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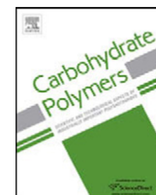

Structural characterization of an anticoagulant-active sulfated polysaccharide isolated from green alga *Monostroma latissimum*

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ABSTRACT

The green alga *Monostroma latissimum* was boiled in hot water to obtain an anticoagulant-active polysaccharide. The crude polysaccharide was further purified on ion-exchange and size-exclusion chromatography to generate a polysaccharide PML of uniform size and charge. PML was a high rhamnose-containing sulfated polysaccharide with an average molecular weight of about 513 kDa. On the basis of detailed one- and two-dimensional nuclear magnetic resonance (1D, 2D NMR) spectroscopic analyses, the chain of the polysaccharide was characterized to consist of (1 → 3)-linked α-L-rhamnopyranose, (1 → 2)-linked α-L-rhamnopyranose and (1 → 2,3)-linked α-L-rhamnopyranose residues in a molar ratio of 4:1:1, and the sulfate groups were substituted at C-2 of the (1 → 3)-linked α-L-rhamnopyranose and C-3 of the (1 → 2)-linked α-L-rhamnopyranose residues. PML had a high anticoagulant activity as evaluated by assays of the activated partial thromboplastin time and thrombin time. The investigation demonstrated that PML appeared to be a sulfated rhamnan with different structural characteristics from other sulfated polysaccharides from Monostromaceae species, and could be a potential source of anticoagulant.
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1. Introduction

Marine green algae often contain bioactive substances with novel functions and structures, and the polysaccharides from green algae are of interest to discovery of new dietary supplements or functional foods (Lee, Hayashi, Hayashi, Sankawa, & Maeda, 1999; Mao et al., 2009; Zhang et al., 2008). Especially, the sulfated polysaccharides from Monostromaceae species show high anticoagulant activity, and represent potential source to be explored (Hayakawa et al., 2000; Maeda, Uehara, Harada, Sekiguchi, & Hiraoka, 1991; Mao et al., 2008).

Until recently, few structural studies have been done on the polysaccharides from Monostromaceae species. Harada and Maeda (1998) reported that a water-soluble sulfated polysaccharide from *Monostroma nitidum* consisted of (1 → 3)-linked rhamnose and sulfate group was located at C-2. A sulfated polysaccharide from *Monostroma latissimum* was found to be composed of (1 → 3)-linked rhamnose and (1 → 2)-linked rhamnose in a molar ratio of 3:2, and the sulfation was substituted at C-3 or C-4 of (1 → 2)-linked rhamnose residues (Lee, Yamagaki, Maeda, & Nakanishi, 1998). A different sulfated polysaccharide from *Monostroma latissimum*, which consisted of (1 → 2)-linked rhamnose with sulfate groups substituted at positions C-3 and/or C-4, was also reported by Mao et al. (2009). However, the major methods currently used in the structural investigations of the sulfated polysaccharides from Monostromaceae were periodate oxidation, Smith degradation and methylation analysis. There were some limitations with these methods though the methylation analysis may offer valuable information about the position of the glycosidic linkage and the site of sulfation (Vieira, Mulloy, & Mourao, 1991). The methylation of sulfated polysaccharide does not always yield reliable proportions of methylated alditols because the steric hindrance of the sulfate esters does not allow complete methylation of these polymers (Mulloy, Ribeiro, Alves, Vieira, & Mourao, 1994; Patankar, Oehninger, Barnett, Williams, & Clark, 1993). Thus, further investigations on the position and proportions of the glycosidic linkage and the sulfation site of the sulfated polysaccharides from Monostromaceae are required. Moreover, the important information on the anomeric configuration of rhamnosyl units could not been obtained by the methylation analysis. An in-depth NMR spectroscopic analysis on the sulfated polysaccharides from Monostromaceae is required, and it can afford important information to identify the anomeric configuration and the ring form of the sugar residues, sites of sulfate esters in sugar residues and linkage patterns between the sugar residues. Furthermore, NMR spectroscopic characterization of sulfated polysaccharides from *M.*

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latissimum will indubitably play an indispensable role in clarifying of its structure–activity relationship.

M. latissimum is widely distributed in China, and has been used as fundamental source of food and drug in traditional Chinese medicine for thousands of years. Recently, the cultivation of green alga, *M. latissimum*, was successfully obtained in China. Thus, the analysis of this species is very important for its application in food and pharmaceutical industry. In the present work, an anticoagulant-active sulfated polysaccharide PML from *M. latissimum* which was cultivated in Yuhuan County was isolated and its structural characteristics were investigated by a combination of chemical and spectroscopic methods, including one- and two-dimensional nuclear magnetic resonance (1D, 2D NMR) spectroscopy techniques.

