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Structural characterization and anticoagulant activity of a sulfated polysaccharide from the green alga *Codium divaricatum*

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ABSTRACT

A sulfated polysaccharide, designated CP2-1, was isolated from the green alga *Codium divaricatum* by water extraction and purified by anion-exchange and size-exclusion chromatography. CP2-1 is a galactan which is highly sulfated and substituted with pyruvic acid ketals. On the basis of chemical and spectroscopic analyses, the backbone of CP2-1 was mainly composed of (1→3)-β-D-galactopyranose residues, branched by single (1→)-β-D-galactopyranose units attached to the main chain at C-4 positions. The degree of branching was estimated to be about 12.2%. Sulfate groups were at C-4 of (1→3)-β-D-galactopyranose and C-6 of non-reducing terminal galactose residues. In addition, the ketals of pyruvic acid were found at 3,4- of non-reducing terminal galactose residues forming a five-membered ring. CP2-1 possessed a high anticoagulant activity as assessed by the activated partial thromboplastin time and thrombin time assays. The investigation demonstrated that CP2-1 was an anticoagulant-active sulfated polysaccharide distinguishing from other sulfated polysaccharides from marine green algae.

1. Introduction

Marine algae often possess sulfated polysaccharides with novel structures and diverse biological activities due to their specific marine environment (Wijesekara, Pangestuti, & Kim, 2011). The novel active sulfated polysaccharides from marine algae hold a great potential application in biology and pharmacology (Fernandez, Arata, & Ciancia, 2014; Usov, 2011; Wijesinghe & Jeon, 2012).

The sulfated polysaccharides isolated from marine algae are known to be one good source of naturally occurring anticoagulant-active substances (Melo, Pereira, Fogue, & Mourao, 2004). Various anticoagulant-active sulfated polysaccharides, especially from marine red and brown algae, have been isolated and characterized (Mourao et al., 1996; Pereira et al., 2005). They contain a variety of sulfated fucans and sulfated galactans, and exhibit high anticoagulant activity. The sulfated polysaccharides from marine algae could be a valid alternative to traditional polysaccharides, such as glycosaminoglycans. However, there are fewer reports of anticoagulant-active polysaccharides from marine green algae than those from marine brown and red algae. In recent years, the anticoagulant-active sulfated polysaccharides from green algae were described. Maeda, Uehara, Harada, Sekiguchi, and Hiraoka (1991) revealed that the anticoagulant polysaccharide from *Monostroma nitidum* yielded a sixfold higher activity than that of heparin. Anticoagulant-active sulfated rhamnans from *M. latissimum* were also characterized (Li et al., 2011; Zhang et al., 2008). A sulfated galactan with high anticoagulant activity was isolated from *Codium cylindricum* (Matsubara et al., 2001). The sulfated polysaccharides from marine green algae represent a potential source of anticoagulant to be explored. In contrast with marine red and brown algal sulfated polysaccharides, the structural component of green algal sulfated polysaccharides responsible for the anticoagulant activity has not been characterized (Lahaye & Robic, 2007). In order to understand better the biological activities of the sulfated polysaccharides, a detailed description of their chemical structures is necessary.

The genus *Codium* (Codiaceae, Codiales, Chlorophyta) are widely distributed in the coastal waters, and the sulfated polysaccharides from *Codium* species have drawn attention (Ciancia et al., 2007; Nika, Mulloy, Carpenter, & Gibbs, 2003). In the genus *Codium*, the presence of sulfated arabinan in *C. latum*, sulfated glucan in *C. pugniforme*, sulfated galactan in *C. yezoense* and sulfated mannan in *C. vermilara* have been described (Bilan, Vinogradova, Shashkov, & Usov, 2006; Fernandez, Estevez, Cerezo, & Ciancia, 2012; Matsubara, Matsuura, Hori, & Miyazawa, 2000). Recently,

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a few detailed structural studies have been done on the sulfated polysaccharides from *Codium* species (Bilan, Vinogradova, Shashkov, & Usov, 2007; Farias et al., 2008). However, the structures of the sulfated polysaccharides from *Codium* species have not yet been fully characterized.

In the present work, a novel anticoagulant-active sulfated polysaccharide was isolated from the green alga *Codium divaricatum*, a species that is abundant on the coast of the Yellow Sea of China, and its structural characterization was investigated by a combination of chemical and spectroscopic methods.

