band in the electrophoretigram (Fig. 2). However, the mobilities of the four fCSs were again different, with an order of fCS-

Table 1
Composition analysis of polysaccharide fractions isolated from sea cucumbers.

Sea cucumbers	Fractions	MW (kDa)	Molar ratio				
			GlcA	GalNAc	Fuc	Gal	Sulfate
P. graeffei	I	73	1.0	0.8	1.5	–	2.6
	II	320	–	–	1.0	–	0.8
H. vagabunda	I	81	1.0	1.1	0.9	–	2.7
	II	340	–	–	1.0	0.1	0.7
S. tremulus	I	100	1.0	0.8	1.2	–	3.0
	II	380	–	–	1.0	–	0.8
I. badionotus	I	109	1.0	0.7	0.9	–	3.1
	II	460	–	–	1.0	–	0.8

"–" not detected (values below 0.01); molar ratio is expressed as relative to GlcA for fraction I and to Fuc for fraction II.

Pg < fCS-*Hv* < fCS-*St* < fCS-*Ib*, although they all migrated faster than chondroitin sulfate A. The averaged molecular weights of the four fCSs, based on gel filtration chromatography on a high performance column, were 73, 81, 100 and 109 kDa for fCS-*Pg*, fCS-*Hv*, fCS-*St* and fCS-*Ib*, respectively.

3.2. Chemical compositions

Monosaccharide composition analysis of the two polysaccharide fractions (fractions I and II, Fig. 1) was carried out by HPLC following acid hydrolysis and derivatization with PMP. Sulfate content was determined by ion chromatography. Three monosaccharides, glucuronic acid (GlcA), *N*-acetylgalactosamine (GalNAc) and fucose (Fuc), were found to be the main components in fractions I whereas fucose was the only monosaccharide identified in fractions II (Table 1). Fractions I were assigned to be the chondroitin sulfate as GlcA and GalNAc were in approximately 1:1 ratio, consistent with the polysaccharide backbone structure, –4GlcAβ1–3GalNAcβ1–. The fucose and sulfate contents in fCSs obtained from different sea cucumbers varied slightly. As shown in Table 1, Fuc is most abundant in fCS-*Pg*, whereas sulfate is most abundant in fCS-*Ib*. Apart from fCS-*Pg*, all other fCSs contain approximately equimolar GlcA, GalNAc and Fuc. As fucose branch is present in every disaccharide unit, indicated by its resistance to chondroitinase digestion (data not shown), a single fucose as the branch at GlcA can be proposed which is similar to the fCS obtained from *S. japonicus* (Yoshida et al., 1992). In fCS-*Pg*, approximately 1.5 moles of fucose per disaccharide unit were found, suggesting either one or two Fuc as the branches, similar to fCS isolated from *L. grisea* (Mourao et al., 1996).

In the IR spectra of the fCSs, the absorptions at 820–860 cm^{-1} (ν_{C-O-S}) and 1240–1260 cm^{-1} ($\nu_{S=O}$) confirmed the presence of sulfates in these polysaccharides (Fig. 3). fCS-*Pg* and fCS-*Hv* showed strong absorption at around 850 cm^{-1} (Fig. 3a and b), indicating the presence of 4-*O*-sulfated Fuc and/or GalNAc according to previous reports on sea weed fucoidans (Bernardi & Springer, 1962; Duarte, Cardoso, Noseda, & Cerezo, 2001). fCS-*St* and fCS-*Ib* exhibited strong absorption at 827 cm^{-1}, indicating the presence of 2,4-*O*-sulfated fucose or 6-*O*-sulfated GalNAc (Bernardi & Springer, 1962; Duarte et al., 2001).

Periodate oxidation of the four fCSs did not show any major changes in molecular masses and monosaccharide compositions (data not shown), suggesting that there was no vicinol diol present in the three monosaccharide residues, GalNAc, GlcA and Fuc, due to substitutions of the hydroxyl groups by either a glycosidic bond or a sulfate.