2. Materials and methods

2.1. Materials

M. latissimum was provided by Yuhuan County, China. It was harvested in April 2005. The raw material was thoroughly washed with tap water, air dried and milled, and then kept in plastic bags at room temperature in a dry environment. Q Sepharose Fast Flow and Sephacryl S-400/HR were from Pharmacia Bioscience (Uppsala, Sweden). Dialysis membranes (flat width 44 mm, molecular weight cut off 3500) were from Lvniao (Yantai, China). Silica Gel 60 high performance TLC plates were from Merck (Darmstadt, Germany). 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). Standard heparin, L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose, D-glucose and D-glucuronic acid were from Sigma (St. Louis, MO, USA). Standard dextrans (*M*w: 5.9, 9.6, 21.1, 47.1, 107, 200, 344, and 708 kDa) were from Showa Denko K.K. (Tokyo, Japan).

2.2. Isolation and purification of the sulfated polysaccharide PML from M. latissimum

The milled algae were dipped into 20 volumes of distilled water and kept at room temperature for 2 h, then filtrated. The residue was dipped into 20 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 against 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. The crude polysaccharide was dissolved in distilled water and fractionated on a Q Sepharose Fast Flow column with a linear gradient of 0–4 mol/L NaCl. The elute was monitored by the phenol–sulfuric acid method. The fraction eluted with 0.5 mol/L NaCl, which was the most abundant fraction, was pooled, dialyzed and further purified on a Sephacryl S-400/HR column eluted with 0.2 mol/L NH_4HCO_3 (pH 8.58). After these steps, a purified sulfated polysaccharide was obtained and named as PML.

2.3. General analysis

Total sugar content was determined by the phenol–sulfuric acid method using rhamnose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was measured by the method of Bradford (1976). Sulfate ester content was estimated according to the method of Therho and Hartiala (1971). Uronic acid content was determined by the carbazole–sulfuric acid method (Bitter & Muir, 1962). Purity and molecular weight was determined by high performance gel permeation chromatography (HPGPC) on a Shodex OHpak SB-804 HQ column, and the column calibration was performed with standard dextrans (*M*w: 5.9, 9.6, 21.1, 47.1, 107, 200, 344, and 708 kDa) (Mao et al., 2009). Cellulose acetate membrane electrophoresis was conducted according to the method described by Nelson, Lyon, Gallagher, Johnson, & Pepys (1991). Monosaccharide compositions were measured by reversed-phase, high performance liquid chromatography (HPLC) after pre-column derivatization and UV detection (Sun et al., 2009).

2.4. Desulfation of the sulfated polysaccharide

Desulfation of the sulfated polysaccharide was performed according to the method of Falshaw and Furneaux (1998). In brief, PML (30 mg) was dissolved in water and passed through an ion-exchange column (731 resin, H^+ form), which was eluted with distilled water. The combined effluent and washes were neutralized with pyridine to pH 7.0 and then lyophilized to give a white powdered pyridinium salt. The product was dissolved in 10 mL of dimethyl sulfoxide (DMSO) 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 diluted with an equal volume of water and adjusted to pH 9.0–9.5 by adding 1 mol/L sodium hydroxide. The desulfated product was recovered by dialysis and then freeze-dried.

2.5. Methylation analysis

The sample was treated according to the method of Hakomori (1964). Polysaccharide (2 mg) was dissolved in DMSO (2 mL), and anhydrous NaH (100–200 mg) was then added. The mixture was stirred at room temperature for 1.5 h. CH_3I (1 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 $CHCl_3$. 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 mol/L trifluoroacetic acid at 105 °C for 6 h, and was converted into its corresponding alditol by reduction with $NaBH_4$ and acetylated. The products were analyzed by gas chromatography–mass spectrometric (GC–MS) equipped with a DB 225MS fused-sillica capillary column using a temperature gradient: first 100–220 °C with a rate of 5 °C/min; then keeping at 220 °C for 15 min. The peaks on the chromatogram were identified from their retention times. GC–MS was performed on an HP6890II instrument.

2.6. Spectroscopy analysis

For IR spectroscopy, sample was mixed with KBr, grounded and pressed into a 1 mm pellet. IR spectrum of the polysaccharide was recorded on a Nicolet Nexus 470 spectrometer. For NMR analysis, the sample was freeze-dried with D_2O (99.97%) three times to remove exchangeable protons, and then dissolved in 0.5 mL of 99.97% D_2O containing acetone as internal standard. ^1H and ^{13}C NMR spectroscopy were performed at 23 °C on a JEOL ECP 600 MHz spectrometer. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^1H and 30.7 ppm for ^{13}C. 2D ^1H–^1H COSY and ^1H–^{13}C HMQC experiments were also carried out using the pulse programs supplied with the Bruker manual.