2. Materials and methods

2.1. Materials

C. divaricatum was collected in May 2011 from the coast of Yantai, Shandong Province, China. The raw material was thoroughly washed with tap water, air-dried, milled using a blender, and then stored at room temperature in a dry environment. Q Sepharose Fast Flow and Sephacryl S-400/HR were from GE Healthcare Life Sciences (Piscataway, NJ, USA). Dialysis membranes (flat width 44 mm, molecular weight cut-off 3500) were from Lvniao (Yantai, China). Pullulan standards (M_w: 5.9, 9.6, 21.1, 47.1, 107, 200, 344, and 708 kDa) were from Showa Denko K.K. (Tokyo, Japan). APTT assay reagent (ellagic acid + bovine phospholipids reagent) and PT assay reagent (rabbit thromboplastin) were from Shanghai Sun (Shanghai, China). TT assay reagent (bovine thrombin) was from Dade Behring (Deerfield, IL, USA). The remaining reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Isolation and purification of the polysaccharide from C. divaricatum

The milled alga was dipped into 30 volumes of distilled water and extracted at room temperature for 4 h. The supernatant was collected by centrifugation at 3600 × *g* for 10 min, concentrated, dialyzed in a cellulose membrane against distilled water at room temperature for three successive days. The retained fraction was recovered, concentrated by rotary evaporation, precipitated by adding four volumes of 95% ethanol (v/v) and dried at 40 °C to obtain a crude polysaccharide. The crude polysaccharide was fractionated by anion-exchange chromatography using a Q Sepharose Fast Flow column (30 cm × 3.5 cm) and eluted with a step-wise gradient of 0, 0.5, 1.0 and 1.5 M NaCl. Total sugar content of the eluate was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions eluted with 1.0 M NaCl were pooled, dialyzed, loaded onto a Sephacryl S-400/HR column (100 cm × 2.5 cm), and eluted with 0.2 M NH_4HCO_3 at a flow rate of 0.3 mL/min. The major fractions were pooled, desalted, freeze-dried and designated as CP2-1.

2.3. Analytical techniques

The homogeneity and molecular weight were determined by high performance gel permeation chromatography (HPGPC) on a Shodex OHpak SB-804 HQ column (7.8 mm × 300 mm, Tokyo, Japan), and the molecular weight was estimated by reference to a calibration curve made by pullulan standards (Li et al., 2011). Total sugar content was assayed by the phenol–sulfuric acid method using galactose as the standard. Protein content was determined as described by Bradford (1976). Sulfate ester content was measured according to the method of Terho and Hartiala (1971). Uronic acid content was assayed by the carbazole–sulfuric acid method using glucuronic acid as standard (Bitter & Muir, 1962). Pyruvic acid content was determined according to the method of Anthon and Barrett (2003).

2.4. Determination of monosaccharide composition and sugar configuration

Monosaccharide compositions were measured by reversed-phase high performance liquid chromatography (HPLC) after pre-column derivatization (Qi et al., 2012). Briefly, polysaccharide was hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 6 h in a sealed tube. 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 M NaOH, and then added to 120 μL of 0.5 M 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 M HC1 solution and vigorously shaken and centrifuged for 5 min. The supernatant was 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). The chromatograms were performed on an Agilent 1260 Infinity HPLC instrument fitted with Agilent XDB-UV detector (254 nm). The mobile phase was a mixture of 0.1 M 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 (L-rhamnose, L-arabinose, L-fucose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, D-galacturonic acid, D-mannuronic acid and *N*-acetyl-β-D-glucosamine). Calculation of the molar ratio of the monosaccharide was carried out on the basis of the ratio of peak areas of monosaccharide and correspondent monosaccharide standard.

Sugar configuration was determined as described by Tanaka, Nakashima, Ueda, Tomii, and Kouno (2007). 5 mg of polysaccharide was hydrolyzed with 2 M trifluoroacetic acid at 105 °C for 6 h. Excess acid was removed with methanol in a rotary evaporator. The hydrolysate was heated with L-cysteine methyl ester in pyridine at 60 °C for 60 min. A solution of the o-tolyl isothiocyanate was added to the mixture, and was further heated at 60 °C for 60 min. The reaction mixture was directly analyzed on an Agilent 1260 Infinity HPLC instrument using an Eclipse XDB-C_{18} column (4.6 mm × 250 mm) and detected by an Agilent XDB-UV detector at 250 nm. Sugar configuration was identified by comparison with reference sugars.

2.5. Desulfation

Desulfation of the sulfated polysaccharide was performed according to the method of Falshaw and Furneaux (1998). Briefly, CP2-1 (30 mg) was dissolved in water and passed through a 732 cation-exchange resin column (H^+ form), which was eluted with distilled water. The combined effluent was adjusted to pH 9.0 with pyridine and then lyophilized to give a white powdered pyridinium salt. The product was dissolved in 10 mL of dimethyl sulfoxide containing 10% (v/v) of anhydrous methanol and 1% pyridine, and then the solution was shaken at 100 °C for 4 h. After the reaction was completed, the mixture was dialyzed against distilled water for several times and then freeze-dried, named as dsCP2-1.

2.6. Methylation analysis

Methylation analysis was performed as described by Harris, Henry, Blakeney, and Stone (1984). 100 mg of anhydrous NaH was added to 1 mL of dimethyl sulfoxide, and the mixture was stirred under N_2 at room temperature. Polysaccharide (2 mg) was added to the mixture until it turned to a gray-green solution. The mixture

was stirred at room temperature for 1.5 h. CH_3I (0.5 mL) was then added to the mixture and stirred for a further 1.5 h. The reaction was terminated with addition of water, and the residue was extracted with CH_2Cl_2. The extract was washed with distilled water and evaporated to dryness. The completion of methylation was confirmed by IR spectroscopy as the disappearance of OH bands. Then methylated polysaccharide was hydrolyzed with 2 M 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 spectrometry (GC–MS) on a HP6890II/5973 instrument (Agilent Technologies Co. Ltd., USA) using a DB 225 fused silica capillary column (0.25 mm × 30 m). Identification of partially methylated alditol acetates was carried out on the basis of retention time and mass fragmentation patterns.

