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# Chemical characteristics and anticoagulant activities of a sulfated polysaccharide and its fragments from *Monostroma latissimum*

Hui-Juan Zhang, Wen-Jun Mao *, Fang Fang, Hong-Yan Li, Hai-Hong Sun, Yin Chen, Xiao-Hui Qi

*Institute of Marine Drug and Food, Ocean University of China, Qingdao, Shandong 266003, China*

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**Abstract**

A sulfated polysaccharide from the green alga *Monostroma latissimum* was extracted in hot water and purified by ion-exchange and size-exclusion chromatography. Five sulfated polysaccharide fragments with different molecular weights were prepared from the sulfated polysaccharide by $H_2O_2$ degradation. The molecular weights of the parent sulfated polysaccharide and its fragments were 725.4, 216.4, 123.7, 61.9, 26.0 and 10.6 kDa, respectively. These sulfated polysaccharide preparations have high contents of rhamnose. Anticoagulant activities of the parent sulfated polysaccharide and its fragments were investigated by studying the activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT) using human plasma. The six sulfated polysaccharide preparations did not affect PT even at the concentration at which APTT and TT were prolonged. The sulfated polysaccharides fragments with molecular weights of 216.4–61.9 kDa had similar anticoagulant activities as the parent sulfated polysaccharide. A decrease in the molecular size of the sulfated polysaccharide fragments dramatically reduced their anticoagulant activities. The results indicated that molecular size had an important effect on the anticoagulant activity of the sulfated polysaccharide obtained from *M. latissimum*, and an even longer chain was necessary to achieve thrombin inhibition.


*Keywords:* *Monostroma latissimum*; Sulfated polysaccharide; Molecular weight; Activated partial thromboplastin time; Thrombin time

## 1. Introduction

The green seaweed *Monostroma latissimum* has been used as fundamental source of human food and drug in traditional Chinese medicine for centuries. It grows in the brackish water area in the upper part of the intertidal zone in the warm waters. One particularly interesting feature of the seaweeds is their richness in polysaccharides, which are useful in topical skin treatments and may have some pharmaceutical applications related to healing of muscle tissue. Sulfated polysaccharides from Monostromaceae also exhibit many biological activities such as anticoagulant, antiviral, antiherpetic and antioxidant activities (Lee, Hayashi, Hayashi, Sankawa, & Maeda, 1999; Lee, Hayashi, Maeda, & Hayashi, 2004; Maeda, Uehara, Harada, Sekiguchi, & Hiraoka, 1991; Wu & Pan, 2004). Especially, polysaccharides from Monostromaceae show potent anticoagulant activity. Maeda et al. (1991) discovered that the active polysaccharide extracts from *Monostroma nitidum* yield a sixfold higher activity than that of heparin. Hayakawa et al. (2000) found that two different sulfated polysaccharides from *M. nitidum* and *M. latissimum* had more potent effect on the inhibition of thrombin than heparin or dermatan sulfate. Studies on the structures of the polysaccharides from Monostromaceae have been limited. Some works showed that the polysaccharides from *M. latissimum* mainly consist of $1 \rightarrow 2$ and $1 \rightarrow 3$ linked rhamnose in a ratio of 3:2 and sulfate was mainly at the C-3 or C-4 position of the $1 \rightarrow 2$ linked rhamnose residues (Lee, Yamagaki, Maeda, & Nakanishi, 1998). Harada and

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

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Maeda (1998) obtained a polysaccharide with similar structure in *M. nitidum*.

Previous studies indicated that molecular weight distributions of polysaccharides had influence on their biological activities (Chen & Wang, 1997; Zhou, Sheng, Yao, & Wang, 2006; Zhou et al., 2004; Zhao et al., 2006). Anticoagulant activity largely depends on the molecular size of the polysaccharides and also relate to monosaccharide composition, sulfate content and position (Shanmugam & Mody, 2000). Jurd, Rogers, Blunden, and McLellan (1995) reported that the sulfated proteoglycan with high molecular weight and sulfated polysaccharides with low molecular weight from the same species have strong anticoagulant activity. Nishino, Aizu, and Nagumo (1991) found that heparin cofactor II-mediated antithrombin activity of fucan sulfate is dependent on both its sulfate content and molecular weight. The low molecular weight fucoidan FF7/3 (50 kDa) combines potent anticoagulant and fibrinolytic properties with only minor platelet activating effects (Durig et al., 1997). The inhibitory effects of fucans on both coagulation and cell proliferation were dependent on their sulfation degree and molecular weight (Ferial, Mosstafa, Corinne, & Catherine, 2000).