2.7. Anticoagulant activity assay

Activated partial thromboplastin time (APTT) clotting assay was carried out by the method of Mourâno 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 mol/L calcium chloride (100 μL) was then added, and the APTT recorded as the time for clot formation in a coagulometer. Thrombin time (TT) 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 60 s. Then, 200 μL of TT assay reagent prewarmed to 37 °C was added and clotting time was recorded. For prothrombin time (PT) clotting assay, 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.

2.8. Statistics

All bioassay results were expressed as means ± standard deviation (SD). All analyses were carried out with GraphPad Instat 3.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Composition analysis of the sulfated polysaccharide PML

The sulfated polysaccharide PML was extracted from *M. latissimum* with hot water, and further purified by a combination of Q Sepharose Fast Flow column and Sephacryl S-400/HR column. PML gave a single and symmetrical sharp peak in HPGPC chromatogram, and its average molecular weight was estimated to be about 513 kDa. The cellulose acetate membrane electrophoresis analysis showed that PML migrated as a single band. The results suggested that PML was homogeneous in molecular size and electrical charge. Monosaccharide composition analysis by HPLC demonstrated that PML consisted of large amounts of rhamnose residues (84.0%), with minor amounts of xylose (4.8%), glucose (4.6%), galactose (2.3%), mannose (1.9%) and glucuronic acid (2.4%). Chemical composition analysis indicated that PML contained 74.1% total sugar and 26.1% sulfate ester, with a minor amount of protein (0.2%). The chemical composition of PML was different from that of the sulfated polysaccharide reported by Mao et al. (2009), especially the average molecular weight. The great variation in molecular weights for the polysaccharides was due to the source of the alga and approach utilized for extraction and fractionation.

3.2. Methylation analysis

Generally, it is difficult for the sulfated polysaccharides to get reliable proportions of methylated alditol due to sulfated esters. Thus a comparative analysis between the sulfated polysaccharide PML and its desulfated product dsPML provided important information for the linkage position assignments of each monosaccharide and the sulfation position. A large amount of 1,3,5-tri-O-acetyl-2,4-di-O-methyl-L-rhamnitol, which originated from the (1 → 3)-linked L-rhamnose residue, was found in PML. 1,2,5-Tri-O-acetyl-3,4-di-O-methyl-L-rhamnitol was also detected, indicating the presence of (1 → 2)-linked L-rhamnose residues. In addition, PML should have a partially branched structure because of the presence of 1,2,3,5-tetra-O-acetyl-4-O-methyl-L-rhamnitol, which originated from the (1 → 2,3)-linked L-rhamnose residues. Moreover, increased amounts of (1 → 3)-linked L-rhamnose and (1 → 2)-linked L-rhamnose residues, and lower amounts of (1 → 2,3)-linked L-rhamnose residues were detected in dsPML than PML. Thus, the sulfate substitutions were deduced to be at the C-2 of (1 → 3)-linked L-rhamnose and/or C-3 of (1 → 2)-linked L-rhamnose residues. The molar ratios of (1 → 3)-linked L-rhamnose, (1 → 2)-linked L-rhamnose and (1 → 2,3)-linked L-rhamnose residues in the polysaccharide were about 3.97:1.02:1.00. The linkage pattern and the proportions of PML were markedly different from that of the sulfated polysaccharide isolated from *M. latissimum* which was collected on the coast of Zhejiang, China. The latter mainly consisted of 1,2-linked L-rhamnose residues with sulfate groups substituted at positions C-3 and /or C-4 (Mao et al., 2009). The result showed that the distinction of the alga source, the artificial breeding and the natural alga, might influence the structural characteristics of the sulfated polysaccharides.

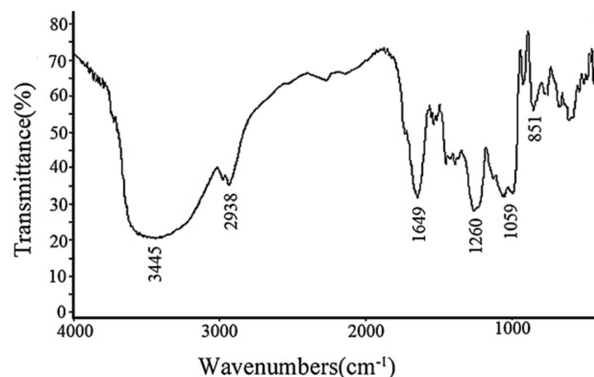

Fig. 1. IR spectrum of the sulfated polysaccharide PML.