2.7. IR spectroscopy analysis

Fourier-transform infrared (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}.

2.8. NMR spectroscopy analysis

^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer. Briefly, 50 mg of polysaccharide was dissolved in 1 mL of D_2O (99.97%) followed by freeze-dried. The process was repeated twice, and the final sample was dissolved in 0.5 mL of 99.97% D_2O. ^1H–^1H correlated spectroscopy (COSY), ^1H–^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC), ^1H–^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments were also carried out. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^1H and 31.07 ppm for ^{13}C.

2.9. Assay of anticoagulant activity

Activated partial thromboplastin time (APTT) clotting assay was carried out according to the method of Mourao et al. (1996) using normal human plasma. In the assay, plasma samples (90 μL) were mixed with different amounts of polysaccharide in 0.9% NaCl (10 μL), and incubated at 37 °C for 60 s and then 100 μL prewarmed APTT assay reagent was added and allowed to incubate at 37 °C for 2 min. Prewarmed 0.25 M calcium chloride (100 μL) was then added, and the APTT recorded as the time for clot formation in a coagulometer.

For thrombin time (TT) clotting assay, citrated normal human plasma (90 μL) 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.

Prothrombin time (PT) clotting assay was performed as follows: citrated normal human plasma (90 μL) 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.

The bioassay results were expressed as means ± standard deviation (*SD*). The experimental data were subjected to an analysis of variance for a completely random design, and three samples were prepared for assays of every attribute.

3. Results and discussion

3.1. Chemical characterization of the sulfated polysaccharide CP2-1

The crude polysaccharide was extracted from the green alga *C. divaricatum* with water, and fractionated by a Q Sepharose Fast Flow column into three fractions. The polysaccharide fraction eluted with 1.0 M NaCl was the most abundant fraction and rich in sulfate ester. The fraction was further purified using a Sephacryl S-400/HR column, and a major fraction CP2-1 was obtained. As shown in Fig. 1A, CP2-1 appeared as a single and symmetrical peak in the HPGPC chromatogram, thus CP2-1 could be a homogeneous polysaccharide. The molecular weight of CP2-1 was estimated as 37.9 kDa on the basis of calibration curve established by pullulan standards (Fig. 1B). The sulfate ester content of CP2-1 was 23.7%, and pyruvic acid content was about 6.6%. No protein and uronic acid were detected. Monosaccharide composition analysis by HPLC showed that CP2-1 mainly consisted of galactose residues (97.8%), with minor amounts of glucose (2.16%). In addition, reversed-phase HPLC analysis showed that galactose of CP2-1 was present in the D-configuration (supplemental Fig. 1). The chemical composition of CP2-1 was markedly different from those of other sulfated polysaccharides from *Codium* species (Ciancia et al., 2007; Matsubara et al., 2000, 2001; Uehara, Takeshita, & Maeda, 1992). CP2-1 also has different chemical composition from the sulfated polysaccharide isolated from *Codium yezoense* reported by Bilan et al. (2006), though they are mainly composed of galactose residues. The latter polysaccharide contained traces of arabinose, xylose, mannose and glucose, with small amounts of protein and uronic acid, D-galactose, sulfate and pyruvate in a molar ratio of about 4:2:1.

The FT-IR spectrum of the sulfated polysaccharide CP2-1 is shown in supplemental Fig. 2a. The bands at 1649 and 1453 cm^{-1} were characteristic of carboxilate groups. The band at 1604 cm^{-1} would correspond to the carboxylic group of the pyruvic acid (Chiovitti et al., 1997; Verhoef, de Waard, Schols, Siika-aho, & Voragen, 2003). The signal at 927 cm^{-1} may be due to the absorbance of β-D-galactopyranose. The signals at 843 and 1253 cm^{-1} were assigned to the bending vibration of C—O—S of sulfate ester in axial position and the stretching vibration of S–O of sulfate group, respectively (Mao et al., 2008). The FT-IR spectrum of the desulfated polysaccharide dsCP2-1 was shown in supplemental Fig. 2b. The completion of desulfation reaction of dsCP2-1 was confirmed as the disappearance of C–O–S and S–O bands.

3.2. Methylation analysis

In order to obtain the information on the linkage pattern and the sulfation position in the sulfated polysaccharide CP2-1, a comparative methylation analysis between the native sulfated polysaccharide CP2-1 and its desulfated product dsCP2-1 was carried out. The identification and proportions of the methylated alditol acetates of CP2-1 and dsCP2-1 were listed in Table 1.

