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Chemical characteristic of an anticoagulant-active sulfated polysaccharide from *Enteromorpha clathrata*

Xiaohui Qi[a], Wenjun Mao[a,*], Yan Gao[b], Yin Chen[a], Yanli Chen[a], Chunqi Zhao[a], Na Li[a], Chunyan Wang[a], Mengxia Yan[a], Cong Lin[a], Jimiao Shan[a]

[a] Key Laboratory of Marine Drugs, Ministry of Education, Institute of Marine Drug and Food, Ocean University of China, 5 Yushan Road, Qingdao 266003, People's Republic of China
[b] National Oceanographic Center of Qingdao, 88 Xuzhou Road, Qingdao 266071, People's Republic of China

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ABSTRACT

A sulfated polysaccharide FEP from marine green alga *Enteromorpha clathrata* was extracted with hot water and further purified by ion-exchange and size-exclusion chromatography. Results of chemical and spectroscopic analyses showed that FEP was a high arabinose-containing sulfated polysaccharide with sulfate ester of 31.0%, and its average molecular weight was about 511 kDa. The backbone of FEP was mainly composed of (1 → 4)-linked β-L-arabinopyranose residues with partially sulfate groups at the C-3 position. In vitro anticoagulant assay indicated that FEP effectively prolonged the activated partial thromboplastin time and thrombin time. The investigation demonstrated that FEP was a novel sulfated polysaccharide with different chemical characteristics from other sulfated polysaccharides from marine algae, and could be a potential source of anticoagulant.

1. Introduction

The leading causes of death are now diseases that involve heart and blood vessels and as a consequence thrombin thrombosis (Melo, Pereira, Fogue, & Mourão, 2004). Heparin is one of the agents more largely used in the therapy and prophylaxis of thrombosis. However, heparin is associated with various side effects, such as hemorrhagic effect, development of thrombocytopenia and incapacity to inhibit thrombin bound to fibrin (Liaw, Becker, Stafford, Fredenburgh, & Weitz, 2001; Warkentin, 1999). In addition, the usual sources of heparin are pig or bovine tissues. These tissues are rich in mast cells, presumably resulting from the high foreign parasite burden in these tissues (Warda, Mao, Toida, & Linhardt, 2003). The appearance of bovine spongiform encephalopathy, 'mad cow disease' and the incidence of prion-related diseases in mammals has limited the use of bovine heparin (Schonberger, 1998). Therefore, the alterative sources of anticoagulant and antithrombotic compounds are required.

One abundant source of new anticoagulant polysaccharides is marine algae. Various anticoagulant-active polysaccharides, especially from marine red and brown algae, have been isolated and characterized (McLellan & Jurd, 1992). In recent years, interest in the sulfated polysaccharides from marine green algae is increasing because of their high anticoagulant activities. Maeda, Uehara, Harada, Sekiguchi, and Hiraoka (1991) revealed that the sulfated polysaccharide from *Monostroma nitidum* yielded a 6-fold higher anticoagulant activity than heparin. A sulfated galactan with high anticoagulant activity was isolated from marine green alga *Codium cylindricum* (Matsubara et al., 2001). Heparinoid-active sulfated rhamnans were also isolated from *Monostroma latissimum* (Li et al., 2011; Zhang et al., 2008). The sulfated polysaccharides from marine green algae represent a potential source of anticoagulant to be explored.

Recently, a few structural studies have been done on sulfated polysaccharides isolated from marine green algae (Lahaye & Robic, 2007). The ulvans from *Ulva* sp. were all based on β-D-glucuronic acid-(1 → 4)-α-L-rhamnose 3-sulfate and α-L-iduronic acid-(1 → 4)-α-L-rhamnose 3-sulfate repeating units, as well as contiguous (1 → 4)-β-D-glucuronic acids possibly occurring either in the ulvan or as a separate glucuronan (Lahaye et al., 1999). The sulfated polysaccharides from *M. nitidum* and *M. latissimum* were mainly composed of (1 → 3)-linked rhamnose and (1 → 2)-linked rhamnose residues, and the sulfate groups were substituted at C-2 of (1 → 3)-linked rhamnose and/or C-3 of (1 → 2)-linked rhamnose residues (Harada & Maeda, 1998; Li et al., 2011). The sulfated polysaccharides from *Codium fragile* and *Codium vermilara* contained linear sulfated arabinans and galactans, (1 → 4)-α-D-glucans and (1 → 4)-β-D-mannans (Ciancia et al., 2007). However, the structures of sulfated polysaccharides from *Enteromorpha* species have

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* Corresponding author. Tel.: +86 532 8203 1560; fax: +86 532 8203 3054.
E-mail address: wenjunmqd@hotmail.com (W. Mao).

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not yet been fully characterized (Kim, Cho, Karnjanapratum, Shin, & You, 2011; Ray, 2006).