3.3. ^1H NMR

^1H NMR spectra (Fig. 4 and Table 2) of the four fCSs were acquired and used for comparison of differences in their fine structures. The signals at 1.21 and 1.89 ppm can be readily assigned to the methyl protons of Fuc (CH_3) and GalNAc (CH_3CO), respectively. The signals between 5.1 and 5.6 ppm were the anomeric protons of variously sulfated fucose residues (Mourao et al., 1996; Yoshida et al., 1992) and apparent difference was observed in this region. fCS-*Pg* showed a major signal at 5.17 ppm and a minor one at 5.23 ppm (Fig. 4a), which were from the 4-*O*-sulfated fucose (Fuc4S), similar to the signals reported on fCS isolated from sea cucumber *L. grisea*

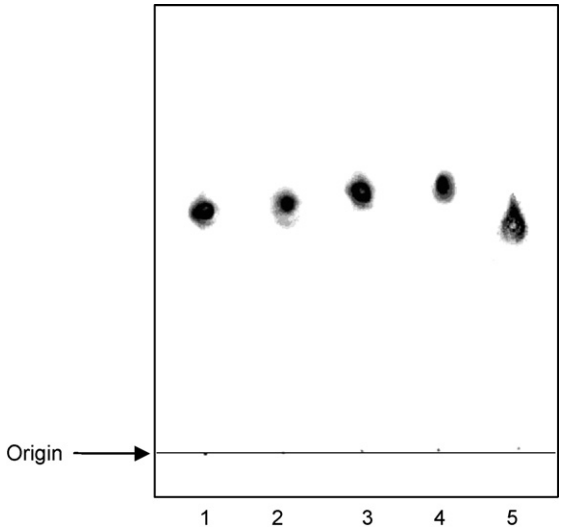

Fig. 2. The cellulose–acetate membrane electrophoresis of purified fCSs—lane 1: fCS-*Pg*; lane 2: fCS-*Hv*; lane 3: fCS-*St*; lane 4: fCS-*Ib*; lane 5: chondroitin sulfate A.

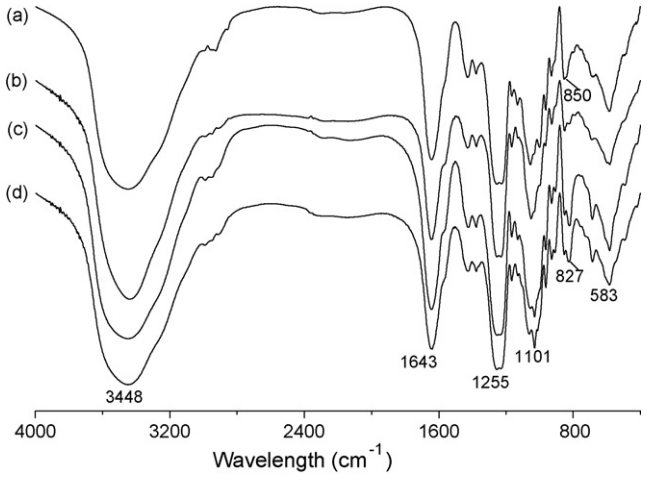

Fig. 3. IR spectra of fucosylated chondroitin sulfates isolated from sea cucumbers: (a) fCS-*Pg*, (b) fCS-*Hv*, (c) fCS-*St*, and (d) fCS-*Ib*.