Large amounts of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methylgalactitol and 1,3,4,5,6-penta-O-acetyl-2-O-methylgalactitol were detected in CP2-1, indicating that CP2-1 was mainly composed of 3,4-disubstituted galactopyranose and 3,4,6-trisubstituted galactopyranose residues if the galactose was in the pyranose form. 1,3,5-Tri-O-acetyl-2,4,6-tri-O-methylgalactitol, which originated from the 3-substituted galactopyranose residue if the galactose was in the pyranose form, was also found. However, the presence of galactopyranose need be further confirmed by NMR spectroscopy analysis.

Compared with the results for CP2-1, increased amounts of 3-substituted galactopyranose and 3,4-disubstituted galactopyranose residues were detected in dsCP2-1, 3,4,6-trisubstituted

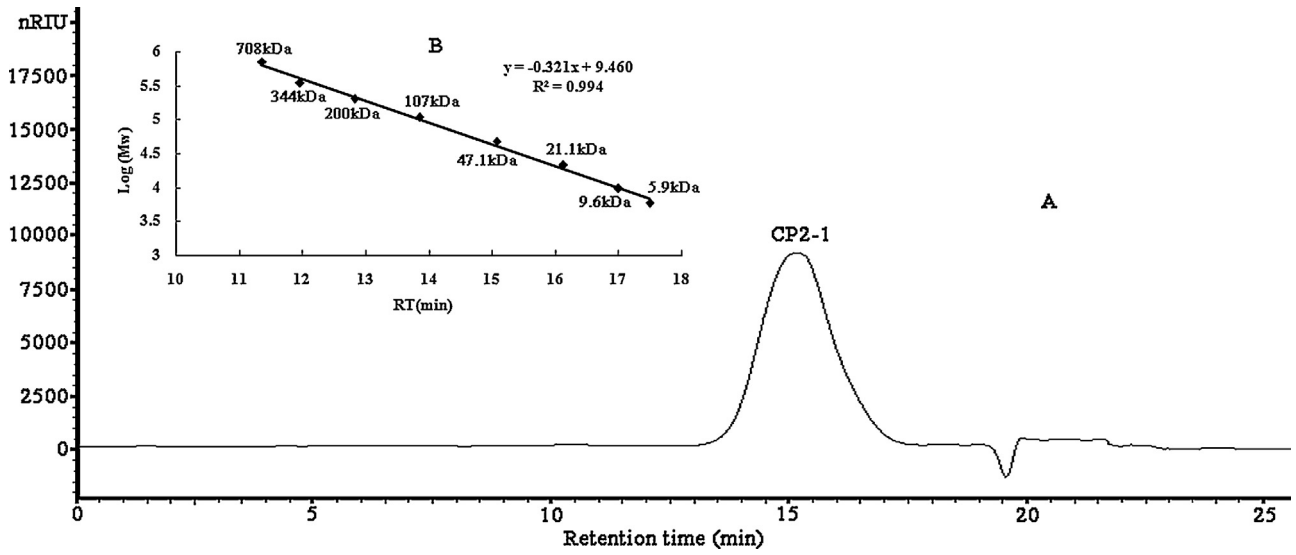

Fig. 1. HPGPC chromatogram of the sulfated polysaccharide CP2-1 and the standard curve of molecular weight. (A) HPGPC chromatogram of CP2-1 and (B) the standard curve of molecular weight.

galactopyranose residue was not detected in dsCP2-1. Thus, the sulfate esters probably occupied the C-4 of 3-substituted galactopyranose and C-6 of 3,4-disubstituted galactopyranose residues. The molar ratios of 3-substituted galactopyranose, 3,4-disubstituted galactopyranose and 3,4,6-trisubstituted galactopyranose residues in the polysaccharide CP2-1 were about 1.00:2.46:2.77. The results suggested that CP2-1 was a sulfated galactan with branching. The degree of branching was calculated to be about 12.2% from the methylation results. 1,3,4,5-Tetra-O-acetyl-2,6-di-O-methylgalactitol in methylation products of dsCP2-1 is formed from pyruvated terminal residues and from branching points. Since no other terminal residues were found, the amount of terminal pyruvated residues should be equal to the branching points. Thus the pyruvic acid content was estimated to be about 6.1%, and the data was slightly lower than the result of quantitative determination.

3.3. NMR spectroscopy analysis

A comparative analysis of 1D, 2D NMR spectra between the native sulfated polysaccharide CP2-1 and its desulfated product dsCP2-1 provided important information for the major glycosidic linkage pattern, sulfation and pyruvation position in the polysaccharide CP2-1.

3.3.1. NMR analysis of the desulfated polysaccharide dsCP2-1

The anomeric region of the ^{1}H NMR spectrum (Fig. 2a) showed two groups of proton signals at 4.72–4.52 ppm. Absence of lower-field signals ($\delta > 5$) was the evidence that the polysaccharide contained β-pyranoses only (Bilan et al., 2007). Other proton signals at 3.58–4.38 ppm were assigned to H2–H6 of the sugar residues. In the ^{13}C NMR spectrum of dsCP2-1 (Fig. 2b), two anomeric carbon signals occurred at 105.5 and 104.7 ppm were assigned to signals of β-galactopyranose because the anomeric carbon signal of β-galactofuranose was at lower field (Nagaoka et al., 1996). The signals at 177.5, 108.7 and 24.7 ppm were attributed to the carbons of COOH, O—C—O and CH_3 groups from pyruvic acid that formed an extra five-membered ring bound to C-3 and C-4 of the galactose residues (Bilan et al., 2007; Shashkov et al., 2000). The signals between 62.3 and 83.5 ppm were assigned to C-2–C-6 of the galactose residues. Three major fragments in dsCP2-1 were deduced as follows (Fig. 2c).