Marine green alga *Enteromorpha clathrata* grows frequently involved in algal proliferation in eutrophicated coastal and lagoon water. It has been used as drug in traditional Chinese medicine for hundreds of years. One particularly interesting feature of the seaweed is its richness in polysaccharides, which are useful in clearing away heat, detoxification and eliminating inflammation. In the present work, an anticoagulant-active sulfated polysaccharide from *E. clathrata* was isolated and its chemical characteristic was investigated by a combination of chemical and spectroscopic methods, including one- and two-dimensional nuclear magnetic resonance (1D, 2D NMR) spectroscopy.

2. Materials and methods

2.1. Materials

E. clathrata was collected on the coast of Fujian, China. The raw material was thoroughly washed with tap water, air dried, milled, and then kept in plastic bags at room temperature in a dry environment. Dialysis membranes (flat width 44 mm, molecular weight cut-off 3500) were from Lvniao (Yantai, China). Q Sepharose Fast Flow and Sephacryl S-400/HR were from Pharmacia Bioscience (Uppsala, Sweden). Standard dextrans (M_w: 5.9, 9.6, 21.1, 47.1, 107, 200 and 708 kDa) were from Showa Denko K.K. (Tokyo, Japan). Standard heparin, D-glucose, L-rhamnose, D-xylose, L-arabinose, D-mannose, L-fucose, D-galactose, D-glucuronic acid, D-galacturonic acid and *N*-acetyl-β-D-glucosamine were from Sigma (St. Louis, MO, USA). Activated partial thromboplastin time (APTT) assay reagent and prothrombin time (PT) assay reagent were from Shanghai Sun (Shanghai, China). Thrombin time (TT) assay reagent was from Dade Behring (Deerfield, IL, USA).

2.2. Isolation and purification of the sulfated polysaccharide

The milled alga (50 g) was dipped into 30 volumes of distilled water and kept at room temperature for 1 h, and then filtrated. The residue was dipped into 30 volumes of distilled water, homogenized and refluxed at 100 °C for 2 h. After cooling to the room temperature, the supernatant was collected by centrifugation, concentrated, dialyzed in a cellulose membrane (molecular weight cut-off 3500) against flowing distilled water at room temperature for three successive days. The retained fraction was recovered, concentrated under reduced pressure, precipitated by adding four volumes of 95% ethanol (v/v) and dried. The protein in the fraction was removed by the method of Sevag (Matthaei, Jone, Martin, & Nirenberg, 1962). The crude polysaccharide was dissolved in distilled water and fractionated on a Q Sepharose Fast Flow column (30 cm × 3 cm) coupled to an AKTA FPLC system, eluted with a step-wise gradient of 0, 1.0, 3.0 and 4.0 mol/L NaCl. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions eluted with 1.0 mol/L NaCl were pooled, dialyzed and further purified on a Sephacryl S-400/HR column (100 cm × 3 cm) eluted with 0.2 mol/L NH_4 HCO_3 at a flow rate of 0.3 mL/min. The major polysaccharide fraction was pooled, freeze-dried and named as FEP.

2.3. General analysis

Total sugar content was determined by the phenol–sulfuric acid method using arabinose as the standard (Dubois et al., 1956). Sulfate ester content was estimated according to Therho and Hartiala (1971). Protein content was quantified as described by Bradford (1976). Purity and molecular weight were determined by high performance gel permeation chromatography (HPGPC) on a Shodex OHpak SB-804 HQ column (0.8 cm × 30 cm), eluted with 0.2 mol/L Na_2SO_4 at a flow rate of 0.5 mL/min. The molecular weight was estimated by reference to a calibration curve made by a set of standard dextrans (M_w: 5.9, 9.6, 21.1, 47.1, 107, 200 and 708 kDa) (Chen et al., 2011). Desulfation of the sulfated polysaccharide was achieved according to Falshaw and Furneaux (1998). The desulfated product was recovered by dialysis, freeze-dried and designated as dsFEP.