The relationship between the structure and activity of sulfated polysaccharide from *M. latissimum* has not been fully characterized. The anticoagulant activities of the sulfated polysaccharides with different molecular weights have not been reported. In this study, a sulfated polysaccharide was isolated from *M. latissimum* and it has high anticoagulant activity. Five sulfated polysaccharide fragments with low molecular weight were prepared from the sulfated polysaccharides by oxidative degradation. The chemical characteristics and anticoagulant activities of the six sulfated polysaccharide preparations from *M. latissimum* were investigated. Relationship between the molecular size of the sulfated polysaccharides and the anticoagulant activities is discussed.

## 2. Experimental

### 2.1. Materials

*Monostroma latissimum* was collected on the coast of Zhejiang province, China. The raw material was thoroughly washed with tap water. The sample was air dried, then kept in plastic bags at room temperature in a dry environment. APTT assay regent (ellagic acid + bovine phospholipids reagent), PT assay regent (rabit thromboplastin) were from Shanghai Sun Co. (China). TT assay reagent (bovine thrombin) was from Dade Behring Inc. (USA).

### 2.2. Isolation and purification of the sulfated polysaccharide from Monostroma latissimum

Dried algae were dipped into 20 volumes of distilled water and kept at room temperature for 2 h, then homogenized and refluxed at 100 °C for 2 h. After cooling, the supernatant was separated from the algae residues by centrifugation. The supernatants were concentrated under reduced pressure, dialyzed in cellulose membrane tubing (molecular weight cut off 8,000) against distilled water for three successive days. The retained fraction was recovered, concentrated under reduced pressure, and precipitated by addition of fourfold volume of 95% (v/v) ethanol and washed twice with absolute ethanol, followed by drying at 40 °C to obtain a crude polysaccharide. The crude extraction was fractionated by a Q Sepharose Fast Flow column with a linear gradient of 0–3 mol/L NaCl. The eluate was determined by the phenol–sulfuric acid method. The fraction containing the most abundant hexose was further purified by a Sephacryl S-400/HR column with 0.2 mol/L sodium acetate buffer. The major fractions were pooled, concentrated, desalted and freeze-dried. A purified sulfated polysaccharide was obtained and named as P.

### 2.3. Depolymerization of the sulfated polysaccharide by $H_2O_2$ hydrolysis

Polysaccharide P was dissolved in distilled water. The solution (1%, w/v) was then sealed in a two-necked bottle and degraded by $H_2O_2$ separately in four different conditions. The reaction times, temperatures and $H_2O_2$ concentrations in the depolymerization process are shown in Table 1. After degradation the reaction was terminated by catalase, and cooled to room temperature followed by lyophilization. The resulting solution was fractionated by a Sephacryl S-400/HR column. Five fractions were obtained and named as P1, P2, P3, P4 and P5, respectively.

### 2.4. Composition analysis

Total sugar content was estimated by the phenol–sulfuric acid analysis using rhamnose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Sulfate content was determined after hydrolysis with 1 mol/L HCl according to the methods of Therho and Hartiala (1971). Uronic acid content was determined by the carbazole–sulfuric acid method using glucuronic acid as standard (Bitter & Muir, 1962). The composition of neutral monosaccharide was measured by gas chromatography after converting them into acetylated aldononitrile derivatives. Briefly, 10 mg of polysaccharide was hydrolyzed in a sealed glass tube with 2 mol/L trifluoroacetic acid (TFA) at 105 °C for 10 h. The hydrolysate was evaporated to dryness. The acid was

