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Preparation and anticoagulant activity of a fucosylated polysaccharide sulfate from a sea cucumber *Acaudina molpadioidea*

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ABSTRACT

A fucosylated polysaccharide sulfate, AMP-2, was purified by DEAE-Sepharose Fast Flow and Sephadex G-100 columns in successive steps from a special sea cucumber in southeastern China. HPLC and cellulose acetate membrane electrophoresis experiments confirmed AMP-2 was a homogenous carbohydrate with a relative molecular weight of ca. 2.4×10^4 Da, and methylation analysis indicated that polysaccharide was composed of 1-substituted-Gal*p*, 1,4-disubstituted-GalN*p*, 1,2-disubstituted-FucS*p*, 1,4,6-trisubstituted-Glc*p* in a molar ratio of ca. 0.5:2.0:1.0:3.0, together with a small amount of different substituted Man*p*. Sulfated derivative and carboxymethylated derivative were prepared using dry pyridine and chlorosulfonic acid, and chloroacetic acid, respectively. Anticoagulant activities *in vitro* investigation showed that sulfated derivative showed a stronger ability than native polysaccharide and carboxymethylated derivative, which might be caused by their different percentages and types of functional groups in their structures.

1. Introduction

Sea cucumber *Acaudina molpadioidea*, belonging to Echinodermata, Holothurioider, has become an important sea food and resource of pharmaceuticals. There are about 1100 types of sea cucumbers all over the world, including 140 types existing in China (Jiang, Yang, & Tai, 2004). As a special sea cucumber living in Southeastern China, *A. molpadioidea* is extremely rich with estimated at least 1 million tons. However, seldom researches has paid their attentions on it (Moll & Roberts, 2002; Zhao et al., 2007, 2009), and the processing industry chain development is also very lagging according to our research group investigation.

Cardiovascular and cerebrovascular disease has become a major cause of death behind cancer. Most of thromboembolism patients require anticoagulation therapy during surgery (Rocha et al., 2005). Un-fractionated heparin and low molecular weight heparin are the current anticoagulant polysaccharide drugs (Moll & Roberts, 2002). However, clinical indicated that heparin was not an ideal anticoagulant due to many limitations such as short half-life, leading to osteoporosis, requiring continuous intravenous injection. Except for mentioned above, heparin can produce the life-threatening syndrome, namely, heparin-induced thromobocytopenia-II (HIT). If the patient was occurred by HIT, 51% of patients will be lead to thromboembolic complications and 37% of patients will die (Wei, 2006). At the same time, commercial production of heparin are refined from pig intestines or cow intestine, which may be infected by viruses (Rocha et al., 2005). Therefore, it has become a research hot-spot to find an alternative anticoagulant and antithrombotic drug.

Previous studies have shown that some mucopolysaccharides extracted from sea cucumber including *Stichopus japonicus*, *Holothuria leucospilota* with a variety of biological effects, such as anticoagulant and anti-thrombosis activities (Glauser, Pereira, Monteiro, & Mourao, 2008; Kariya, Sakai, Kaneko, Suzuki, & Kyogashima, 2002; Suzuki, Kitazato, Takamatsu, & Saito, 1991; Zancan & Mourao, 2004), anti-tumor (Lu & Wang, 2009; Zhang et al., 2009), inhibit bone stromal hyperplasia (Kariya et al., 2004), protection of neural tissue (Zhang et al., 2010), of which the anticoagulant and antithrombotic effect is the most prominent function. More fortunately, some research reported that sea cucumber polysaccharides cannot be degraded by enzyme, which can degrade the fucose-polysaccharide from mammalian, and the sea cucumber polysaccharide exhibited a sound anticoagulant effect when it was taken by oral in mouse trials. These evidences provide a potential of producing an oral anticoagulant with sea cucumber polysaccharides (Fonseca & Mourao, 2006). At the same time, the potential agent can make up for limitations of some oral anticoagulations,

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such as Warfarin. So, it might be a good opportunity and an effective way to develop a new anticoagulant to overcome the shortcomings of existing anticoagulant using southeastern China sea cucumber polysaccharide as raw materials.