2.4. Analysis of monosaccharide composition

Monosaccharide composition was measured by reversed-phase high performance liquid chromatography (HPLC) after pre-column derivatization (Sun et al., 2009). Briefly, polysaccharide was hydrolyzed with 2 mol/L trifluoroacetic acid at 100 °C for 6 h in a sealed tube. The completeness of hydrolysis was confirmed by thin-layer chromatography. Excess acid was removed by co-distillation with methanol for four times after the hydrolysis was completed. 1 mg of dry hydrolysate was dissolved in 100 μL of 0.3 mol/L NaOH, and then added to 120 μL of 0.5 mol/L methanol solution of 1-phenyl-3-methyl-5-pyrazolone (PMP) at 70 °C for 1 h. Finally, the mixture was added 100 μL of 0.3 mol/L HCl solution and vigorously shaken and centrifuged for 5 min. The supernatant, containing the labeled carbohydrates, was finally filtered through 0.22 μm nylon membranes (Westborough, MA, USA) and 10 μL of the resulting solution was injected into the XDB-C_{18} column (4.6 mm × 250 mm) fitted with UV detector (245 nm). The mobile phase was a mixture of 0.1 mol/L KH_2PO_4 (pH 6.7)–acetonitrile (83:17). The flow rate was 1.0 mL/min and column temperature was 30 °C. Sugar identification was done by comparison with reference sugars (D-glucose, L-rhamnose, D-xylose, L-arabinose, D-mannose, L-fucose, D-galactose, D-glucuronic acid, D-galacturonic acid and *N*-acetyl-β-D-glucosamine). Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide. The complete hydrolysate of FEP was also analyzed by Whatman 3 mm paper chromatography and developing with the solvent system of 1-butanol:pyridine:water (6:4:3) (Harada & Maeda, 1998). The arabinose was obtained by extracting with water and its optical rotation value was measured at 20 °C with a P-1020 digital polarimeter, using a 10-cm light path length at 589 nm (Mao et al., 2008).

2.5. Methylation analysis

Methylation analysis was performed according to Hakomori (1964) with some modification. Briefly, 2.0 mg of polysaccharide was dissolved in 1.0 mL of dimethyl sulfoxide, and then 100 mg of anhydrous sodium hydride was added. The mixture was stirred at room temperature for 1 h. 1.0 mL of CH_3I was added to the mixture and stirred for 2 h. Finally, the reaction was terminated with addition of distilled water, and the residue was extracted with CH_3Cl. The extract was washed with distilled water and evaporated to dryness. The methylated polysaccharide recovered after dialysis and freeze-drying was hydrolyzed with 2 mol/L trifluoroacetic acid at 105 °C for 6 h. The methylated sugar residues were converted to partially methylated alditol acetates by reduction with $NaBH_4$, followed by acetylation with acetic anhydride. The products were analyzed by gas chromatography–mass spectrometric (GC–MS) on a HP6890II/5973 instrument using a DB 225 fused silica capillary column (0.25 mm × 30 m) (Agilent Technologies Co. Ltd., USA). The temperature was increased from 100 to 220 °C at a rate of 5 °C/min then maintained at 220 °C for 15 min. Identification of partially methylated alditol acetates was carried out on the basis of the retention time and its mass fragmentation patterns.

2.6. IR spectroscopy

FTIR spectrum of the polysaccharide was measured on a Nicolet Nexus 470 spectrometer. The polysaccharide was mixed with KBr powder, ground and pressed into a 1-mm pellet for FTIR measurements in the frequency range of 4000–500 cm^{-1}.

2.7. NMR spectroscopy

^{1}H NMR and ^{13}C NMR spectra were recorded at 23 °C using a JEOL JNM-ECP 600 MHz spectrometer. 40 mg of polysaccharide was deuterium exchanged by three successive freeze-drying steps in 99% D_2O and then dissolved in 0.5 mL of 99.98% D_2O. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. ^{1}H–^{1}H correlated spectroscopy (COSY), ^{1}H–^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC) and ^{1}H–^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) and ^{1}H–^{1}H nuclear overhauser effect spectroscopy (NOESY) experiments were also carried out.

2.8. Assay of anticoagulant activity

APTT assay was carried according to Mourâo et al. (1996). In brief, 90 μL of citrated normal human plasma samples and 10 μL of sample solution at concentrations of 0, 10, 20, 50 and 100 μg/mL were incubated at 37 °C for 60 s, 100 μL of prewarmed APTT assay reagent was added, and the reaction was allowed to proceed at 37 °C for 2 min. Prewarmed 100 μL of 0.25 mol/L calcium chloride was added. APTT was recorded as the time for clot formation. For TT assay, 90 μL of citrated normal human plasma was mixed with 10 μL of a solution of polysaccharide and incubated at 37 °C for 60 s. Then, 200 μL of TT assay reagent prewarmed to 37 °C was added and clotting time was recorded. PT assay was measured as follows: 90 μL of citrated normal human plasma was mixed with 10 μL of a solution of polysaccharide and incubated at 37 °C for 1 min. Then, 200 μL of PT assay reagent pre-incubated at 37 °C for 10 min was added and clotting time was recorded. All clotting assays were performed in triplicate. The results were expressed as means ± standard deviation (*SD*).