Table 1
Conditions for $H_2O_2$ degradation of the sulfated polysaccharide from *Monostroma latissimum*

| Fractions | Temperature (°C) | $H_2O_2$ concentration (%) | Time (h) |
|---|---|---|---|
| P1 | 30 | 2.5 | 2 |
| P2, P3 and P4 | 50 | 1.5 | 5 |
| P5 | 50 | 2.5 | 7 |

removed under reduced pressure by repeated co-evaporations with methanol. The hydrolysates were then converted into alditol acetates according to conventional procedures. After adding 10 mg hydroxylammonium and 3 mg inositol (as internal reference), the mixture was dissolved in 0.5 mL pyridine and incubated at 90 °C for 30 min. The mixture was cooled to room temperature. Acetic anhydride (0.5 mL) was then added to the mixture and the solution was incubated at 90 °C for another 30 min. The following neutral monosaccharides were used as references: rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose. Gas chromatography was performed on an HP5890II instrument. The viscosities of samples (0.2 g in 20 mL distilled water, 1% w/v) were determined at 25 °C using an Ubbelohde Viscometer. The relative viscosity $\eta_r$ was obtained by the following equation, $\eta_r = T/T_0$, where $T$ is the solution flow time (degraded samples in 1% w/v distilled water), $T_0$ is the solvent flow time (distilled water).

### 2.5. Measurement of molecular weight

Molecular weights were determined by high performance gel permeation chromatography. The column (A Shodex OHpak SB-804 HQ column, Japan) was maintained at 35 °C and the mobile phase was 0.1 mol/L $Na_2SO_4$ buffer at a flow rate of 0.5 mL/min. The samples were dissolved in 0.1 mol/L $Na_2SO_4$ to reach a final concentration of 0.5% (w/v) and the sample solution was filtered through 0.45 μm filter membrane before injection (20 μL). Detection was at 35 °C with a refractive index detector (Agilent 1100 Series). Column calibration was performed with standard dextrans (Mw: 5.9, 22.8, 47.3, 112, 212, 404 and 788 kDa, respectively, purchased from Fluka). Calculation of the molecular weights of samples was carried out using the Angilent GPC software (USA).

### 2.6. Methylation analysis

Each sample was treated twice according to the method of Hakomori (1964) with some modification. Each sample (2 mg) was dissolved in DMSO (2 mL) and anhydrous NaH (100–200 mg) were then added. The mixture was stirred at room temperature for 1.5 h. Then $CH_3I$ was added to the mixture and stirred for a further 1.5 h. After the reaction was terminated with addition of water, 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. Methylated samples were hydrolyzed with 2 mol/L TFA at 105 °C for 6 h. The methylated products were converted into their corresponding alditols by reduction with $NaBH_4$ and acetylated. The products were analyzed by GC–MS on DB 225 using a temperature gradient: first 100–240 °C with a rate of 5 °C/min; then keeping at 240 °C for 15 min.

### 2.7. Spectroscopy analysis

For IR spectroscopy, samples were mixed with KBr, grounded and pressed into a 1 mm pellet. IR spectra of polysaccharides were recorded on a Nicolet Nexus 470 spectrometer.$^{13}$C NMR spectroscopy was performed at 25 °C on a JEOL ECP 600 MHz spectrometer. Samples were dissolved in 99.97% $D_2O$. Chemical shifts were calculated based on an internal DDS ($\delta^{13}$C: 57.0 ppm) and expressed in parts per million (ppm).

### 2.8. Anticoagulant activity

All coagulation assays were performed with a coagulometer. Activated partial thromboplastin time (APTT) clotting assay was carried out by the method of Mourâno et al. (1996). Human plasma samples (90 μL) were mixed with 10 μL of a solution of different amounts of polysaccharide in 0.9% NaCl and incubated at 37 °C for 60 s before addition of 100 μL of pre-warmed APTT assay reagent and incubation at 37 °C for 2 min. Pre-warmed calcium chloride (100 μL, 0.25 mol/L) was then added and the APTT was recorded as the time for clot formation in a coagulometer.

Prothrombin time (PT) assay was as follows. Citrated normal human plasma (90 μL) was mixed with 10 μL of a solution of algal 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.

Thrombin time (TT) assay was performed as follows. Citrated normal human plasma (90 μL) was mixed with 10 μL of a solution of algal 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.