2. Materials and methods

2.1. Materials and reagents

The sea cucumber *A. molpadioidea* was provided by Ninghai East Ocean Economic Development Co., Ltd., Zhejiang Province, P.R. China. Trypsin (262 U/g), pepsin (3000–35,000 NFU/mg) were bought from Sigma Company (Shanghai, China). The media of DEAE Sepharose Fast Flow and Sephadex G-10 were purchased from Pharmacia Company (Shanghai, China). All other reagents were of analytical grade from Sinopharm Chemical Reagent Co., Ltd.

2.2. General analysis

The polysaccharides were detected by the phenol–sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). IR spectra were recorded on a Bruker EQUIXOX55 spectrometer from 4000 to 400 cm^{-1}, and the frequency of scanning was 4 cm^{-1} (Ye et al., 2009).

2.3. Purification of native fucosylated polysaccharide sulfate

The detailed protocol of purification native polysaccharide from *A. molpadioidea* was as follows: the dry bodies of sea cucumber were soaked with tap-water for 16 h in order to desalt and demineralization, and were chopped into small slices and digested with 6 vol 0.5 mol/L K_2CO_3 at 60 °C for 1.5 h. After that, the pH of digested solution was adjusted to 1.12 with 6 mol/L HCl. Then, the solution was digested with 0.95% pepsin for 5.4 h at 56 °C. At this point, the crude polysaccharide productivity was 1.59%. The crude polysaccharide was dissolved in distilled water (concentration: 50 mg/mL) and applied to a DEAE-Sepharose Fast Flow column. The column was eluted first with distilled water and then with a gradient NaCl solution using a flow rate of 3 mL/min and fractions of 2 mL/tube. The polysaccharide in the eluted fractions was detected using phenol–sulfuric acid method (DuBois et al., 1956). The second polysaccharide-positive peak eluted at ca. 0.35 mol/L NaCl was pooled, dialyzed and lyophilized. As a next step, the Sephadex G-100 was used for further purifying and the fractions eluted at around at K_{av} of 0.45 were pooled with productivity of ca. 0.48%, and was designated as AMP-2.

2.4. Homogeneity and molecular weight

HPLC and cellulose acetate membrane electrophoresis (CAME) were used for evaluating homogeneities of polysaccharide AMP-2 (1) HPLC (Wang, Huang, Nakamura, Burchard, & Hallett, 2005): the measurement were completed with HPLC on a linked column of TSK PW 4000 and PW 3000, using 0.3 mol/L $NaNO_3$ and 0.1 mol/L NaH_2PO_4 as mobile phase. The concentration of polysaccharide AMP-2 was prepared to be of 1 mg/mL, and the inject volume was 15 μL. Column and detector temperatures were set at 30 °C, and the flow rate was 1.0 mL/min prior to degas with ultrasound. (2) CAME: cellulose acetate film (2 cm × 8 cm) was immersed in 0.025 mol/L borate buffer solution for 20 min. To get rid of excess buffer, the film was then sandwiched within two layers filter papers. The experiment was carried out under voltage of 100 V, current electricity of 0.4 mA/cm for 35 min. Carbohydrate bands were visualized by Toluidine Blue staining. The molecular weight of the polysaccharide AMP-2 was determined by HPLC equipped with a ZORBAX PSM 1000 GPC-SEC gel filtration column, and the elution was monitored by a RI detector. The columns were calibrated with Dextran T-3, 10, 70, 500, 2000 as standards.