3. Results and discussion

3.1. Composition analysis of the sulfated polysaccharide

The sulfated polysaccharide FEP from *E. clathrata* was extracted with hot water, and further purified by a combination of ion-exchange chromatography on a Q Sepharose Fast Flow column and gel filtration chromatography on a Sephacryl S-400/HR column (Fig. 1). The yield of FEP from crude polysaccharide (10.3 g/100 g dry alga) was about 61.9%. FEP appeared as a single and symmetrical peak in the HPGPC chromatogram, and its average molecular weight was about 511 kDa (Fig. 2). Monosaccharide composition analysis by reversed-phase HPLC demonstrated that FEP consisted of large amounts of arabinose (80.5%), with small amounts of rhamnose (10.7%), galactose (4.8%) and glucuronic acid (4.0%) (Fig. 3). The optical rotation of the arabinose hydrate was +104.8°, the value is in agreement with that for L-arabinose hydrate (Uehara, Takeshita, & Maeda, 1992). FEP contained 31.0% sulfate ester, with a minor amount of protein (0.4%). The chemical composition of FEP was markedly different from those of other sulfated polysaccharides from *Enteromorpha* species (Kim et al., 2011; Ray, 2006).

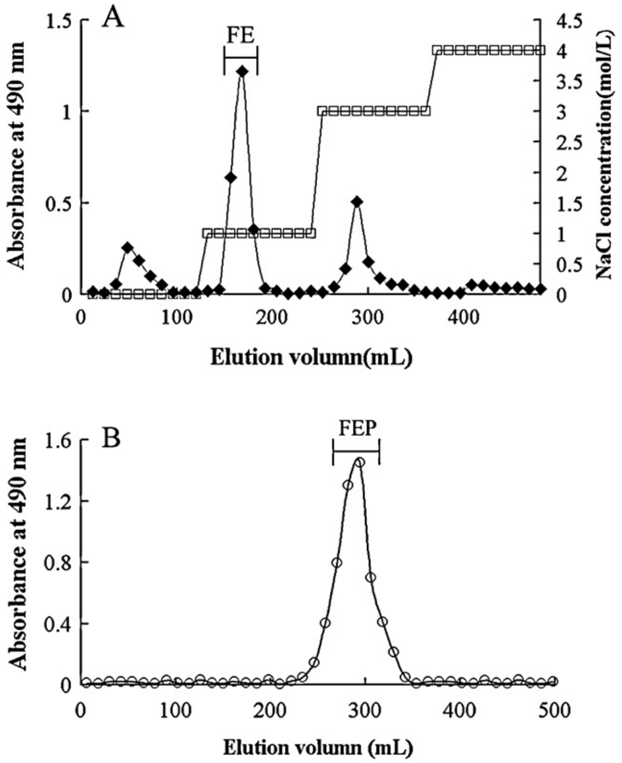

Fig. 1. Isolation and purification of the sulfated polysaccharide from marine green alga *E. clathrata*. (A) The crude polysaccharides were applied to a Q Sepharose Fast Flow column. The fraction eluted with 1.0 mol/L NaCl was pooled and named as FE; (B) FE was applied to a Sephacryl S-400/HR column and eluted with 0.2 mol/L NH_4HCO_3. The peak fractions containing the polysaccharides were pooled and named as FEP.

3.2. Methylation analysis

In general, methylation analysis is useful to determine the linkages and the sulfate substitution position of sugar residues by comparing the results of native and desulfated polysaccharides. The yield of desulfated polysaccharide dsFEP from FEP was 62.7%. The completeness of methylation of FEP and dsFEP was confirmed by IR spectroscopy as the disappearance of OH bands (Supplementary Fig. S1). As shown in Table 1, FEP mainly consisted of (1 → 3,4)-linked arabinose with small amounts of (1 → 4)-linked arabinose, (1 → 3)-linked rhamnose and (1 → 3,4)-linked rhamnose residues. Compared with the results for FEP, the amounts of (1 → 4)-linked arabinose distinctively increased, and the amounts of (1 → 3,4)-linked arabinose significantly decreased in dsFEP. Therefore, the sulfate substitution was at C-3 of (1 → 4)-linked arabinose. In addition, high amounts of (1 → 3)-linked rhamnose residues and low amounts of (1 → 3,4)-linked rhamnose residues were also detected

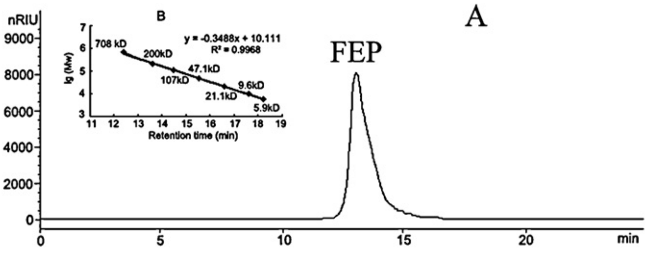

Fig. 2. HPGPC chromatogram of the sulfated polysaccharide FEP and the standard curve of molecular weight. (A) HPGPC chromatogram of FEP on a Shodex OHpak KB-804 column (0.8 cm × 30 cm); and (B) the standard curve of molecular weight.

Table 1
Results of methylation analyses of FEP and dsFEP.