The ^{1}H–^{1}H COSY spectrum (supplemental Fig. 3a) of dsCP2-1 gave various proton correlations of the sugar residues. The ^{1}H–^{13}C HMQC spectrum of dsCP2-1 (supplemental Fig. 3b) afforded some of the correlations between proton and carbon signals with the sugar residues. The anomeric proton signal of A at 4.72 ppm was correlated to the anomeric carbon signal at 104.7 ppm. The ^{1}H–^{13}C HMQC spectrum revealed the substitution of A at C-3 and C-4 due to the downfield shifts of H-3/C-3 and H-4/C-4, thus A was assigned to the 3,4-O-(1-carboxyethylidene)-(1→)-β-galactopyranose residue (Ravenscroft, Parolis, & Parolis, 1994). The anomeric proton signal of B at 4.71 ppm was correlated to the anomeric carbon signal at 105.5 ppm. The correlated signals H-3/C-3 in B further indicated that B was the (1→3)-β-galactopyranose residue (Pereira et al., 2005). The anomeric proton signal of C at 4.52 ppm was correlated to the anomeric carbon signal at 104.7 ppm. Combined with methylation analysis and the chemical shift data of similarly substituted sugar residues (Nie et al., 2013), C was attributed to the chain 3,4-disubstitued β-galactopyranose residue.

In the ^{1}H–^{13}C HMBC (supplemental Fig. 3c) and ^{1}H–^{1}H NOESY (supplemental Fig. 3d) spectra of dsCP2-1, the presence of cross signals H-1 (A)/C-3 (B) and H-1 (B)/C-3 (B) indicated the presence of the linkage 3,4-O-(1-carboxyethylidene)-β-Gal*p*-(1→3)-β-Gal*p*-(1→ and →3)-β-Gal*p*-(1→3)-β-Gal*p*-(1→. The cross signal H-1

Table 1
Identification of the O-methylated alditol acetates derived from CP2-1 and dsCP2-1.

Methylated alditol acetate	Molar ratio		Linkage pattern
	CP2-1	dsCP2-1	
1,3,5-Tri-O-acetyl-2,4,6-tri-O-methylgalactitol	1.00	2.72	→3)-Gal*p*-(1→
1,3,4,5-Tetra-O-acetyl-2,6-di-O-methylgalactitol	2.46	3.53	→3,4)-Gal*p*-(1→
1,3,4,5,6-Penta-O-acetyl-2-O-methylgalactitol	2.77	nd[a]	→3,4,6)-Gal*p*-(1→

[a] Not detected.

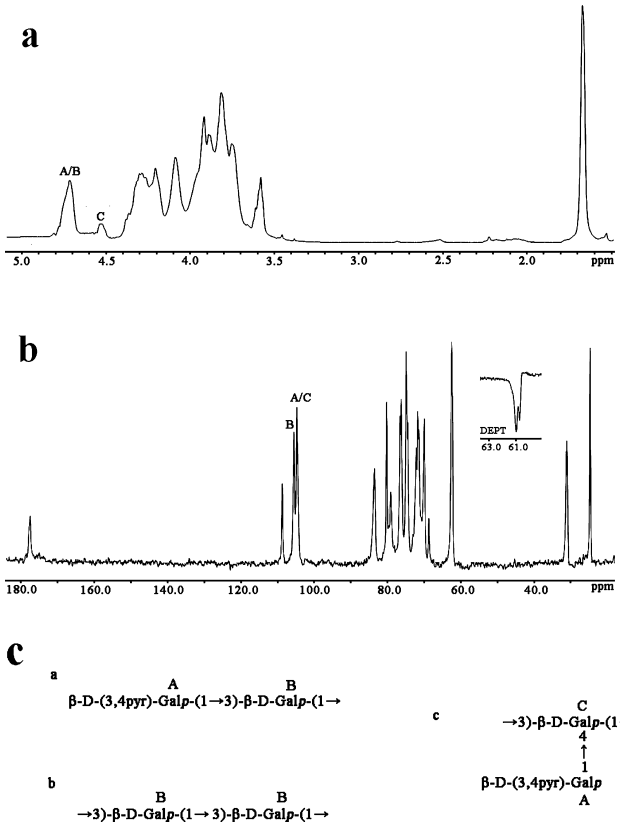

Fig. 2. 1D NMR spectra of the sulfated polysaccharide dsCP2-1. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer using acetone as internal standard. (a) ^{1}H NMR spectrum; (b) ^{13}C NMR and DEPT spectra; and (c) proposed structural fragments of dsCP2-1. (A–C) Correspond to β-(3,4-Pyr)-D-Gal*p*-(1→, →3)-β-D-Gal*p*-(1→, →3,4)-β-D-Gal*p*-(1→, respectively. Gal*p*: galactopyranose. (a) 3,4-O-(1-carboxyethylidene)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→; (b) →3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→; (c) 3,4-O-(1-carboxyethylidene)-β-D-Gal*p*-(1→3,4)-β-D-Gal*p*-(1→.