3.3. IR spectroscopy analysis

The FTIR spectrum of the sulfated polysaccharide PML showed two bands corresponding to sulfate esters (Fig. 1). The signals at 851 and 1260 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., 2009). The signal at 3445 cm^{-1} was derived from the stretching vibration of O–H, and the signal at 2938 cm^{-1} was attributed to the stretching vibration of C–H. In addition, the signal at 1649 cm^{-1} was due to the bound water, and the signal at 1059 cm^{-1} was derived from the stretching vibration of C–O.

3.4. NMR spectroscopy analysis

In order to obtain the information on the major glycosidic linkage pattern and the sulfation position in the sulfated polysaccharide PML, a comparative analysis of 1D, 2D NMR spectra between the native sulfated polysaccharide PML and its desulfated product dsPML was proceeded.

3.4.1. NMR analysis of the desulfated polysaccharide dsPML

In the ^{1}H NMR spectrum of dsPML (Fig. 2a), the anomeric resonances between 5.38 and 5.07 ppm were assigned to α-L-rhamnopyranose residues. In the anomeric region, three major signals appeared at δ 5.25, 5.23 and 5.07 ppm, and had relative integrals of 1:1:4. The signals between δ 4.40–3.30 ppm were assigned to H-2–H-5 of the rhamnose residues, and the signal at δ 1.35 ppm was attributed to the proton of CH_3 group of the rhamnose residues. In the anomeric region of the ^{13}C NMR spectrum (Fig. 2b), three main carbon resonances occurred at δ 103.3, 102.0 and 100.4 ppm. The signals between δ 81.7–70.4 ppm were attributed to the C-2–C-5 of the rhamnose residues, and the signal at δ 17.8 ppm was assigned to the C-6 of the rhamnose. The α-anomeric configuration of rhamnopyranose residues was also deduced from H-5 signal at δ 3.80 ppm and C-5 signal at δ 70.4 ppm (Cassolato et al., 2008).