Retention time (min)	Methylated alditol acetate	Primary mass fragments (*m*/*z*)	Mol (%)		Deduced linkage
			FEP	dsFEP	
22.45	1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl-rhamnitol	89, 101, 117, 131, 159, 201, 233	2.5	8.4	→3)-Rha*p*-(1→
23.06	1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methyl -arabinitol	87, 101, 117, 129, 161, 189	10.5	79.5	→4)-Ara*p*-(1→
24.18	1,3,4,5-Tetra-*O*-acetyl-2-*O*-methyl -rhamnitol	87, 99, 113, 117, 129, 159, 173, 201, 275	10.1	4.2	→3,4)-Rha*p*-(1→
25.62	1,3,4,5-Tetra-*O*-acetyl-2-*O*-methyl-arabinitol	87, 99, 117, 129, 159, 201, 261	76.9	7.9	→3,4)-Ara*p*-(1→

in dsFEP, suggesting that the sulfate groups were substituted at C-4 of (1 → 3)-linked rhamnose residues.

3.3. IR spectroscopy

As shown in Fig. 4, the band at 3413 cm^{-1} was derived from the stretching vibration of O—H, and the signal at 2925 cm^{-1} was attributed to the stretching vibration of C—H. The band at 1647 cm^{-1} was due to the bending vibration of O—H, and the signal at 1243 cm^{-1} was assigned to the stretching vibration of S—O of sulfate group (Mao et al., 2008). The signal at 1080 cm^{-1} was attributed to the stretch vibration of C—O and change angle vibration of O—H. In addition, the absorption at 868 cm^{-1} suggests the presence of a β-anomeric configuration for arabinopyranose (Zhang, 1999, chap. 4). The signal at 828 cm^{-1} was assigned to the bending vibration of C—O—S of sulfate ester in axial position.

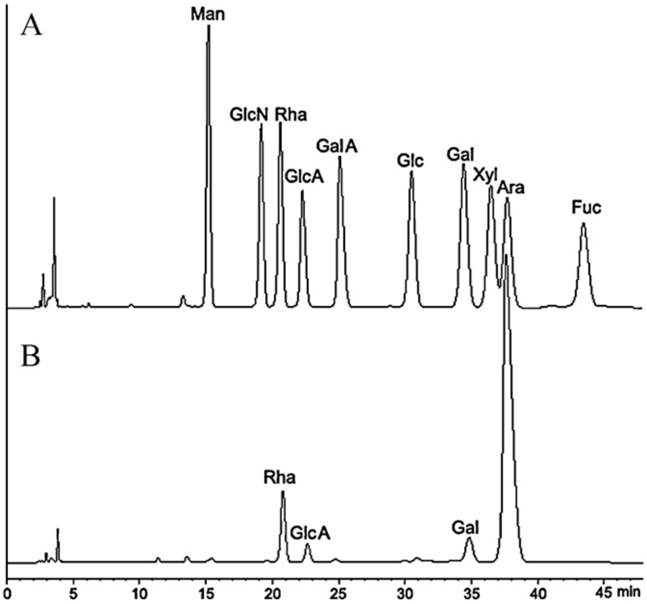

Fig. 3. HPLC analyses of monosaccharide standards and FEP. (A) Monosaccharide standards and (B) FEP.

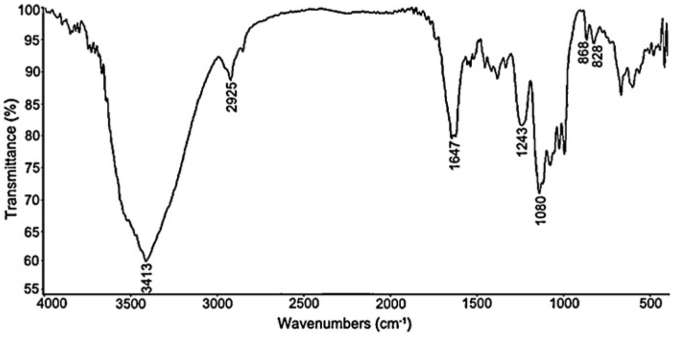

Fig. 4. IR spectrum of the sulfated polysaccharide FEP.

3.4. NMR spectroscopy

In the ^{1}H NMR spectrum of FEP (Fig. 5A), a major anomeric proton signal at 5.18 ppm was assigned to β-L-arabinopyranose residues (Fischer et al., 2004; Glushka et al., 2003; Ishii, Ono, Ohnishi-Kameyama, & Maeda, 2005). The chemical shifts from 3.87 to 4.71 ppm were attributed to protons of the C-2–C-5 of the arabinopyranose residues, and the signal at 1.32 ppm was assigned to the proton of CH$_3$ group of the rhamnopyranose residues. In the anomeric region of the ^{13}C NMR spectrum (Fig. 5B), a major carbon resonance occurred at 98.3 ppm. The signals between 61.4 and 77.1 ppm were assigned to the C-2–C-5 of the arabinopyranose residues, and the signal at 18.3 ppm was attributed to the C-6 of the rhamnopyranose residues.