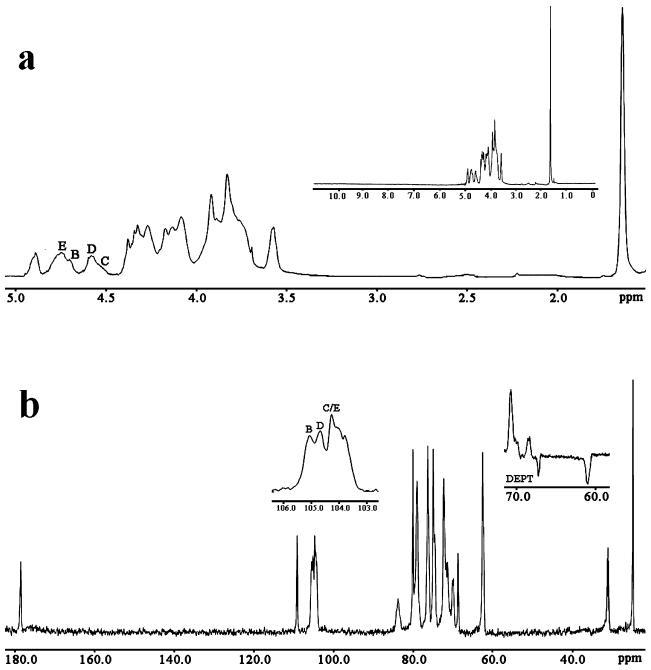

Fig. 3. 1D NMR spectra of the sulfated polysaccharide CP2-1. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer using acetone as internal standard. (a) ^{1}H NMR spectrum and (b) ^{13}C NMR and DEPT spectra. (B)–(E) Correspond to →3)-β-D-Gal*p*-(1→, →3,4)-β-D-Gal*p*-(1→, →3)-β-(4SO_4)-D-Gal*p*-(1→, β-(3,4-Pyr,6SO_4)-D-Gal*p*-(1→, respectively. Gal*p*: galactopyranose.

(A)/H-4 (C) indicated that the H-1 of 3,4-O-(1-carboxyethylidene)-β-Gal*p* was connected to C-4 site of (1→3,4)-β-Gal*p*. The cross signal H-3 (A)/C (O—C—O) confirmed the assignment of residue A (Ravenscroft et al., 1994). Correlation peaks H/C 1.67/108.7 and 1.67/177.5 proved that the major methyl group belongs to a pyruvic acid residue (Bilan et al., 2007). Assignments of the other signals were completed by means of the ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC spectra. The results suggested that dsCP2-1 consisted of 3,4-O-(1-carboxyethylidene)-(1→)-β-galactopyranose, (1→3)-β-galactopyranose and (1→3,4)-β-galactopyranose residues. Signal positions of proton (δ_H 1.67) and carbon (δ_C 24.7) of the methyl group evidenced that the five-membered ketals are in the *S* configuration (Garegg et al., 1980; Shashkov et al., 2000).

3.3.2. NMR analysis of the sulfated polysaccharide CP2-1

In the ^{1}H NMR spectrum of CP2-1 (Fig. 3a), four major anomeric proton signals at 4.75, 4.71, 4.58 and 4.52 ppm were found, and had relative integrals of 2.78:1.00:1.72:0.75. The ^{1}H-signals between 4.4 and 5.0 ppm contained a mixture of signals of H-1 from the β-galactopyranose residues and of H-4 from the sulfated sugar rings. The signal H-4 exhibited a down-field shift (−0.6 ppm) was due to sulfation of C-4 (Pomin et al., 2005). The signal at 2.225 ppm was due to ^{1}H of internal standard acetone. From the ^{13}C NMR spectrum of CP2-1 (Fig. 3b), three major anomeric carbon signals occurred at 105.5, 105.1 and 104.7 ppm.