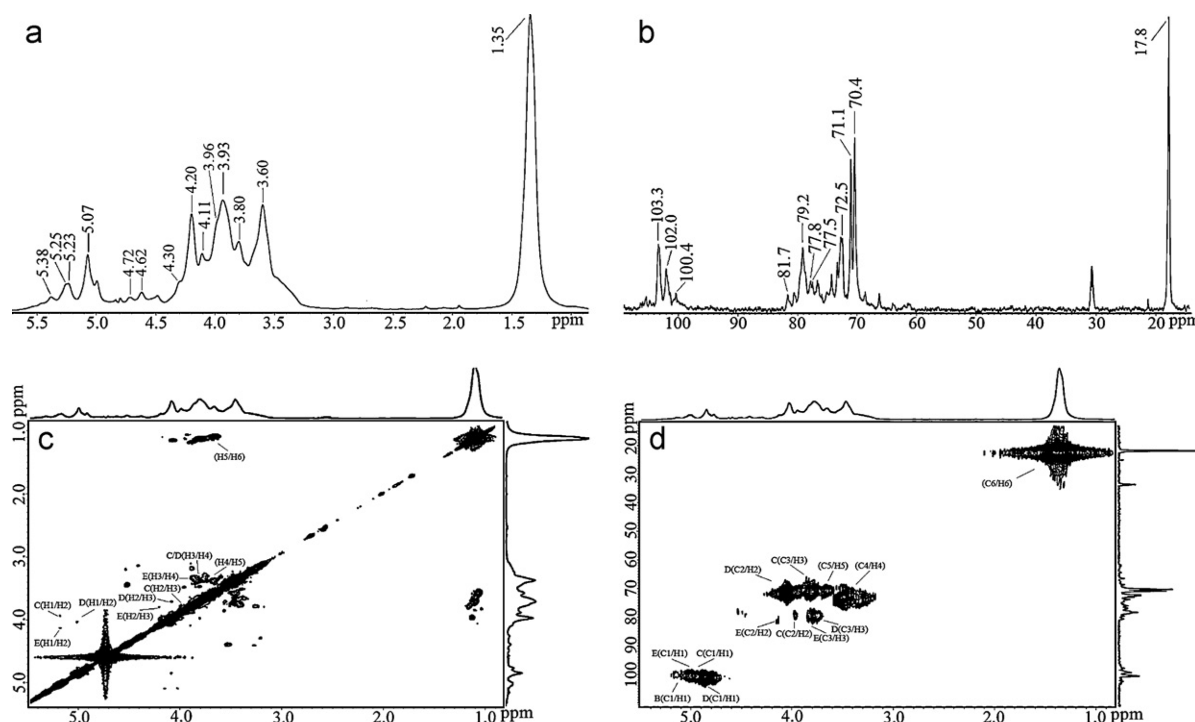

Fig. 2. NMR spectra of the polysaccharide dsPML. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer using acetone as internal standard. (a) ^{1}H NMR spectrum; (b) ^{13}C NMR spectrum; (c) ^{1}H–^{1}H COSY spectrum; (d) ^{1}H–^{13}C HMQC spectrum. C–E correspond to →2)-α-L-Rhap-(1→, →3)-α-L-Rhap-(1→, and →2,3)-α-L-Rhap-(1→, respectively. Rhap: rhamnopyranose.

The ^{1}H–^{1}H COSY spectrum gave various proton correlations of the sugar residues (Fig. 2c). From the ^{1}H–^{13}C HMQC spectrum (Fig. 2d), the anomeric proton signal at δ 5.07 ppm was found to be correlated to the anomeric carbon signal at δ 103.3 ppm, which was assigned to the (1 → 3)-linked rhamnopyranose residues. The anomeric proton signals at δ 5.23 and 5.25 ppm, which were assigned to the (1 → 2)-linked and (1 → 2,3)-linked rhamnopyranose residues, were related to the anomeric carbon signal at δ 102.0 ppm. The assignments for the rhamnose residues are in good agreement with the values reported for the rhamnose residues present in the O-polysaccharide chains of the lipopolysaccharide from *Pseudomonas syringae* pv. *garcae* NCPPB 2708 and *P. syringae* pathovar tomato GSPB 483 (Knirel, Ovod, Zdorovenko, Gvozdyak, & Krohn, 1998; Ovod, Zdorovenko, Shashkov, Kocharova, & Knirel, 2004). It is worthy to note that C-1 signal at δ 100.4 ppm was correlated with the H-1 resonance at δ 5.38 ppm, which was assigned to the (1 → 2)-linked rhamnopyranose residues with sulfation at C-3. The result indicated that the desulfation of dsPML was not absolute. However, compared with the NMR spectra of PML, the signals at δ 4.62 and 4.72 ppm which were substituted by the sulfated groups, with the correlated carbon signals at δ 77.5 and 77.8 ppm, were very weak. The weak signals suggested that the sulfate esters in dsPML have been greatly reduced, and the effect of the desulfation was significant.

Assignments of the other signals were completed by means of the ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC spectra, and also by comparison with the chemical shift data of similarly substituted sugar residues (Gargiulo et al., 2008; Gaur, Galbraith, & Wilkinson, 1998; Senchenkova, Shashkov, Laux, Knirel, & Rudolph, 1999). From the ^{1}H–^{13}C HMQC spectrum, the correlated signals of H-3 at δ 3.93 ppm and C-3 at δ 79.2 ppm were ascribed to the linkage sites of the (1 → 3)-linked rhamnopyranose, and the shoulder signals of the H-3 at δ 3.96 ppm with its related C-3 at δ 79.2 ppm suggested that the protons had different environments in the structure, and it was assigned to the (1 → 2,3)-linked rhamnopyranose. The related signals of H-2 at δ 4.11 ppm and C-2 at δ 79.2 ppm were attributed to the linkage sites of the (1 → 2)-linked rhamnopyranose.

The results confirmed the presence of three types of (1 → 3)-linked α-L-rhamnopyranose, (1 → 2)-linked α-L-rhamnopyranose and (1 → 2,3)-linked α-L-rhamnopyranose residues.

3.4.2. NMR analysis of the sulfated polysaccharide PML

In the ^{1}H NMR spectrum of PML, the anomeric proton signals between 5.46 and 5.08 ppm were assigned to α-L-rhamnopyranose residues (Fig. 3a). The H-1 resonances of at δ 5.46, 5.35, 5.25, 5.23, and 5.08 ppm had relative integrals of 3:1:2:1:5. The ^{1}H NMR and ^{13}C NMR (Fig. 3b) spectra of the sulfated polysaccharide PML showed two anomeric nuclei signals with high strength, which was clearly different from those of dsPML. The ^{1}H–^{1}H COSY (Fig. 3c) and ^{1}H–^{13}C HMQC (Fig. 3d) spectra of PML contain well-defined cross-peaks for the two anomeric nuclei. The anomeric proton signal at δ 5.46 ppm with its correlated anomeric carbon signal at δ 100.6 ppm was assigned to the (1 → 3)-linked 2-sulfated-rhamnopyranose. The anomeric proton signal at δ 5.35 ppm was related to the anomeric carbon signal at δ 100.8 ppm, and was ascribed to the (1 → 2)-linked 3-sulfated-rhamnopyranose residues. Additionally the anomeric carbon signal at δ 103.1 ppm showed correlation with the anomeric proton signal at δ 5.08 ppm, and the signals were assigned to the (1 → 3)-linked rhamnopyranose residues. The anomeric carbon signal at δ 101.5 ppm was correlated with the anomeric proton signals at δ 5.23 and 5.25 ppm, and the signals were ascribed to the (1 → 2)-linked rhamnopyranose and (1 → 2,3)-linked rhamnopyranose residues, respectively.