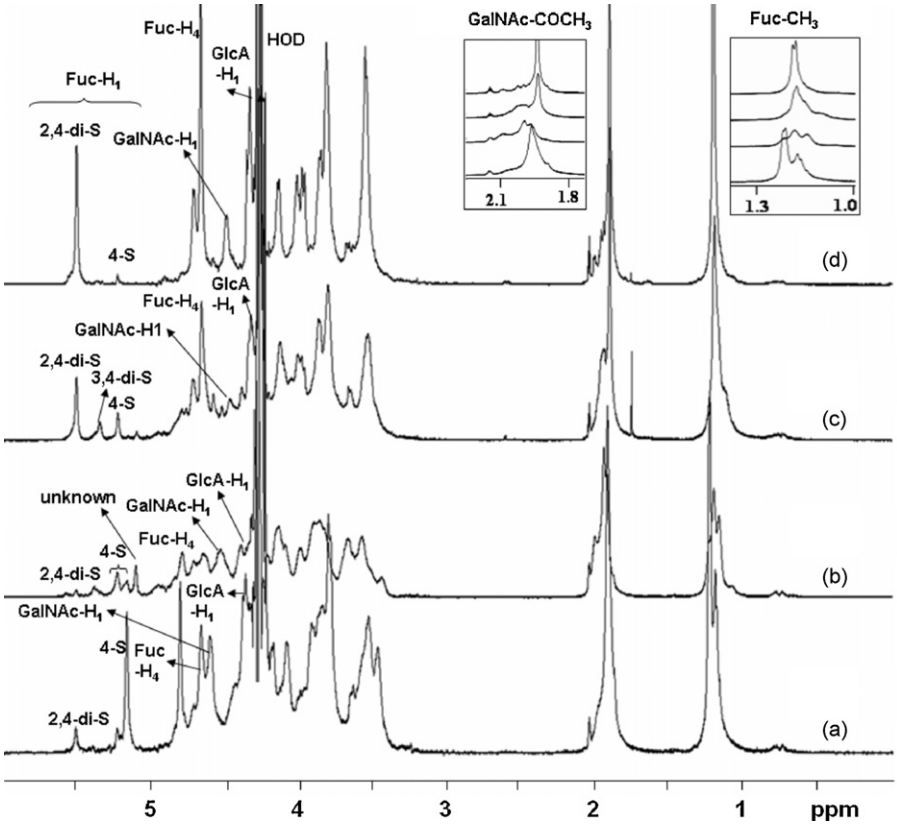

Fig. 4. ^{1}H NMR spectra of fucosylated chondroitin sulfates isolated from sea cucumbers: (a) fCS-*Pg*, (b) fCS-*Hv*, (c) fCS-*St*, and (d) fCS-*Ib*.

(Mourao et al., 1996). The minor peak at 5.56 ppm in the spectrum of fCS-*Pg* was assigned to a 2,4-*O*-disulfated fucose (Fuc2,4S), in agreement with the signal found in the spectra of fCSs isolated from *S. japonicus* and *L. grisea* (Mourao et al., 1996; Yoshida et al., 1992). fCS-*Ib* also exhibited signals at 5.23 and 5.56 ppm (Fig. 4d), indicating the presence of Fuc4S and Fuc2,4S, but with the latter as the dominant component. The spectrum of fCS-*St* showed three clear signals in the anomeric region (Fig. 4c). In addition to the peaks at 5.23 and 5.56 ppm (from Fuc4S and Fuc2,4S, respectively), the signal at 5.33 ppm was assigned to a 3,4-*O*-disulfated fucose (Fuc3,4S), based on the studies of fCSs obtained from *S. japonicus* and *L. grisea* (Mourao et al., 1996; Yoshida et al., 1992). The anomeric proton signals of fCS-*Hv* (Fig. 4b) were rather weak and complicated. The peaks at 5.17 and 5.23 ppm were from Fuc4S, and those at 5.33 and 5.56 ppm were from Fuc3,4S and Fuc2,4S, respectively. The signal at 5.09 ppm was tentatively assigned to a non-sulfated fucose (Fuc0S).

Different sulfation patterns of the fucose branches also affected the chemical shifts of methyl protons of Fuc and GalNAc. In the Fuc methyl region (1.0–1.3 ppm), fCS-*Pg* (Fig. 4a) showed two signals at 1.23 and 1.19 ppm and these were assigned to the methyl protons of Fuc4S and Fuc2,4S, respectively, as the ratio of the peak areas was similar to that of the anomeric signals at 5.23 and 5.56 ppm. fCS-*Ib* gave a major methyl proton signal at 1.19 ppm (Fig. 4d) which can be readily assigned to a Fuc2,4S. Both fCS-*Hv* and fCS-*St* exhibited complicated Fuc methyl peaks (Fig. 4b and c) similar to their anomeric proton signals, indicating complex sulfation patterns of the Fuc. The methyl proton signals of GalNAc (1.8–2.0 ppm) were also different among the four polysaccharides. A major peak at 1.89–1.93 ppm was found in the spectra of fCS-*Pg*, -*St* and -*Ib*, indicating that they shared a similar sulfation pattern on the GalNAc of the disaccharide backbone. However, fCS-*Hv* gave more complex GalNAc methyl signals due to its complex sulfation pattern of the GalNAc (see below for discussion).

The assignment can be corroborated by their COSY (Fig. 5 and Supplementary Figs. 1 and 2) and TOCSY spectra (Table 3). As shown in the COSY spectrum (Fig. 5), fCS-*St* gave three clearly distinguishable signals from the sulfated Fuc in the anomeric region. The COSY spectrum showed cross-peaks between anomeric protons H-1 and H-2 of Fuc, *e.g.* a1/a2 from Fuc2,4S, b1/b2 from Fuc3,4S and c1/c2 from Fuc4S (Fig. 5 and Table 3). The TOCSY spectrum (not shown) extended the assignment to the remaining proton signals, *e.g.* H-3, H-4 and H-6 (Table 3). In some cases H-5 was too weak to be

Table 2
Sulfation patterns of the fucose-branched chondroitin sulfates isolated from sea cucumbers.