## 3. Results and discussion

### 3.1. Chemical characteristics of the sulfated polysaccharide and its fragments from Monostroma latissimum

A polysaccharide was extracted from the green alga *M. latissimum* in hot water and purified by ion-exchange and size-exclusion chromatography. The chemical compositions of the polysaccharide are given in Table 2. The polysaccharide was found to contain mainly rhamnose with small amounts of glucose and xylose and trace amounts of galactose and mannose. Its sulfate content was high (21.20%) and the molecular weight was 725.4 kDa. The results demonstrated that the polysaccharide from *M. latissimum* had a difference chemical composition to *Chlorophyta* from other species, was high rhamnose-containing sulfated polysaccharide.

The sulfated polysaccharide from *M. latissimum* was hydrolyzed into smaller fragments with different molecular weights by $H_2O_2$ degradation. The major factors affecting

Table 2
Chemical compositions and molecular weights of the sulfated polysaccharide and its fragments from *Monostroma latissimum*

| Sample | Neutral monosaccharide content (mol %) | | | | | Uronic acid content (%) | Sulfate content (%) | Molecular weight (kDa) |
|---|---|---|---|---|---|---|---|---|
| | Rha | Glu | Xyl | Man | Gal | | | |
| P | 78.65 | 11.49 | 7.83 | 2.03 | nd | 10.77 | 21.20 | 725.4 |
| P1 | 84.94 | 8.55 | 4.79 | nd | 1.72 | 13.55 | 22.71 | 216.4 |
| P2 | 84.65 | 8.33 | 5.05 | nd | 1.97 | 11.25 | 24.73 | 123.7 |
| P3 | 85.77 | 7.29 | 5.72 | nd | 1.22 | 12.06 | 25.48 | 61.9 |
| P4 | 80.35 | 10.07 | 5.28 | 1.43 | 2.87 | 14.58 | 27.28 | 26.0 |
| P5 | 78.28 | 10.02 | 8.60 | 1.66 | 1.44 | 13.50 | 24.30 | 10.6 |

*Note.* Rha, rhamnose; Glu, glucose; Xyl, xylose; Man, mannose; Gal, galactose; nd, below detection limit (0.001).

the rate of degradation were investigated and these were shown in Fig. 1. The sulfated polysaccharides fragments with different molecular size could be prepared by changing the reaction time, temperature and $H_2O_2$ concentration. As expected, higher concentrations of $H_2O_2$ and temperature lead to a higher extent of degradation. The relative viscosity reduced after degradation with the temperature increased, $H_2O_2$ concentration increased and time lengthened. Temperature was the main factor. Comparing with temperature, effect of $H_2O_2$ concentration was minus. Five sulfated polysaccharide fragments with lower molecular weights were produced by oxidative degradation. As shown in Table 2, the molecular weights of the sulfated polysaccharide fragments were 216.4, 123.7, 61.9, 26.0 and 10.6 kDa, respectively, as determined by high performance gel permeation chromatography (Fig. 2). Moreover, chemical and instrumental analysis indicated that chemical components and structure of the sulfated polysaccharide fragments are similar to that of the parent sulfated polysaccharide.

IR spectra of the sulfated polysaccharide preparations showed several bands corresponding to sulfate ester (Fig. 3): the peaks at 838 and 1260 $cm^{-1}$ are derived from the bending vibration of C–O–S of sulfate in axial position and the stretching vibration of S–O of sulfate, respectively. The signals at 3411 and 1049 $cm^{-1}$ are from stretching vibration of O–H and C–O, respectively. In addition, signals at 1615 $cm^{-1}$ was due to the asymmetric stretch vibration of $COO^-$ of uronic acids; 2935 $cm^{-1}$, the stretch vibration of C–H; 1451 $cm^{-1}$, the symmetric stretch vibration of $COO^-$ and the stretch vibration of C–O within COOH. IR spectroscopy indicated that the parent sulfated polysaccharide and its fragments are structurally similar.

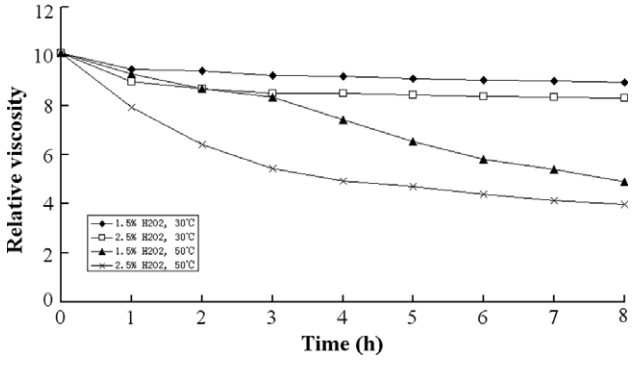


Fig. 1. Effects of the reaction time, temperature and $H_2O_2$ concentration on depolymerization of the sulfated polysaccharide from *Monostroma latissimum*. Degradation of the sulfated polysaccharide is assessed by its relative viscosity.