The ^{1}H–^{13}C HMQC and ^{1}H–^{1}H COSY spectra further afforded some of the correlations between carbon and proton signals with the sugar residues, and elaborated the sites of sulfate esters in sugar residues and the linkage patterns between the sugar residues. The major signals appeared at δ 78.0 and 79.1 ppm were due to the C-2 and C-3 substituted rhamnose residues. The H-2 signal at δ 4.73 ppm and its correlated C-2 signal at δ 78.0 ppm

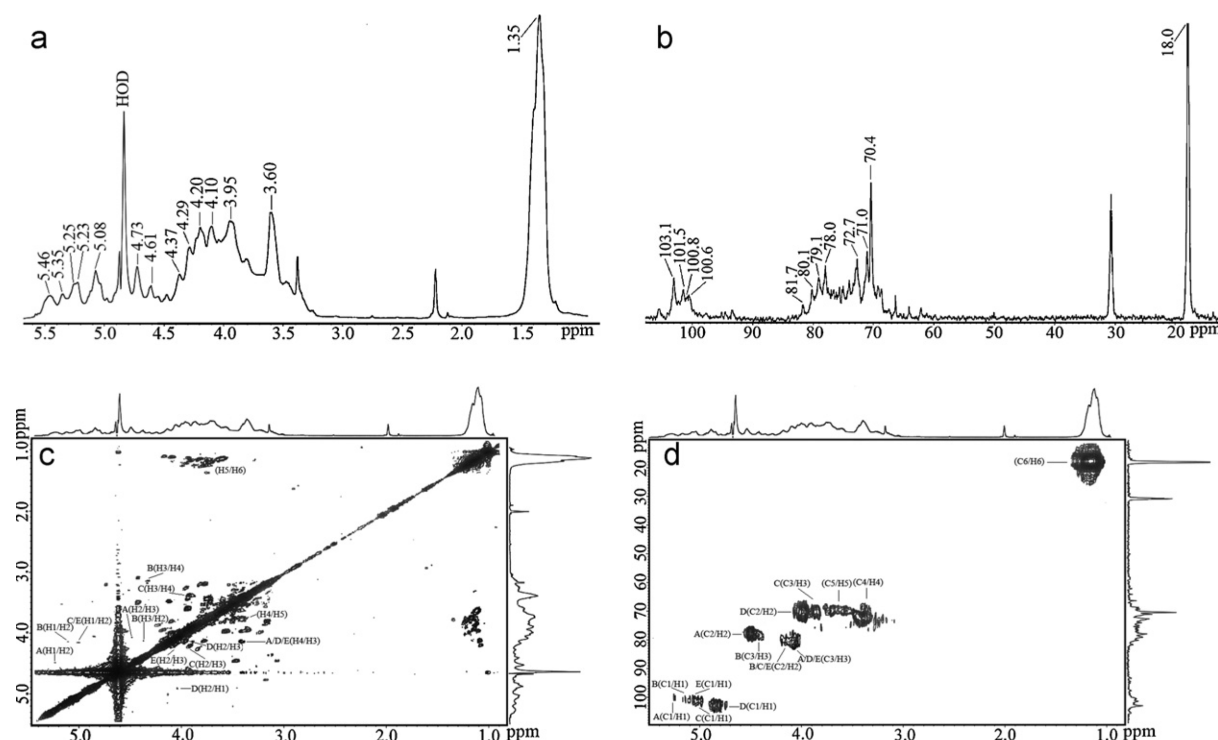

Fig. 3. NMR spectra of the polysaccharide PML. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer using acetone as internal standard. (a) ^{1}H NMR spectrum; (b) ^{13}C NMR spectrum; (c) ^{1}H–^{1}H COSY spectrum; (d) ^{1}H–^{13}C HMQC spectrum. A–E correspond to →3)-α-L-Rha*p*(2SO$_4$)-(1→, →2)-α-L-Rha*p*(3SO$_4$)-(1→, →2)-α-L-Rha*p*-(1→, →3)-α-L-Rha*p*-(1→, and →2,3)-α-L-Rha*p*-(1→, respectively. Rha*p*: rhamnopyranose.