^{1}H–^{1}H COSY spectrum (Fig. 5C) of FEP allowed the assignment of most of the signals of the proton spin systems. The ^{1}H–^{13}C HMQC spectrum afforded some of the correlations between carbon and proton signals with the sugar residues, and elaborated the site of sulfate ester in sugar residues and the linkage patterns between sugar residues. In the ^{1}H–^{13}C HMQC spectrum (Fig. 5D) of FEP, the anomeric proton signal at 5.18 ppm was correlated to the anomeric carbon signal at 98.3 ppm, which was attributed to the (1 → 4)-linked 3-sulfated L-arabinopyranose residues. In addition, the H-3 signal at 4.71 ppm showed the correlation with the C-3 signal at 77.1 ppm. The H-4 signal at 4.43 ppm was correlated with the C-4 signal at 74.7 ppm. Downfield shifted signals at C-3 and C-4 indicated that the substitution positions were at the C-3 and C-4 of arabinopyranose residues (Fischer et al., 2004). The results suggested the presence of (1 → 4)-linked arabinopyranose residues with sulfate groups at the C-3 position.

The ^{1}H–^{13}C HMBC spectrum (Fig. 5E) further confirmed some of the correlations between carbon and proton signals within the sugar residues, and the sequence of the sugar residues. The anomeric proton signal at 5.18 ppm was correlated with the C-4 signal at 74.7 ppm in the ^{1}H–^{13}C HMBC spectrum. The anomeric proton signal at 5.18 ppm was related to the H-4 signal at 4.43 ppm in the ^{1}H–^{1}H NOESY spectrum (Fig. 5F). Thus, the (1 → 4)-linked pattern between arabinopyranose residues was proved.

All the results demonstrated that the backbone of FEP was mainly composed of (1 → 4)-linked β-L-arabinopyranose residues, and the sulfation position was at C-3. The assignments of ^{1}H and ^{13}C signals of the arabinopyranose residues were shown in Table 2. Because of the strong signals of (1 → 4)-linked 3-sulfated L-arabinopyranose residues, other sugar signals were relatively weak and overlapped. The weak resonance at 5.28 ppm could be attributed to the anomeric proton of (1 → 3)-linked 4-sulfated α-rhamnopyranose residues (Cassolato et al., 2008; Li et al., 2011). Other signals of the rhamnopyranose were difficult to identify.

The structural characteristics of FEP were markedly different from those of the sulfated polysaccharide from *Enteromorpha compress* (Ray, 2006), and the latter was composed of (1 → 4)- and (1 → 2,4)-linked rhamnopyranosyl, (1 → 4)-linked xylopyranosyl, (1 → 4)- and terminally linked glucuronosyl residues, and sulfate