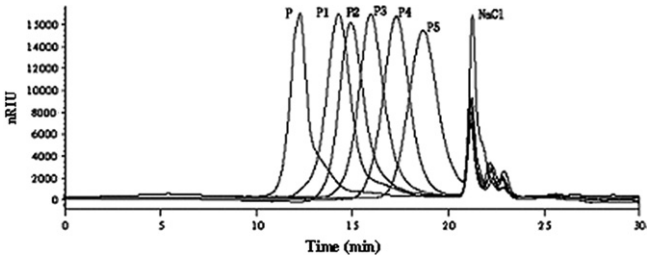


Fig. 2. HPGPC profiles of the sulfated polysaccharide and its fragments from *Monostroma latissimum*.

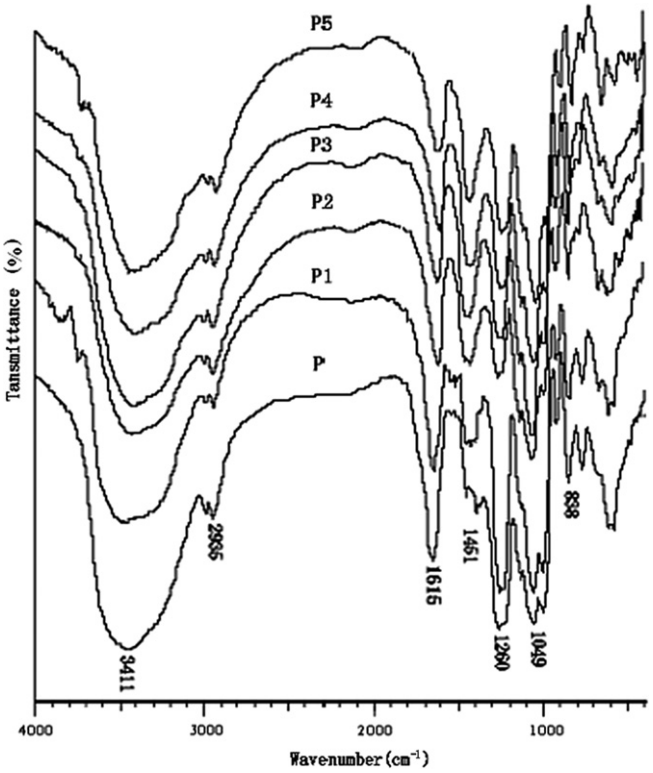


Fig. 3. IR spectra of the sulfated polysaccharide and its fragments from *Monostroma latissimum*.

The structures of the sulfated polysaccharide P and its fragment P3 as a representative of the fragments were further studied by $^{13}C$ NMR spectroscopy. Signals at 104.5 and 102.7 ppm corresponded to C-1 of glucuronic acid and rhamnose. Signals at 19.6 ppm corresponded to C-6 of rhamnose. These data showed that the basic structure of P3 was similar to P.

The methylation analysis demonstrated that the parent sulfated polysaccharide and its fragment mainly included 1,3-; 1,2-; 1,2,3-; 1,2,3,4- and 1,2,4-linked rhamnose (Table 3). The linkage patterns of two polysaccharides are similar. These results indicated that the sulfated polysaccharide fragments with different molecular weights could be obtained by changing the reaction time, temperature and $H_2O_2$ concentration in the depolymerization process. The method of $H_2O_2$ depolymerization could effectively break glycosidic linkages in the sulfated polysaccharide isolated from *M. latissimum* without destroying the basic chemical structure of the sulfated polysaccharide.

### 3.2. Anticoagulant activity of the sulfated polysaccharide and its fragments

The complicated structure and the high molecular weight of the polysaccharide from *M. latissimum* make the anticoagulant mechanism study difficult. For this reason, we prepared well-characterized low molecular weight fractions, which may give some important information necessary to understand their interactions with biological targets and their resulting biological activities.