2.5. Monosaccharide analysis

AMP-2 was hydrolyzed with 2 mol/L trichloroacetic acid at 120 °C for 6 h and was vacuum-dried about 40 °C. Trichloroacetic acid was removed by addition of 3–4 mL of methanol and evaporating repeatedly (3–4 times) using a rotary evaporator. To the dry samples, 10 mL of hydroxylamine hydrochloride, 0.5 mL of anhydrous pyridine were added and mixed. The mixed solution was heated in 90 °C water bath. After cooling, acetylation reaction was conducted by addition of 0.5 mL of acetic anhydride for 30 min at 90 °C, and the reaction products was evaporated to dryness in a Speed-Vac. The acetylated samples were dissolved in chloroform and were analyzed by GC. The conditions of gas chromatography applied to analyze monosaccharide of polysaccharide AMP-2 was as follows: GC analysis was performed with HP-5 column and a temperature program consisted of an initial temperature of 165 °C, increased to 170 °C at a speed of 1 °C min^{-1} and held 1 min, then to 175 °C at 1 °C min^{-1} and held 1 min, finally to 190 °C at a speed of 1 °C min^{-1}. Split ratio was 1:30 in a triage injection mode, and the temperature of injection port and detector were 235 °C and 280 °C, respectively. 1 μL samples were injected to machine for analysis using the standard monosaccharides including Sorbose, Fructose, Rhamnose, Arabinose, Xylose, Glucose, Galactose; Mannose as references prior to acetylation.

2.6. Methylation reaction and GC–MS

AMP-2 was methylated using the method of Anumula, and Taylor (Anumula & Taylor, 1992) with a small modification. Sample (2 mg) was dried with P_2O_5 and dissolved in DMSO (1 mL) and methylated with a solution of NaOH/DMSO (1 mL) and CH_3I (0.5 mL). The reaction mixture was extracted with $CHCl_3$, the organic phase was washed with 3 vol MilliQ water, and the solvent was then removed by evaporation. The per-methylated polysaccharide (Fig. 1b) was hydrolyzed by treatment with HCO_2H (88%, 0.5 mL), MilliQ water (0.1 mL) and TFA (0.05 mL) for 16 h at 100 °C. The partially methylated sugars in the hydrolysate were reduced with $NaBH_4$, acetylated by Ac_2O, and the partially methylated alditol acetates (PMAAs) were extracted into an equal volume of chloroform. The organic phase was washed with 4 vol MilliQ water and evaporated to dryness under vacuum. PMAAs were re-dissolved in chloroform to ~1 nmol/μL concentration and 2 μL samples were analyzed by GC–MS using a DB-5MS column (30 m × 0.25 mm × 0.25 μm) and a temperature program of 180–270 °C at 20 °C min^{-1}, with holding at 270 °C for 25 min. The absolute configurations of the monosaccharides were determined as described by Vliegenthart et al. using (+)-2-butanol.

2.7. Preparation of AMP-2 derivatives

2.7.1. Preparation of sulfated derivative

The sulfating agent was prepared using dry pyridine and chlorosulfonic acid (Yang, Gao, Han, & Tan, 2005; Yoshida et al., 1995) with a minor modification as follows: 15 mL fresh-distilled pyridine was added into a 250 mL three-neck flask with condenser and mixing devices, and the flask was cooled down under sodium chloride ice bath, then 5 mL chlorosulfonic acid was added into flask with a very slow speed. At this point, a large amount of light yellow solid will be found in the flask.100 mg AMP-2 was suspended in 20 mL of fresh-distilled dimethylformamide (DMF), and was ultrasonicated about 20 min before pouring into a three neck flask. Quickly put the flask into a 50 °C water bath and reacted 5 h as soon as the polysaccharide AMP-2 and DMF solution was poured into flask. After the reaction