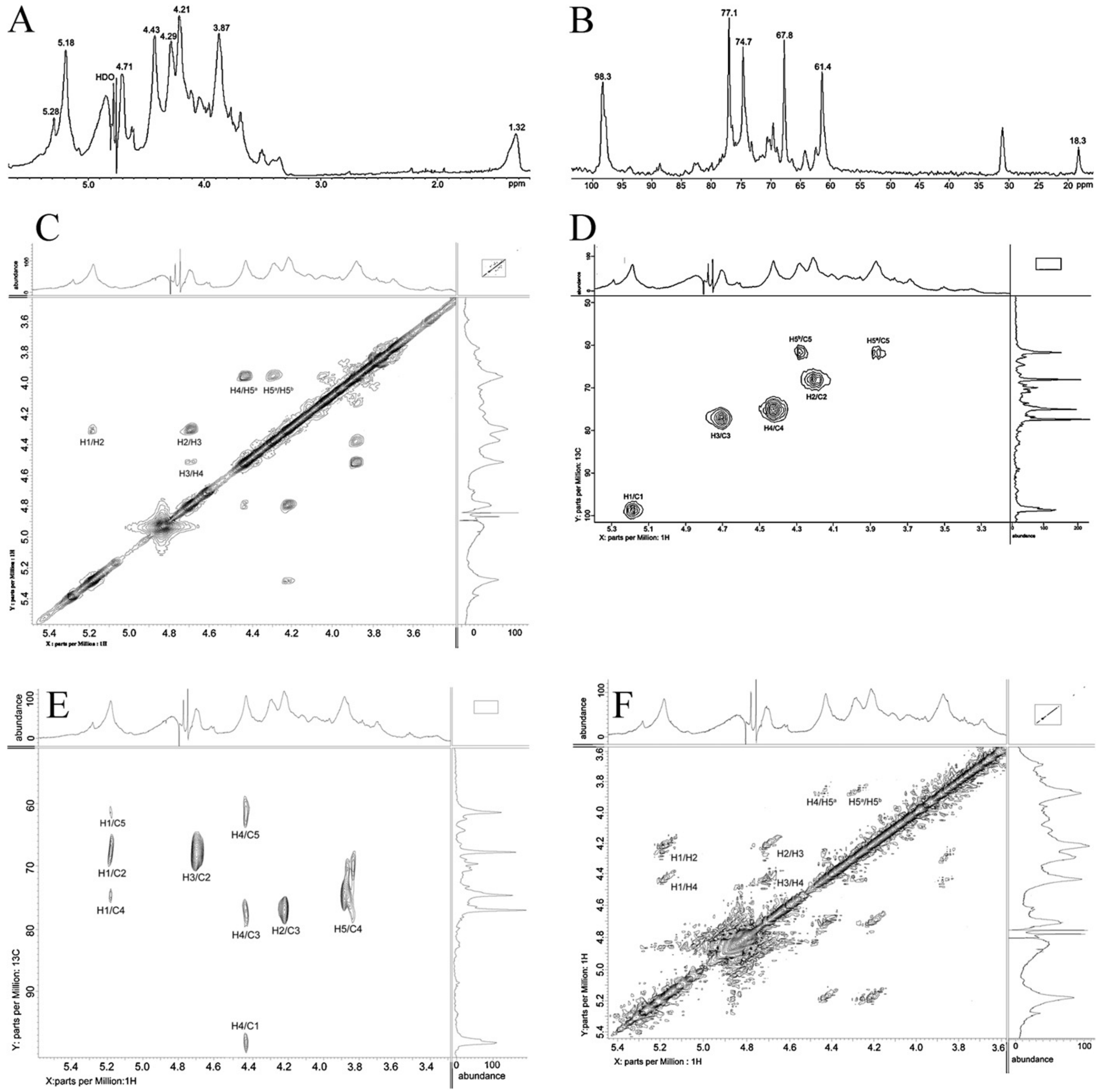

Fig. 5. NMR spectra of the sulfated polysaccharide FEP. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer using acetone as internal standard. Chemical shifts are referenced to internal acetone at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. (A) ^{1}H NMR spectrum; (B) ^{13}C NMR spectrum; (C) ^{1}H–^{1}H COSY spectrum; (D) ^{1}H–^{13}C HMQC spectrum; (E) ^{1}H–^{13}C HMBC spectrum; and (F) ^{1}H–^{1}H NOESY spectrum.

Table 2
^{1}H and ^{13}C chemical shifts for the sulfated polysaccharide FEP.

Residue[d]	Chemical shifts (ppm)[c]				
	H1/C1	H2/C2	H3/C3	H4/C4	H5[a], H5[b]/C5
→4)-β-L-Ara*p*($3SO_4$)-1→	5.18/98.3	4.21/67.8	4.71/77.1	4.43/74.7	4.29,3.87/61.4

[a] Equatorial bond.
[b] Axial bond.
[c] The spectra were recorded using a JEOL JNM-ECP 600 MHz spectrometer. Chemical shifts are referenced to internal acetone at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C.
[d] Ara*p*: arabinopyranose.

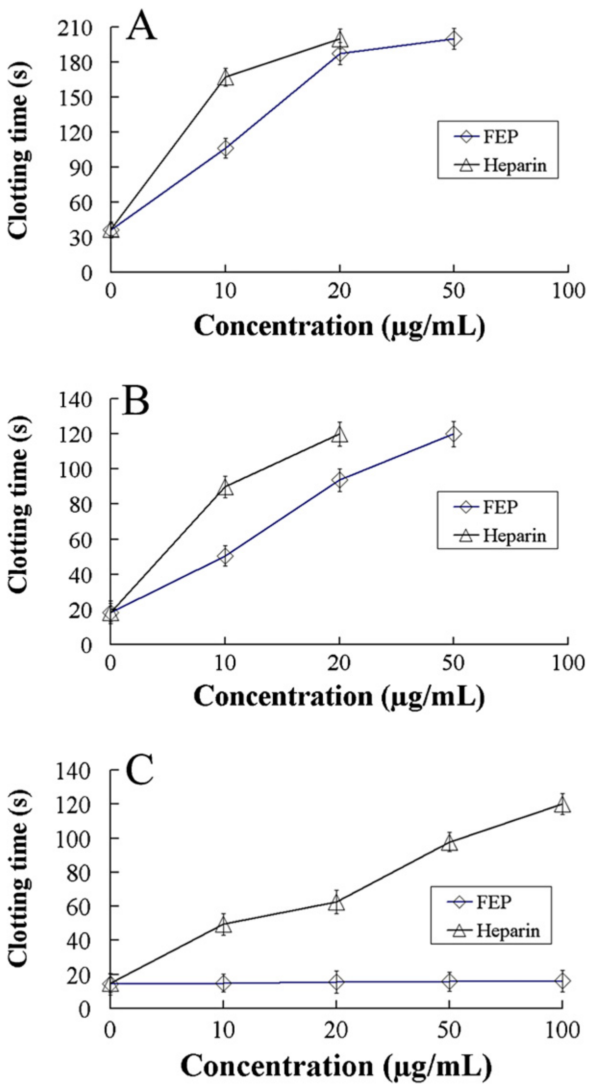

Fig. 6. Analysis of the anticoagulant activity by APTT, TT and PT on the sulfated polysaccharide FEP. Three samples were prepared for assays of every attribute. (A) APTT; (B) TT; and (C) PT.