Samples	Fuc0S	Fuc4S	Fuc2,4S	Fuc3,4S
fCS-*Pg*	–	81.6	18.4	–
fCS-*Hv*	25.6	50.2	15.8	8.4
fCS-*St*	–	24.8	22.4	52.8
fCS-*Ib*	–	4.1	95.9	–

The chemical shifts of Fuc0S, Fuc4S, Fuc2,4S, and Fuc3,4S was 5.09, 5.17/5.23, 5.56 and 5.33 ppm, respectively; "–", not detected. The relative percentage of different sulfation pattern of Fuc was based on anomeric proton-NMR peak area.

Table 3
^{1}H NMR chemical shifts of the fucose residues in the fCSs.

	Fuc0S	Fuc4S	Fuc2,4S	Fuc3,4S
H-1	5.03	5.17 (major) or 5.23 (minor)	5.56	5.33
H-2	3.96	3.71	4.36	4.28
H-3	4.01	3.90	4.05	4.43
H-4	3.96	4.56	4.68	4.62
H-5	4.35	–	4.38	–
H-6	1.21	1.23	1.19	1.19

The chemical shifts of Fuc0S were from a standard non-sulfated monosaccharide fucose, cited from literature (Ribeiro et al., 1994); "–", not assigned.

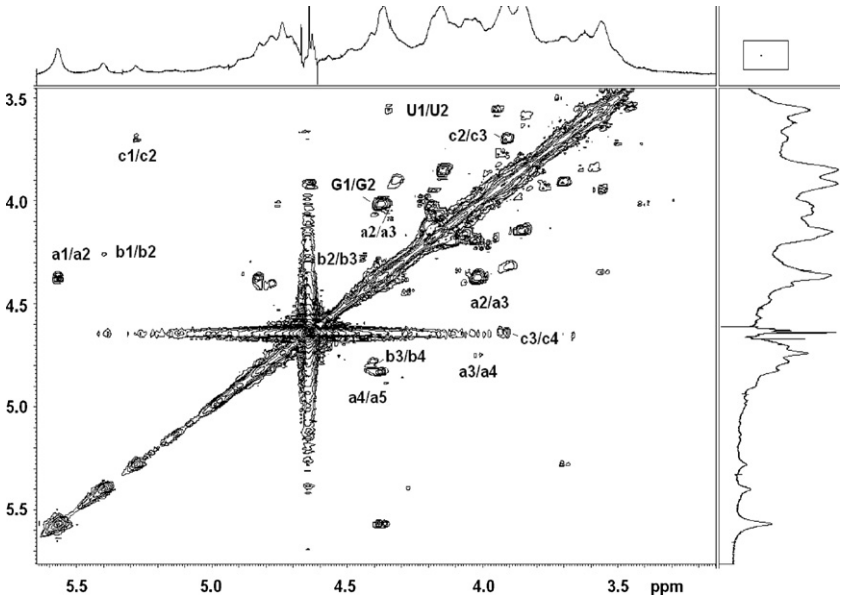

Fig. 5. COSY spectrum of fCS-*St*. Proton signals designated with a, b, and c refer to those produced by Fuc2,4S, Fuc3,4S, and Fuc4S, respectively; proton signals designated with G and U refer to GalNAc and GlcA, respectively.

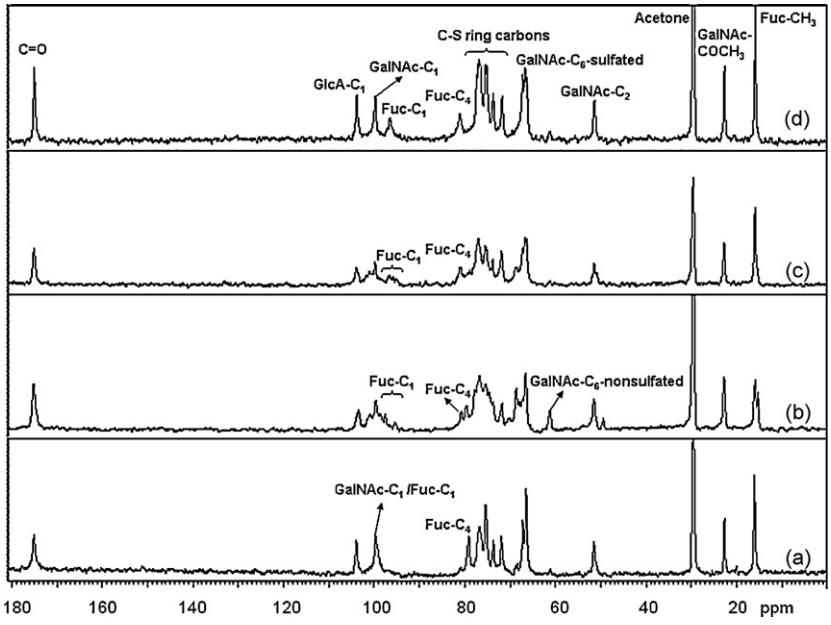

Fig. 6. ^{13}C NMR spectra of fucosylated chondroitin sulfates isolated from sea cucumbers: (a) fCS-*Pg*, (b) fCS-*Hv*, (c) fCS-*St*, and (d) fCS-*Ib*.