Anticoagulant activities of the sulfated polysaccharide preparations with different molecular weights were evaluated by APTT, PT and TT assays. APTT and TT were effectively prolonged, but no clotting inhibition was observed in PT assay at the same concentrations (Figs. 4–6). The signals for clotting time of the sulfated polysaccharide fragment with a lower molecular weight (P1, P2 and P3) becomes excessively saturated with high concentration level (200 μg/mL for APTT and 100 and 200 μg/mL for TT), at 200 and 120 s points in Figs. 4 and 5. Prolongation of APTT usually suggests inhibition of the intrinsic and/or common pathway, whereas prolongation of TT indicates inhibition of thrombin activity or fibrin polymerization. Thus, the sulfated polysaccharides isolated from *M. latissimum* inhibited both the intrinsic and or common pathways of coagulation and thrombin activity or conversion of fibrinogen to fibrin (Matsubara et al., 2001). No effect of the anticoagulant on PT suggests that the sulfated polysaccharides from *M. latissimum* did not inhibit extrinsic pathway of coagulation.

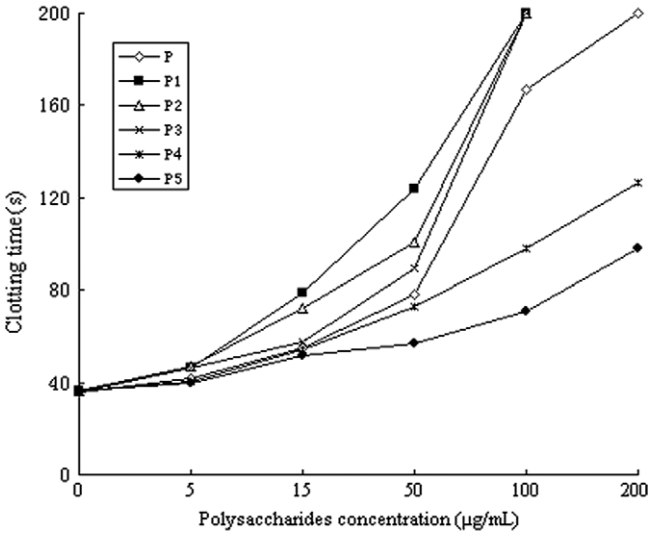


Fig. 4. An analysis of the anticoagulant activity by APTT on the sulfated polysaccharide and its fragments from *Monostroma latissimum*. Results representative of four separated experiments.

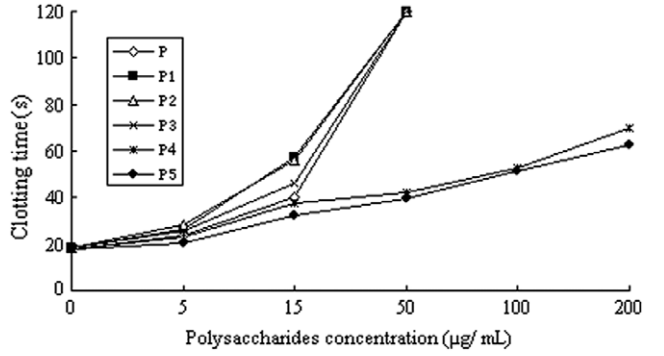


Fig. 5. An analysis of anticoagulant activity by TT on the sulfated polysaccharide and its fragments from *Monostroma latissimum*. Results representative of four separated experiments.

In addition, the sulfated polysaccharides with different molecular weights showed different anticoagulant activities. The sulfated polysaccharides fragments with higher molecular weight ranging from 216.4–61.9 kDa had slightly higher anticoagulant activities than their parent sulfated