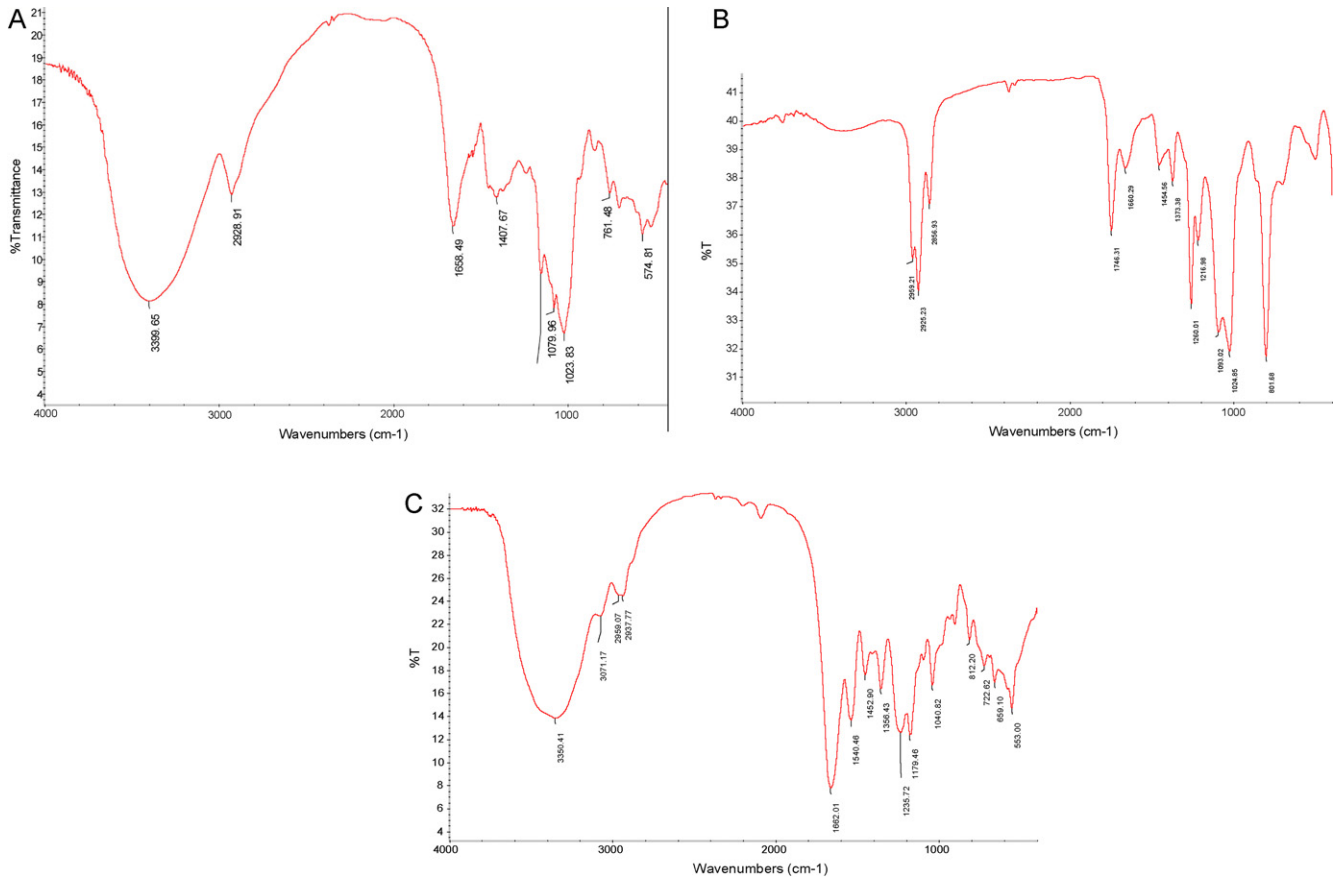

Fig. 1. FT-IR spectra for *A. molpadioidea* native polysaccharide AMP-2 and its derivatives: (a) FT-IR spectrum for native AMP-2; (b) FT-IR spectrum for methylated derivative; (c) FT-IR spectrum for sulfated derivative.

was finished, the mixture was cooled and neutralized with 4 mol/L NaOH solution. Finally, the product was dialyzed against distilled water for 72 h. The dialyzate was concentrated and freeze-dried and designated as AMP-3. The sulfur content (S%) in the sulfated sample was determined according to method described by Schoniger (1956), and the degree of substitution (DS), which refers to the average number of sulfate residues on each monosaccharide residue, was established on the basis of the sulfur content and calculated in line with the following formula described by Zhang, Zhang, Wang, and Cheung (2003).

$$DS = \frac{162 \times S\%}{32 - 80 \times S\%}$$

2.7.2. Preparation of carboxymethylated derivative

Carboxymethylated derivative was prepared according to the method described by Bao, Zhen, Ruan, and Fang (2002) with a modification. Briefly, 500 mg AMP-2 was mixed with 10 mL 75% ethanol, and 500 mg NaOH powder was added after 30 min stirring, then stirred another 50 min for alkaline. Subsequently, chloroacetic acid (0.24 g) was added to the mixture and reaction temperature was varied to 50 °C and maintained for 3 h. Then, 200 mg NaOH and a small volume of distilled water were added into the mixture. After 1 h later, the mixture was cooled, neutralized, dialyzed against distilled water, and lyophilized, finally designated as AMP-4. The carboxymethylated group percentage and degree of substitution were measured according to the method described by Wang, Yu, and Mao (2009).