Table 4
^{1}H and ^{13}C chemical shifts of fCS-*Ib* oligosaccharide fragment and a standard CSE.

Samples	fCS-*Ib* oligosaccharide			CSE	
	GlcA(β)	GalNAc4,6S(β)	Fuc2,4S(α)	GlcA(β)	GalNAc4,6S(β)
H_1 (C_1)	4.34 (104.6) *J* = 7.6	4.38 (101.2) *J* = 6.8	5.56 (96.5) *J* = 3.6	4.49 (104.2)	4.65 (102.1)
H_2 (C_2)	3.56 (73.7)	3.88 (54.4)	**4.34 (75.7)**	3.41 (73.0)	4.07 (52.2)
H_3 (C_3)	3.64 (77.4)	3.88 (75.3)	3.97 (67.3)	3.60 (74.7)	4.07 (76.1)
H_4 (C_4)	3.78 (75.4)	**4.57 (76.8)**	**4.64 (82.4)**	3.80 (82.6)	4.80 (76.6)
H_5 (C_5)	3.76 (71.9)	3.97 (68.8)	4.60 (67.6)	3.68 (77.5)	4.10 (73.3)
H_6 (C_6)	3.41 (73.0)	**4.21/4.09 (67.8)**	1.10 (15.7)	–	4.30 (68.5)
CH_3	–	1.90 (23.0)	–	–	2.04
C=O	– (174.2)	– (175.3)	–	–	–((174.6)

Chemical shifts of CSE are cited from literature (Yoshida et al., 1992), and chemical shifts of the sulfation sites of fCS-*Ib* oligosaccharide fragments are highlighted in bold.