were attributed to the (1 → 3)-linked 2-sulfated-rhamnopyranose residues. The H-3 signal at δ 4.61 ppm and C-3 signal at δ 79.1 ppm were assigned to the (1 → 2)-linked 3-sulfated-rhamnopyranose residues. The influence of sulfation on these resonances was shown by the weak signals in the NMR spectra of dsPML. The results showed the presence of several types of sulfated (1 → 3)-linked α-L-rhamnopyranose and (1 → 2)-linked α-L-rhamnopyranose residues. The H-3 signal at δ 4.29 ppm was correlated with C-3 signal at δ 81.7 ppm, and were assigned to the (1 → 3)-linked rhamnopyranose and (1 → 2,3)-linked rhamnopyranose residues. The proton signal at δ 4.37 ppm with corresponding carbon signal at δ 80.1 ppm were ascribed to H-2 and C-2 of the (1 → 2)-linked and (1 → 2,3)-linked rhamnopyranose units, respectively.

All the analysis results allowed the identification of most of the ^{1}H and ^{13}C signals of the sugar residues (Table 1). Structures of the main repeating disaccharides in the sulfated polysaccharide PML were established as follows (Fig. 4).

3.5. Anticoagulant activity of the sulfated polysaccharide PML

As listed in Table 2, the sulfated polysaccharide PML showed increasing clotting times in the APTT and TT assays that were proportional to its concentration. APTT and TT were effectively prolonged by PML, and the signals for clotting time of the sulfated polysaccharide PML became excessively saturated, which were 16 μg/mL for APTT and TT, respectively. The effect of PML on PT was significantly different from that of heparin, and no clotting inhibition was observed in PT assay even at the concentration at which APTT and TT were prolonged. The results suggested that the sulfated polysaccharide PML 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. The anticoagulant activity of PML was compared with that of heparin, and the result showed that the anticoagulant activity of PML was higher than that of heparin, and low concentrations were required to obtain the same effect as with heparin.

The sulfated polysaccharide PML demonstrated higher anticoagulant activity than other sulfated polysaccharides isolated from *M. latissimum* (Mao et al., 2009; Zhang et al., 2008). However, the anticoagulant activity of PML was lower than that of rhamnan sulfate obtained from *Monostroma nitidum* reported by Harada

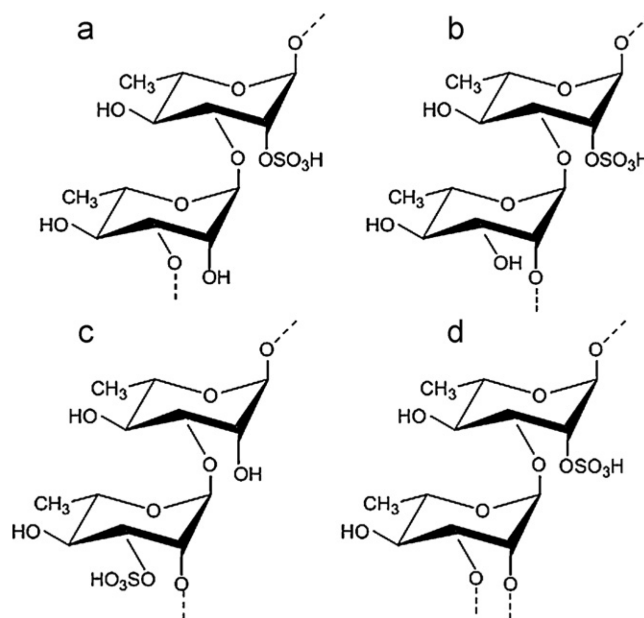

Fig. 4. Structures of the main repeating disaccharides in the sulfated polysaccharide PML. (a) →3)-α-L-Rha*p*(2SO$_4$)-(1 → 3)-α-L-Rha*p*-(1→; (b) →3)-α-L-Rha*p*(2SO$_4$)-(1 → 2)-α-L-Rha*p*-(1→; (c) →3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*(3SO$_4$)-(1→; (d) →3)-α-L-Rha*p*(2SO$_4$)-(1 → 2,3)-α-L-Rha*p*-(1→. Rha*p*: rhamnopyranose.

Table 1
Chemical shifts assignments of NMR spectra of the sulfated polysaccharide PML.