2.8. Anti-coagulant activity assay in vitro

The anti-coagulant ability of AMP-2, AMP-3 and AMP-4 were assayed *in vitro* using Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), Thrombin Time (TT) as indicators according to the methods provided by the biological reagents provider, Sun Biochemical Corp. Before assay, 1 volume of different concentration of native sulfated fucose-containing polysaccharide (AMP-2) or its derivatives (AMP-3 and AMP-4) were added to 4 volumes of the platelet-poor human plasma. The APTT, TT or PT values of various sample concentrations were measured by comparing to the results of control assays in which neither AMP-2 nor its derivatives were added. In this research, all the data were the mean of three parallel assays with discrepancies among them less than 1 s (Huang, Du, Yang, & Fan, 2003).

3. Results and discussion

3.1. General analysis

Cellulose acetate membrane electrophoresis experiment showed that AMP-2 exhibited one band, and HPLC figure was confirmed one sharp peak. Both of these experiments confirmed that AMP-2 was pure, and the molecular weight of AMP-2 was estimated to be 2.4×10^4 Da. The FTIR spectra of AMP-2 and its methylated derivative were showed in Fig. 1. From Fig. 1a, we can find a small peak at ca. 1250 cm^{-1} (did not indicated) contributed by S=O group. In Fig. 1b, the peak of 1250 cm^{-1} became sharply and clearly after methylation by $CHCl_3$, which further confirmed

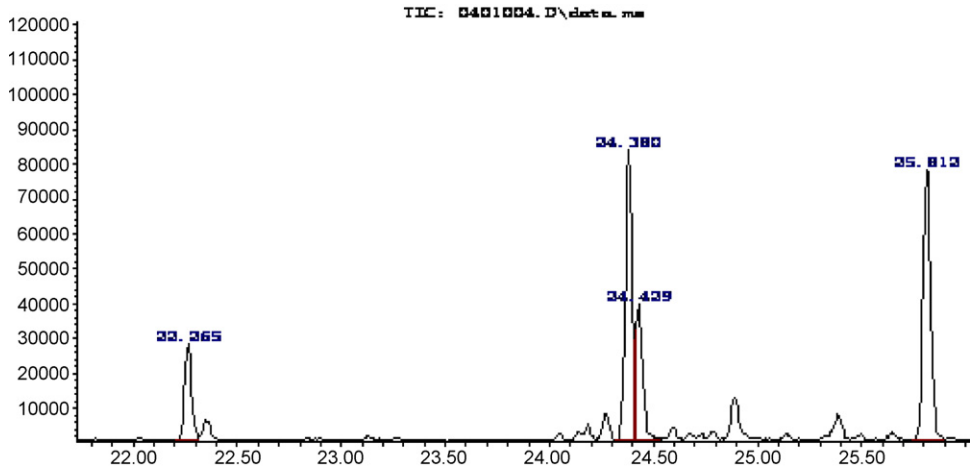

Fig. 2. Total ion spectrum of PMAAs prepared from AMP-2.

the S=O group existing in AMP-2. Gas chromatography was applied to identify monosaccharide composition of AMP-2, and the result indicated that AMP-2 mainly was consisted of fucose, glucose, galactose and galactosamine in a molar ratio of ca. 1:3:0.5:2, along with a minor amount of fructose and mannose. The FTIR spectrum of AMP-2 was showed as follows.