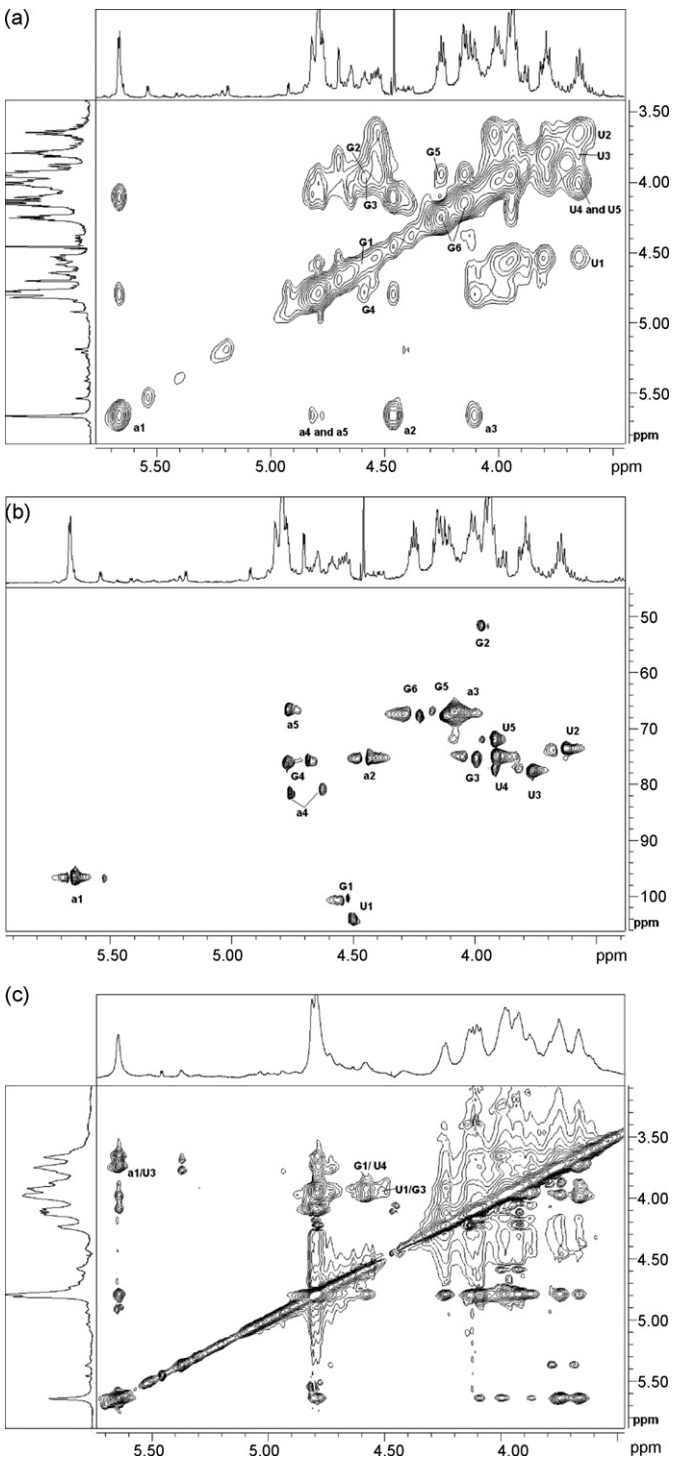

Fig. 7. The 2D NMR spectra of the oligosaccharides prepared from fCS-Ib using peroxide degradation (Petit et al., 2006): (a) TOCSY; (b) HMQC; (c) NEOSY. Signals designated with "a" refer to those produced by Fuc2,4S; signals designated with G and U refer to GalNAc and GlcA, respectively.

detected in the COSY and TOCSY spectra due mainly to the small value of Fuc $J_{H\text{-}4,H\text{-}5}$.

Sulfate substitutions were assigned by careful comparison of each proton chemical shift of the Fuc residues in the polysaccharide chains with that of a standard monosaccharide fucose (Table 3) as it is not possible to obtain a non-sulfated fCS polysaccharide because of the lability of the fucose branches during acid hydrolysis used for removal of the sulfate groups (Kariya et al., 2002; Mourao et al., 1996; Yoshida et al., 1992). The position of sulfate was deduced from its down-field shift (Table 3). The H-1 signals of GlcA and GalNAc in backbone of fCSs were at 4.46–4.52 and 4.58–4.62 ppm, slightly different from the previous report on the structure of fCS from *S. japonicus* (Yoshida et al., 1992) and this was likely to have been caused by the different experimental conditions.

3.4. ^{13}C NMR

^{13}C NMR spectra of fCSs (Fig. 6) did not show any apparent difference in the sulfation patterns of the fucose branches. However, the major signals exhibited were useful to assign the chondroitin sulfate backbone sequence as these carbon signals were very similar to those present in the spectrum of a standard chondroitin sulfate E (CSE) (Yoshida et al., 1992) and in a fCS from *S. japonicus* (Yoshida et al., 1992) which contains the CSE backbone. In CSE, the GalNAc is sulfated at both 4- and 6-positions. A signal at 67.5 ppm, present in all four spectra, was from the sulfated C-6 of the GalNAc. The sulfated C-4 signal was difficult to assign as it was in the overlapping signal region 70–80 ppm. fCS-*Hv* gave a complex spectrum (Fig. 6b) and an additional signal at 62.5 ppm indicated a non-sulfated C-6 in the GalNAc.

In the anomeric signal region, fCS-*Ib* (Fig. 6d) showed three clear signals at 97.6, 99.8 and 104.1 ppm, derived from the three constituent monosaccharides Fuc, GalNAc and GlcA, respectively, consistent with the previous report (Mourao et al., 1996). However, although the GalNAc and GlcA signals were also clearly recognizable in the spectra of the rest of the fCSs (Fig. 6a–c), anomeric signals of Fuc were rather complicated as the sulfation patterns were more complex in these fCSs than that in fCS-*Ib*.

3.5. NMR of fCS oligosaccharide fragment

The assignment of a CSE backbone, –[4GlcAβ1–3GalNAc(4,6S)β1]– of the fCS polysaccharides was corroborated by 2D NMR (Fig. 7 and Table 4) using fCS-*Ib* oligosaccharide fragment (~3,000 Da) as a representative, which was prepared by partial depolymerization using peroxide oxidative degradation (Petit et al., 2006) and fractionation by gel filtration chromatography. Assignments of ^{1}H and ^{13}C chemical shifts of the backbone residues –4GlcAβ1– and –3GalNAcβ1– were made from the TOCSY (Fig. 7a) and HMQC spectra (Fig. 7b). The β anomeric configuration of both GlcA and GalNAc can be deduced from the H-1/H-2 coupling constants 6.8 and 7.6 Hz, respectively (Table 4). The down-field shifts of H-4 and H-6 of GalNAc (Table 4) suggested sulfations at the 4-*O*- and 6-*O*-positions. Careful comparison with the spectral data of the standard CSE (Yoshida et al., 1992) (Table 4) provided further evidence of a CSE backbone. The correlation signals G1/U4 and U1/G3 in the NOESY spectrum (Fig. 7c) unambiguously identified GalNAc linked to the C-4 positions of the GlcA, while the latter linked to the C-3 positions of GalNAc.