The ^{1}H–^{1}H COSY spectrum (supplemental Fig. 4a) of CP2-1 gave various proton correlations of the sugar residues. In the ^{1}H–^{13}C HMQC spectrum of CP2-1 (supplemental Fig. 4b), the anomeric proton signal of E at 4.75 ppm was related to the anomeric carbon signal at 104.7 ppm, and E was assigned to the 3,4-O-(1-carboxyethylidene)-6-sulfated-β-galactopyranose residue (Duarte, Noseda, Cardoso, Tulio, & Cerezo, 2002). The anomeric proton signal of B at 4.70 ppm was correlated to the anomeric carbon signal at 105.5 ppm, and B was ascribed to the (1→3)-β-galactopyranose residue. The anomeric carbon signal of D at 105.1 ppm was correlated with the anomeric proton signal at 4.58 ppm, and D was assigned to the (1→3)-β-galactopyranose residue with sulfation at C-4 (Farias et al., 2008). The smaller anomeric proton signal of C at 4.52 ppm was correlated to the anomeric carbon signal at 104.7 ppm, thus C was ascribed to the (1→3,4)-β-galactopyranose residue. In addition, the H-6 signal at 4.44 ppm and its correlated C-6 signal at 68.7 ppm were attributed to the 3,4-O-(1-carboxyethylidene)-6-sulfated-β-galactopyranose residue (Duarte et al., 2002). The H-4 signal at 4.90 ppm and C-4 signal at 79.0 ppm were assigned to the (1→3)-4-sulfated-β-galactopyranose residue. No critical cross signals were found in the ^{1}H–^{13}C HMBC spectrum (supplemental Fig. 4c).

All the analysis results allowed the identification of most of the ^{1}H and ^{13}C signals of the sugar residues (Table 2). CP2-1 was characterized to be composed of 3,4-O-(1-carboxyethylidene)-6-sulfated-β-D-galactopyranose, (1→3)-β-D-galactopyranose, (1→3)-4-sulfated-β-D-galactopyranose and (1→3,4)-β-D-galactopyranose residues.

The sulfated polysaccharide CP2-1 isolated from the green alga *C. divaricatum* had different structural characteristics from other sulfated galactans from marine seaweeds (Fenoradosoa et al., 2009; Pereira et al., 2005). Some pyruvated galactan sulfates were found in red algal polysaccharides, but they generally contained 3-substituted 4,6-*O*-(1-carboxyethylidene)-D-galactopyranose residues (Chiovitti et al., 1997). The carrageenans and agaran from the red algae *Coccotylus truncates*, *Solieria chordalis*

Table 2
^{1}H and ^{13}C chemical shifts for the polysaccharides CP2-1 and dsCP2-1.

Galactose residues[a]	Chemical shifts (ppm)[b]					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	4.72/104.7	3.61/74.4	4.30/80.2	4.21/76.3	4.09/74.9	3.99/62.3
B	4.71/105.5	3.82/71.7	3.92/83.5	4.27/69.9	3.58/74.9	3.71/62.5
C	4.52/104.7	3.75/71.4	3.92/76.3	4.09/74.9	3.72/76.3	3.89/62.5
D	4.58/105.1	3.78/72.3	4.09/80.1	4.89/79.0	3.58/74.9	3.69/62.5
E	4.75/104.7	3.57/74.9	4.27/80.1	4.17/76.1	4.33/71.7	4.44/68.8

[a] A: β-(3,4-Pyr)-D-Gal*p*-(1→, B: →3)-β-D-Gal*p*-(1→, C: →3,4)-β-D-Gal*p*-(1→, D: →3)-β-(4SO_4)-D-Gal*p*-(1→, and E: β-(3,4-Pyr,6SO_4)-D-Gal*p*-(1→.
[b] The spectra were recorded using a JEOL 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.

Table 3
Analysis of the anticoagulant activity by APTT, TT and PT on the sulfated polysaccharide CP2-1.

Clotting time[a] (s)	Sample	Concentration (μg/mL)					
		0	10	50	100	150	200
APTT	CP2-1	35.3 ± 2.9	47.9 ± 2.6	86.8 ± 3.2	102.7 ± 3.5	164.3 ± 4.2	>200
	Heparin	36.4 ± 2.4	138.7 ± 3.5	>200			
TT	CP2-1	18.5 ± 2.2	29.2 ± 2.6	68.7 ± 3.3	88.3 ± 3.1	>120	
	Heparin	18.2 ± 2.3	69.2 ± 3.2	>120			
PT	CP2-1	14.3 ± 1.5	14.5 ± 1.2	15.2 ± 2.0	15.6 ± 1.8	15.9 ± 2.1	16.3 ± 2.3
	Heparin	14.2 ± 1.3	49.4	97.8	>120		

[a] All clotting assays were performed in triplicate. The results were expressed as means ± SD.

and *Palisada flagellifera* contained characteristic galactose units and 4′,6′-pyruvated β-D-galactopyranosyl residues. The pyruvate formed six-membered cyclic ketals with the positions O-4 and O-6 of the β-D-galactoses (Ferreira et al., 2012; Stephanie, Eric, Sophie, Christian, & Yu, 2010; Tuvikene et al., 2009).