3.2. Methylation analysis

The positions of the O-acetyl and O-methyl groups on the PMAAs polysaccharide derivatives were determined by GC–MS. The total ion spectrum of PMAAs is shown in Fig. 2, and the main fragments for PMAAs are listed in Table 1. The peak areas corresponding to each of the PMAAs were divided by the appropriate response factor, and the resulting quotients were normalized with reference to the first peak, 2,3,4,6-Me_4-Gal*p*. The third column in Table 1 provided the molar ratios of glycosyl linkages in AMP-2. Glycosyl-composition analysis was consistent with the monosaccharide composition analysis detected by GC mentioned above. Methylation and GC–MS analysis revealed that AMP-2 was main composed of 1-substituted-Gal*p*, 1,4-disubstituted-GalN*p* (in which the NH_2 group attached to 2 position of glycosyl residue), 1,2-disubstituted-Fuc*Sp* (in which the SO_4^{2-} group attached to 2-position of glycosyl residue), 1,4,6-trisubstituted-Glc*p* in a molar ratio of ca. 0.5:2.0:1.0:3.0, together with a small amount of different substituted Man*p*.

3.3. AMP-2 modification and characterization

Sulfated derivative was prepared according to the method described above. In the FT-IR spectrum of sulfated derivative (Fig. 1c), compared with that of the native polysaccharide, two peaks found to become more bigger, one at 812 cm^{-1} representing a symmetrical C–O–S vibration associated with a SO_4 group and the other at 1235 cm^{-1} due to the presence of the bonds of S=O, indicating that the sulfation reaction had occurred. The sulfur content (S%) in the sulfated sample was determined according to method described by Schoniger (1956), and the degree of substitution (DS), which refers to the average number of sulfate residues on each monosaccharide residue, was calculated in line with method described by Zhang et al. (2003). The results indicated that the sulfate group percentage was 21.6%, which was higher than sulfuric acid group percentage in native polysaccharide (7.8%), and DS was 2.37. Carboxymethylated derivative was prepared according to the method described by Bao et al. (2002), and the carboxymethylated group percentage was measured using the method described by Wang et al. (2009). The result indicated that DS of carboxymethylated group was 0.96.

3.4. In vitro anticoagulant activities

The anticoagulant activities of native sulfated fucose-containing polysaccharide and its derivatives were measured by APTT, TT and PT, meanwhile the heparin was used as positive control. APTT is used to evaluate the coagulation factors including VIII, IX, XI, XII and prekallikrein in the intrinsic blood coagulation pathway. PT is used to characterize the extrinsic coagulation factors, and TT is as an indicator of blood coagulation status that transforming fibrinogen into fibrin (Zhou, Hong, Shu, Ni, & Qin, 2009).

As shown in Table 2, the anticoagulant activities of fucosylated polysaccharide sulfate, its derivatives and heparin characterized different effects in the anticoagulation process. Although activity less than heparin, AMP-3 exhibited a little stronger anticoagulant activity than AMP-2 and AMP-4 with the raise of concentration in APTT experiment and exhibited a significant different ($P < 0.5$) from negative control and native polysaccharide AMP-2, suggesting that AMP-3 may usually express anticoagulant activity correlating with the extrinsic coagulation process. Meanwhile, AMP-2 and AMP-4 could also increase anticoagulant activity, which was characterized by APTT, PT and TT (Table 2). In previous researches, Borsig et al. has also reported a polysaccharide of fucosylated chondroitin sulfate (FucCS) isolated from another sea cucumber *Ludwigothurea grisea*, composed of a chondroitin sulfate backbone substituted at the 3-position of the β-D-glucuronic acid residues with 2,4-disulfated

Table 1
Results of a methylation analysis of AMP-2 by GC–MS.

Methylated sugar	Linkages	Molar ratios	Major mass fragment (*m*/*z*)
2,3,4,6-Me_4-Gal*p*	1-Linked-Gal*p*	0.5	43, 71, 87, 101, 117, 129, 145, 161, 161, 205
3,6-Me_2-GalN*p*	1,4-Linked-GalN*p*	2.0	43, 71, 87, 99, 101, 117, 142, 161, 203, 233
3,4,5-Me_4-Fuc*Sp*	1,2-Linked-Fuc*Sp*	1.0	43, 87, 115, 129, 149, 172, 183, 214, 275
2,3-Me_2-Glc*p*	1,4,6-Linked-Glc*p*	3.0	43, 71, 87, 101, 117, 129, 145, 161, 233

Table 2
APTT, TT, and PT of human platelet-poor plasma containing AMP-2, AMP-3, AMP-4 and heparin.