Rhamonsyl residues[a]	Chemical shift (ppm)					
	H_1/C_1	H_2/C_2	H_3/C_3	H_4/C_4	H_5/C_5	H_6/C_6
A	5.46/100.6	4.73/78.0	4.26/81.7	3.60/72.7	3.95/70.4	1.35/18.0
B	5.35/100.8	4.37/80.1	4.61/79.1	3.60/72.7	3.95/70.4	1.35/18.0
C	5.23/101.5	4.37/80.1	4.10/71.0	3.60/72.7	3.95/70.4	1.35/18.0
D	5.08/103.1	4.20/71.0	1.29/81.7	3.60/72.7	3.95/70.4	1.35/18.0
E	5.25/101.5	4.37/801	4.29/81.7	3.60/72.7	3.95/70.4	1.35/18.0

[a] A–E correspond to →3)-α-L-Rha*p*-(2SO_4)-(1→, →2)-α-L-Rha*p*-(3SO_4)-(1→, →2)-α-L-Rha*p*-(1→, →3)-α-L-Rha*p*-(1→, and →2,3)-α-L-Rha*p*-(1→, respectively.

Table 2
Analysis of the anticoagulant activity by APTT, TT and PT on the sulfated polysaccharide PML.

Sample	Concentration (μg/mL)	Clotting time[a] (s)		
		APTT	TT	PT
PML	0	35.2 ± 3.1	18.2 ± 2.5	14.1 ± 1.9
	2	58.6 ± 3.5	35.6 ± 2.8	14.6 ± 1.5
	4	87.8 ± 4.3	55.3 ± 3.6	14.9 ± 1.7
	8	158.4 ± 4.7	103.6 ± 3.2	15.5 ± 2.3
	16	>200	>120	15.7 ± 2.6
Heparin	0	36.4 ± 2.5	18.5 ± 2.4	14.2 ± 1.8
	2	41.5 ± 2.9	29.3 ± 2.2	46.4 ± 2.3
	4	78.2 ± 3.8	40.4 ± 3.5	53.4 ± 2.6
	8	136.7 ± 4.7	68.2 ± 4.3	69.4 ± 3.7
	16	>200	>120	90.2 ± 4.3

[a] Each clotting time represents the average of triplicate experiments. Data are shown as means ± SD in s.

and Maeda (1998), in that both were composed of (1 → 3)- and (1 → 2)-linked rhamnose residues, and sulfate ester for the latter, estimated at DS 0.7, were substituted at C-2 of (1 → 3)-linked rhamnose residues. The results suggested that anticoagulant activity largely depended on the sulfate content and position, and the linkage patterns of rhamnose residues. The nature of the sulfation position could markedly affect the anticoagulant activity of sulfated rhamnan. The detailed mechanisms of the anticoagulant activity of *Monostroma* sulfated polysaccharides are unknown. Complex relationships existed between the structure and anticoagulant properties of the sulfated polysaccharides (Melo, Pereira, Fogue, & Mourão, 2004; Shanmugam & Mody, 2000). Further investigations in the relationship between the fine structure and anticoagulant activity for the different species of *Monostroma* sulfated polysaccharide are required.

4. Conclusion

A sulfated polysaccharide PML with high anticoagulant activity was successfully isolated from *M. latissimum*. Based on a detailed 1D, 2D NMR analysis, PML was characterized to be composed of (1 → 3)-linked α-L-rhamnopyranose, (1 → 2)-linked α-L-rhamnopyranose and (1 → 2,3)-linked α-L-rhamnopyranose residues, and sulfate groups were substituted at positions C-2 of the (1 → 3)-linked α-L-rhamnopyranose and C-3 of the (1 → 2)-linked α-L-rhamnopyranose residues, which makes it a sulfated rhamnan distinguishing it from other sulfated polysaccharides from Monostromaceae species. Four major disaccharides units: (→3)-α-L-Rha*p*(2SO_4)-(1 → 3)-α-L-Rha*p*-(1→], [→3)-α-L-Rha*p*(2SO_4)-(1 → 2)-α-L-Rha*p*-(1→], [→3)-α-L-Rha*p*-(1 → 2)-α-L-Rha*p*(3SO_4)-(1→], and [→3)-α-L-Rha*p*(2SO_4)-(1 → 2,3)-α-L-Rha*p*-(1→], distributed in the polysaccharide chain. PML could be a potential source of anticoagulant and required further investigation as a candidate for use in food supplements or ingredients in the pharmaceutical industry. An in-depth spectroscopic study on comparative structural characterization for different sulfated polysaccharides from Monostromaceae will play an important role in the understanding of the mechanism of anticoagulant activity and the structure–activity relationship.

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