The major sulfation pattern in the fucose branch of fCS-*Ib* can also be readily assigned as Fuc2,4S (Table 4) by the 2D NMR spectrum of the oligosaccharide fragment, consistent with the assignment made from the NMR analysis of the polysaccharide.

3.6. Anticoagulant activities

Anticoagulant activities of fCSs were assessed by measuring the activated partial thromboplastin time (APTT) and thrombin time (TT), and the results are shown in Table 5. All four fCSs had profound anticoagulant activities as indicated by the significant prolongation of APTT and TT, even at low concentrations of fCSs (Fig. 8). The potencies of fCS-*Ib* and fCS-*St* were similar to that of the standard unfractionated heparin in the APTT and TT assays, whereas fCS-*Pg* and fCS-*Hv* had much lower anticoagulant activities. In order to

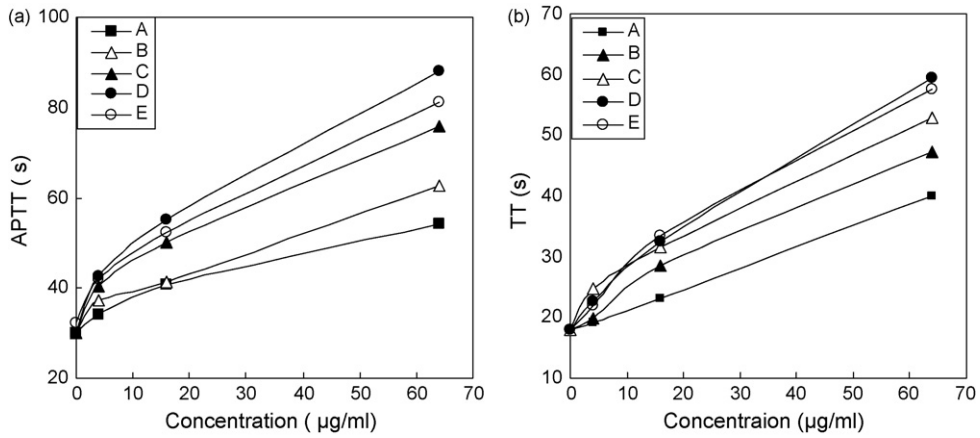

Fig. 8. Anticoagulant activities of fucosylated chondroitin sulfates from different sea cucumbers: (a) APTT of fCSs and heparin; (b) TT of fCSs and heparin; (A) fCS-*Pg*; (B) fCS-*Hv*; (C) fCS-*St*; (D) fCS-*Ib*; (E) unfractionated heparin (14 kDa, 150 IU/mg).

Table 5
Anticoagulant properties of the fCSs purified from different sea cucumbers.

Samples	APTT	TT
fCS-*Pg*	35 IU/mg	78 IU/mg
fCS-*Hv*	42 IU/mg	90 IU/mg
fCS-*St*	135 IU/mg	132 IU/mg
fCS-*Ib*	183 IU/mg	157 IU/mg
Unfractionated heparin	150 IU/mg	150 IU/mg

Clotting times were recorded as described under Section 2. The activity is expressed as international units/mg using a parallel standard curve based on the International Heparin Standard (150 units/mg).

obtain the same prolongation time on APTT, 4.3-fold (w/w) of fCS-*Pg* was required compared to unfractionated heparin. In the case of fCS-*Hv*, which contains a slightly higher content of Fuc2,4S, 3.2-fold was required. fCS-*St* contains a significant amount of Fuc2,4S in its branch in addition to Fuc4S and Fuc3,4S, and its anticoagulant activity increased significantly. The ATPP (135 IU/mg) and TT (132 IU/mg) of fCS-*St* were comparable to those of the unfractionated heparin (Table 5). fCS-*Ib* contains Fuc2,4S branch only and had the best activity among all four fCSs investigated, as shown by 183 IU/mg in the APTT assay and 157 IU/mg in the TT assay, which were even higher than those of unfractionated heparin.

PT assay was also carried out but none of the fCSs showed any effect on PT at the concentrations (4–64 μg/mL) tested. The lack of PT activity of the fCSs was similar to heparin and other sulfated fucans (Mourao et al., 2001). It has been known that APTT determines an interference with the intrinsic coagulation process while TT represents the last coagulation step of the thrombin-mediated fibrin formation. Therefore, the anticoagulant effects of fCSs were considered to affect the multi-coagulation processes.