Sample	APTT(s) at different concentrations (mg/mL)			PT(s) at different concentrations (mg/mL)			TT(s) at different concentrations (mg/mL)		
	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0
Negative control	34.3 ± 0.6	–	–	11.8 ± 1.2	–	–	18.7 ± 0.9	–	–
AMP-2	35.1 ± 0.3	35.7 ± 0.4	36.9 ± 0.8	12.3 ± 0.4	13.1 ± 0.6	14.3 ± 1.3	20.3 ± 1.7	21.1 ± 1.5	24.1 ± 0.7
AMP-3	36.3 ± 0.8	38.8 ± 1.2	46.4 ± 1.4	12.6 ± 1.3	13.7 ± 1.7	14.8 ± 0.7	21.4 ± 0.7	23.8 ± 0.9	24.9 ± 1.0
AMP-4	35.2 ± 0.3	36.1 ± 1.8	36.8 ± 2.7	12.1 ± 0.6	12.7 ± 0.9	14.1 ± 1.1	20.8 ± 1.4	21.4 ± 2.3	23.7 ± 3.1
Heparin	>60.0 ± 3.7	–	–	>15.0 ± 2.7	–	–	>40.0 ± 3.3	–	–

Note: all the data were the mean of three parallel assays. – indicates the value was not evaluated.

α-L-fucopyranosyl branches, was a potent inhibitor of P- and L-selectin binding to immobilized sialyl Lewisx and LS180 carcinoma cell attachment to immobilized P- and L-selectins. And, inhibition ability exhibited in a concentration-dependent manner. Interestingly, removal of the sulfated fucose branches on the FucCS abolished the inhibitory effect *in vitro* and *in vivo*. Our results are consistence with their findings further confirmed sulfated group playing an important role in anticoagulant biological activity (Borsig et al., 2007).

4. Conclusions

A native fucosylated polysaccharide sulfate was purified from a special sea cucumber, *A. molpadioidea*, with a relative molecular weight of ca. 2.4×10^4 Da, and the monosaccharide composition analysis using GC–MS indicated this polysaccharide was composed of 1-substituted-Gal*p*, 1,4-disubstituted-GalN*p* (in which the NH_2 group attached to 2 position of glycosyl residue), 1,2-disubstituted-Fuc*Sp* (in which the SO_4^{2-} group attached to 2-position of glycosyl residue), 1,4,6-trisubstituted-Glc*p* in a molar ratio of ca. 0.5:2.0:1.0:3.0, together with a small amount of different substituted Man*p*. Upon to now, the polysaccharide from this sea cucumber in eastern China is the first time to be characterized. Simultaneously, its sulfated derivative and carboxymethylated derivative were prepared using dry pyridine and chlorosulfonic acid, and chloroacetic acid respectively. The results indicated that the sulfated group percentage was 21.6% (DS of 2.37), which was higher than sulfated group percentage in native polysaccharide (7.8%), and DS of carboxymethylated group was 0.96. At the same time, the anticoagulant activities *in vitro* investigation indicated that sulfated derivative showed a stronger ability than native polysaccharide and carboxymethylated derivative. These phenomena indicated that a little stronger anticoagulant ability of AMP-3 than that of AMP-2 and AMP-4 might be attributed to higher sulfated group percentage in AMP-3 than ones in AMP-2 and AMP-4, and the slight anticoagulant activities of AMP-2 and AMP-4 might be attributed to fucosylated groups in these compounds. Therefore, the different anticoagulant activities of these compounds might be caused by their different percentages and types of functional groups in their structures. The mild anticoagulant activities of our compounds suggested that they might be useful for therapeutical purposes, particularly for the research of antithrombotic drugs.

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