Table 3
Methylation analysis of the sulfated polysaccharide P and its fragment P3

| Retention time (min) | Methylation product | Molar ratio | | Linkage pattern |
|---|---|---|---|---|
| | | P | P3 | |
| 21.34 | 1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl-L-Rha | 1 | 1 | →3) Rha(l→ |
| 20.98 | 1,2,5-Tri-*O*-acetyl-3,4-di-*O*-methyl-L-Rha | 2.66 | 3.50 | →2) Rha(l→ |
| 23.34 | 1,2,3,5-Tetro-*O*-acetyl-4-*O*-methyl-L-Rha | 0.52 | 1.19 | →2,3) Rha(l→ |
| 23.90 | 1,2,4,5-Tetra-*O*-acetyl-3-*O*-methyl-L-Rha | 1.80 | 1.30 | →2,4) Rha(l→ |
| 24.32 | 1,2,3,4,5-Penta-*O*-acet-L-Rha | 1.87 | 2.30 | →2,3,4) Rha(l→ |

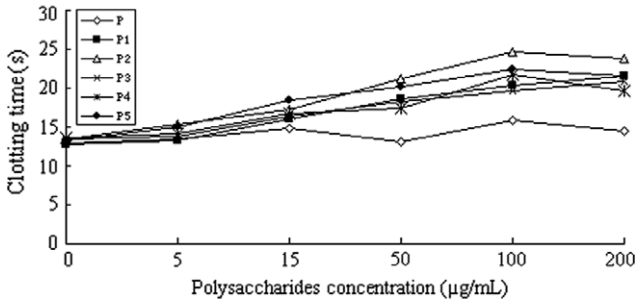


Fig. 6. An analysis of the anticoagulant activity by PT on the sulfated polysaccharide and its fragments from *Monostroma latissimum*. Results representative of four separated experiments.

polysaccharide at the same concentration. The sulfated polysaccharide fragment with a lower molecular weight (P4) had a lower anticoagulant activity. The sulfated polysaccharide fragment with the lowest molecular weight (P5) had the lowest anticoagulant activity. The similar results were observed in the TT assay. These results suggest that the molecular size has a profound effect on the anticoagulant activity of the sulfated polysaccharides from *M. latissimum*, and plays an important role in anticoagulant action. The sulfated polysaccharides from *M. latissimum* require longer chains to achieve complete thrombin inhibition. In the study, heparin was used as a positive control. The anticoagulant activities of the sulfated polysaccharides and its fragments were weaker than that of heparin.

The relationship between structure and anticoagulant activity has been previously investigated in detail for galactan and fucans (Colliec et al., 1991; Hayakawa et al., 2000; Pereira, Mulloy, & Mourão, 1999). Melo, Pereira, Fogue, and Mourão (2004) found that the paradigm of heparin–antithrombin interaction couldn't be extended to other sulfated polysaccharides. Each type of polysaccharide may form a particular complex with the plasma inhibitor and the target protease. The structural requirements for the interaction of sulfated polysaccharides with coagulation inhibitors and their target proteases are not merely a consequence of their charge density. The structural basis of this interaction is complex because it involves naturally heterogeneous polysaccharides but depends on the distribution of sulfate groups and on monosaccharides composition. An in-depth knowledge of the sulfated polysaccharides chemical characteristics is a prerequisite to the understanding of their biological activity.

## 4. Conclusion

The chemical composition of cell wall polysaccharides from *M. latissimum* possesses a high rhamnose-containing sulfated polysaccharide, which differs from the other Chlorophyta (Lahaye, Inizan, & Vigouroux, 1998; Lahaye & Ray, 1996). Five sulfated polysaccharide fragments with different molecular weights were first prepared from the sulfated polysaccharide obtained from *M. latissimum* by $H_2O_2$ degradation. The molecular weights of the parent sulfated polysaccharide and its fragments were 725.4, 216.4, 123.7, 61.9, 26.0 and 10.6 kDa, respectively. The chemical compositions and structures of the sulfated polysaccharide fragments are similar to the parent sulfated polysaccharide isolated from *M. latissimum*. The six sulfated polysaccharide preparations inhibited both the intrinsic and/or common pathways of coagulation and thrombin activity or conversion of fibrinogen to fibrin. The molecular size had important effect on the anticoagulant activity of the sulfated polysaccharide obtained from *M. latissimum* and a longer chain was necessary to achieve thrombin inhibition. Further structural studies on the sulfated polysaccharides isolated from *M. latissimum* will play an indispensable role in the understanding of the mechanism of anticoagulant activity. The detailed mechanism of anticoagulant action of the sulfated polysaccharide from *M. latissimum* is currently still being investigated.

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