groups were mainly located at C-3 of (1→4)-linked rhamnose residues and C-2 of (1→4)-linked xylose residues. In addition, FEP demonstrated different structural characteristics from other sulfated arabinans from marine green algae (Ciancia et al., 2007; Uehara et al., 1992). The sulfated polysaccharide isolated from *C. latum* was reported to be a (1→5)-linked furanosidic arabinan with 20.7% sulfate (Uehara et al., 1992). Ciancia et al. (2007) found that the sulfated arabinan from *C. vermilara* was mainly composed of β-L-arabinopyranose residues, and sulfate groups were substituted at C-2 and C-4. The data suggested that marine green algae could be a potential source of sulfated polysaccharides with novel structures.

3.5. *Assay of anticoagulant activity*

Anticoagulant activity of the sulfated polysaccharide FEP was evaluated by the classical coagulation assays APTT, PT and TT using heparin as a reference. The anticoagulant activity of FEP was concentration-dependent. APTT was effectively prolonged by FEP, and the signal for clotting time was more than 200 s at 50 μg/mL (Fig. 6A). The prolongation of APTT indicates inhibition of the intrinsic and/or common pathways of coagulation. Moreover, FEP also strongly extended the TT, and the signal for clotting time was more than 120 s at 50 μg/mL (Fig. 6B). TT is a measure of conversion of fibrinogen to fibrin by thrombin, and an increase in the TT suggests either thrombin inhibition or impaired fibrin polymerization. The anticoagulant activity of FEP was weaker than that of heparin. The effects of FEP on PT were significantly different from that of heparin, and lack of prolongation effect of FEP on PT was discovered (Fig. 6C). No prolongation of PT demonstrates no inhibition of the extrinsic pathway of coagulation. The results suggested that the sulfated polysaccharide FEP inhibited both the intrinsic and/or common pathways of coagulation and thrombin activity or conversion of fibrinogen to fibrin, and did not inhibit the extrinsic pathway of coagulation. Extrinsic pathway activation leads to fibrin clot formation in response to tissue injury while red thrombus or clot in the absence of tissue injury is the result of the intrinsic pathway.

It was noted that the sulfated polysaccharide FEP had higher anticoagulant activity than the sulfated polysaccharides from *M. latissimum* (Zhang et al., 2008). FEP also exhibited higher anticoagulant activity than the crude acidic polysaccharide from brown alga *Laminaria cichorioides* as evaluated by APTT assay (Yoon, Pyun, Hwang, & Mourão, 2007). Pereira et al. (2005) found that the sulfated galactan from the red alga *Gelidium crinale* had a lower anticoagulant activity than a similar polysaccharide from *Botryocladia occidentalis* with different sulfation patterns. The data suggested that monosaccharide composition and the sulfate content and position could influence the interaction of polysaccharide with coagulation inhibitors and their target proteases. In addition, it was observed that the sulfated polysaccharide FEP demonstrated lower anticoagulant activity than the sulfated polysaccharide from *C. latum*, possibly due to different linkage patterns of the arabinose residues in the polysaccharides (Uehara et al., 1992). The differences of anticoagulant activities between the sulfated polysaccharides were directly due to their structural features discrepancy (Pereira, Mulloy, & Mourão, 1999). Further investigation on the structure–activity relationship of the sulfated polysaccharide is required.

4. Conclusion

The water-soluble polysaccharide FEP was isolated from marine green alga *E. clathrata*. On the basis of detailed chemical and spectroscopic analyses, FEP was characterized to be mainly composed of (1→4)-linked β-L-arabinopyranose residues with partially sulfate groups at the C-3 position. FEP was a novel high arabinose-containing sulfated polysaccharide with different chemical characteristics from the sulfated polysaccharides isolated from *Enteromorpha* species and other sulfated arabinans. FEP had high anticoagulant activity and specially interfered with the coagulation cascade at several stages. FEP may be a potential source of anticoagulant polysaccharide with novel structure. Complex relationships existed between the structure and anticoagulant property of the sulfated polysaccharides. Further analysis on the structure–activity relationship of the sulfated polysaccharides will play an important role in the understanding of anticoagulant property. An in-depth investigation on the mechanism of anticoagulant activity of the sulfated polysaccharide is in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.07.077.

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