So far, pyruvylated galactan sulfates were seldom found in green algal polysaccharides. Bilan et al. (2007) firstly reported a highly pyruvylated galactan sulfate from *C. yezoense*, which contained (1→3)-β-D-galactopyranose residues connected by (1→6) linkages, sulfate groups were mainly at C-4 and in minor amounts at C-6 of the β-D-galactose residues. Pyruvate was forming mainly five-membered ketals with C-3 and C-4 of nonreducing terminal galactose residues, the minor part of pyruvate formed six membered ketals with C-4 and C-6. In addition, the polysaccharide from the green alga *C. isthmocladum* is a predominant linear 4-sulfated (1→3) galactan structure with some units glycosylated or sulfated at C-6. Pyruvate groups are also found, forming five-membered ketals as 3,4-O-(1′carboxy)-ethylidene-β-D-galactose residues (Farias et al., 2008). Recently, Lee, Ohta, Hayashi, and Hayashi (2010) reported the presence of pyruvylated galactan sulfate in *C. fragile*, which is a highly branched polysaccharide consisting of 3-substituted, 3,6-substituted, and non-reducing terminal D-galactose residues with pyruvate and sulfate groups. Ciancia et al. (2007) also reported a polysaccharide from *C. fragile* with some similar features to those of CP2-1, in terms of linkage and pyruvic ketal substitution. But the polysaccharide from *C. fragile* contains, besides 3-linked β-D-galactopyranose residues, a rather high amount of β-L-arabinopyranose residues. Compared with the sulfated galactans from the genus *Codium*, the sulfated galactan CP2-1 from *C. divaricatum* had different structural characteristics. CP2-1 had a small degree of branching with single (1→)-β-D-galactopyranose units attached to the main chain at C-4 positions. Sulfate groups were at C-4 of the (1→3)-β-D-galactopyranose and C-6 of the non-reducing terminal galactose residues simultaneously substituted by pyruvic acid ketals. The present result suggested that the marine green algae could be a potential source of sulfated polysaccharides with unique structures, and be worth being further investigated.

3.4. Anticoagulant activity of the sulfated polysaccharide CP2-1

Anticoagulant activity of the sulfated polysaccharide was evaluated by APTT, TT and PT assays using heparin as a reference. As shown in Table 3, the anticoagulant activity of CP2-1 was concentration-dependent. APTT activity by CP2-1 slowly increased and clotting time was more than 200 s at 200 μg/mL. The prolongation of APTT indicates inhibition of the intrinsic and/or common pathways of coagulant. While APTT activity by heparin quickly increased and clotting time was more than 200 s at 50 μg/mL. The result suggested that CP2-1 may have a lower bleeding risk than heparin at same therapeutic doses and could be a promising antithrombotic agent. The sulfated polysaccharide from the green alga *Caulerpa cupressoides* possessed *in vivo* anti- and prothrombotic effects (Rodrigues et al., 2011). In addition, CP2-1 effectively extended the TT, and the signal for clotting time was more than 120 s at 150 μg/mL. The TT revealed the inhibition of thrombin activity or fibrin polymerization as thrombin inhibition-dependent clotting time. TT activity of CP2-1 was lower than that of heparin, and high concentrations were required to obtain the same effect as with heparin. The effects of CP2-1 on PT were significantly different from that of heparin, and lack of prolongation effect of CP2-1 on PT was discovered. PT is the extrinsic pathway-dependent clotting time. No effect of the anticoagulant on PT suggests that the sulfated polysaccharide CP2-1 did not inhibit extrinsic pathway of coagulation. Thus, the sulfated polysaccharide CP2-1 from *C. divaricatum* inhibited both the intrinsic and/or common pathways of coagulation and thrombin activity or conversion of fibrinogen to fibrin.

It was observed that the sulfated polysaccharide CP2-1 demonstrated similar anticoagulant activity to the sulfated polysaccharides from *C. fragile* and *C. vermilara*, which consisted of 3-linked β-D-galactopyranose and β-L-arabinopyranose residues, they are highly sulfated and substituted with pyruvic acid ketals (Ciancia et al., 2007). In addition, it is interesting to note that CP2-1 had lower anticoagulant activity than the sulfated galactan from *C. cylindricum* (Matsubara et al., 2001), in that both were mainly composed of galactose residues, and sulfate ester for the latter, estimated at 13.1%. 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 sulfated galactans express anticoagulant activity not merely as a function of charge density. The structural basis of this activity certainly depends on the monosaccharide composition, sites of sulfation and/or of the glycosidic linkage, and also on the proportion and/or the distribution of sulfation pattern (Pereira, Melo, & Mourao, 2002). The differences of anticoagulant activities between the sulfated polysaccharides were directly due to their structural features discrepancy. Complex relationships existed between the structure and anticoagulant property of the sulfated polysaccharides. Structural requirements for the interaction of sulfated polysaccharide with coagulation inhibitors and their target proteases were stereo-specific (Melo et al., 2004). The structure and anticoagulant activity relationship of the sulfated polysaccharides from the different species of *Codium* is complex, and requires further in-depth study.

4. Conclusion

A pyruvated galactan sulfate, CP2-1, was obtained from the green alga *C. divaricatum*. The backbone of CP2-1 consists of (1→3)-β-D-galactopyranose residues with a small degree of branching. Pyruvate is forming a five-membered cyclic ketal with O-3 and O-4 of non-reducing terminal galactose residues. CP2-1 has a high anticoagulant activity, and may be a potential source of anticoagulant polysaccharide with novel structure. Further analysis on the structure–activity relationship of the marine algal sulfated galactans will play an important role in the understanding of anticoagulant property. The detailed mechanism of anticoagulant action of CP2-1 is currently still being investigated.

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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.2014.12.036.

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