The anticoagulant activities of the fucosylated chondroitin sulfates were conferred mainly by the sulfated fucose branches according to a previous report (Mourao et al., 2001). As all the fCSs investigated here have a similar backbone structure except fCS-*Hv*, we attribute the difference in anticoagulant activities of fCSs to the difference in their sulfation patterns of the fucose branch. fCS-*St* and fCS-*Ib*, which contain mainly 2,4-*O*-disulfated fucose residues gave the strongest anticoagulant activity.

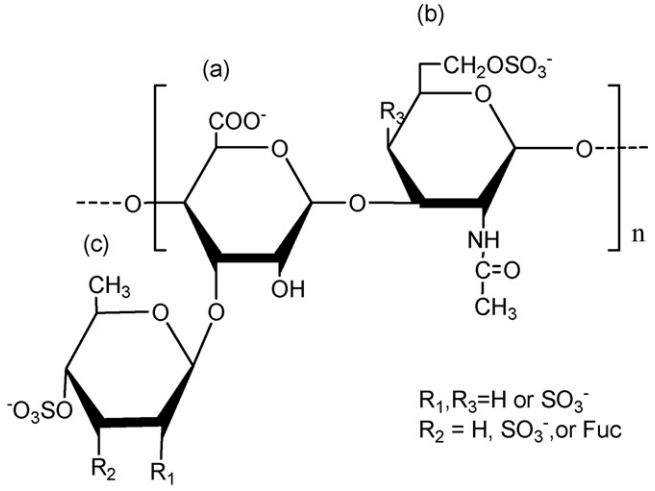

Fig. 9. Preponderant structures of fucosylated chondroitin sulfates isolated from sea cucumbers. The backbone of the polysaccharide is made up by repeating disaccharide units of alternating β-D-glucuronic acid (*a*) and *N*-acetyl-β-D-glucosamine (*b*), the same sequence as mammalian chondroitin sulfate. However, in the case of the sea cucumber chondroitin sulfate, the β-D-glucuronic acid residues bear different sulfated fucose branches (*c*) at its 3-*O*-position. Most of the *N*-acetyl-β-D-galactosamine residues are 4,6-*O*-disufated; however, a small proportion is sulfated at different positions. The sulfation patterns of the fucose branches are also different for chondroitin sulfates obtained from different sea cucumbers.

4. Conclusion

We have isolated fucosylated chondroitin sulfates from four sea cucumbers obtained from seawaters in three different geographical zones. Detailed 1D and 2D NMR analyses indicated that the main difference in their structures is the sulfation pattern of their fucose branches (Fig. 9). Anticoagulant activities of these fCSs, as assessed by APTT and TT, are related to the sulfation pattern of the fucose branch. The 2,4-*O*-disulfation is particularly important for anticoagulant activity. This is in agreement with previous reports that a specific sulfation pattern can be important for activity, *e.g.* the role of 3-*O*-sulfation of heparin in its interaction with anti-thrombin III. Our present study together with other work highlights a close relationship between structure and anticoagulant activity of sulfated polysaccharides.

The present results indicated that fCSs of *P. graeffei* and *H. vagabunda* obtained from the torrid zone (the tropics) contain more Fuc4S branch; fCS of *I. badionotus* obtained from the temperate zone contains mainly Fuc2,4S branch; whereas fCS of *S. tremulus* obtained from the frigid zone has more Fuc3,4S branch. Interestingly, other published results on fCSs and our work on nine additional fCSs (data not shown) isolated from sea cucumbers in various seawaters of tropical, temperate, and frigid zones seem to follow the same trend, *e.g.* fCS of *L. grisea* collected from Guanabara Bay near the tropical zone contains mainly Fuc4S branch (Mourao et

al., 1996; Vieira et al., 1991), whereas fCS of *S. japonicus* collected in the temperate zone appear to have more Fuc2,4S branches (Kariya et al., 1990, 1997). Further work may help to understand if there is a close relationship between the geographical area of the habitat of the sea cucumbers and the sulfation patterns of the fucose branches.

Acknowledgements

This work was supported in part by the National Key Technology R&D Program of China (2008BAD94B05), the National Natural Science Foundation of China (30871944), National High-Tech Research and Development Project of China (2007AA09180), and an International Collaboration Program grant from the Ministry of Science and Technology (2007DFA30980) and. We thank Xiuli Zhang (Ocean University China) for NMR experiments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.08.040.

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