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Heparinoid-active two sulfated polysaccharides isolated from marine green algae *Monostroma nitidum*

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ABSTRACT

Two sulfated polysaccharides WF1 and WF3 were isolated from marine green algae *Monostroma nitidum*, and their structural characteristics were determined. Anticoagulant activities of WF1 and WF3 were evaluated by assays of the activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), antithrombin and anticoagulation factor Xa activities. The results showed that WF1 and WF3 had similar high contents of rhamnose, whereas their sulfate contents, sulfation positions, molecular sizes and linkage patterns of rhamnose residues were different. The bioassay results demonstrated that WF1 and WF3 had high anticoagulant activities, and were potent thrombin inhibitors mediated by heparin cofactor II, especially WF3. They also hastened thrombin and coagulation factor Xa inhibition by potentiating antithrombin III, but at a lower effectiveness. The differences of anticoagulant activities between WF1 and WF3 were directly due to their structural features discrepancy.

1. Introduction

Naturally occurring or synthetic polysaccharides having similar biological activities to those of heparin are referred to as heparinoids. The polysaccharides isolated from marine algae are known to be one abundant source of heparinoids. Various anticoagulant-active polysaccharides, especially from marine red and brown algae, have been isolated and characterized (McLellan & Jurd, 1992). They contain a variety of sulfated galactans and sulfated fucans, and exhibit high anticoagulant activity. However, there are fewer reports of anticoagulant-active polysaccharides from marine green algae than those from brown and red algae. Jurd, Rogers, Blunden, and McLellan (1995) reported that the anticoagulant-active polysaccharides isolated from *Codium fragile ssp. atlanticum* were xyloarabinogalactans. Matsubara, Matsuura, Hori, and Miyazawa (2000) found anticoagulant activity in the extract of *Codium pugniforms* and isolated a highly sulfated galactoarabinoglucan. A sulfated galactan with anticoagulant activity was also isolated from green algae *Codium cylindricum* (Matsubara et al., 2001). Mao et al. (2006) discovered that the sulfated polysaccharide from *Ulva conglobata* showed high anticoagulant activity, and was mainly consisted of rhamnose with variable content of glucose and fucose. The anticoagulant polysaccharide from *Monostroma nitidum* yielded a 6-fold higher activity than that of heparin (Maeda et al., 1991). Hayakawa et al. (2000) found that two different sulfated polysaccharides from *Monostroma* species had more potent effect on the inhibition of thrombin than heparin or dermatan sulfate. Zhang et al. (2008) reported that a sulfated polysaccharide and its fragments from *M. latissimum* had high anticoagulant activities. With today's interest in new renewable sources of chemicals and polymers, the marine green algae represent potential source to be explored. Further work on the polysaccharides isolated from various marine green algae will aid in the development of new drugs and health foods.

The green seaweed *M. nitidum* grows in the warm water and is cultivated as edible algae. In this study, two sulfated polysaccharides WF1 and WF3 were isolated from marine green algae *M. nitidum*, and their chemical characteristics and anticoagulant activities were investigated.

2. Experimental

2.1. Materials

Monostroma nitidum was collected on the coast of Zhejiang Province, China. The raw material was thoroughly washed with tap water. The sample was air dried and kept in plastic bags at room temperature in a dry environment. APTT assay reagent (ellagic acid + bovine phospholipids reagent) and PT assay reagent (rabbit thromboplastin) were from Shanghai Sun (China). TT assay reagent (bovine thrombin) was from Dade Behring (USA). Thrombin, coagulation factor Xa, antithrombin III, heparin cofactor II and thrombin generation colorimetric substrate were purchased

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from Calbiochem (Germany). Coagulation factor Xa colorimetric substrate was from Sigma (USA). Standard heparin was purchased from Sigma (USA). All other reagents used were analytical grade.

2.2. Isolation and purification of the sulfated polysaccharide from M. nitidum

Dried algae were dipped into 20 volumes of distilled water and kept at room temperature for 2 h. The algae were then homogenized and the solution was 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 8000) against distilled water for three successive days. The retained fraction was recovered, concentrated under reduced pressure, and precipitated by addition of 4-fold 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 extract was fractionated by a Q Sepharose Fast Flow column with distilled water, 1.0 mol/L NaCl and 3.0 mol/L NaCl. Total sugar content of the eluate was determined by the phenol-sulfuric acid method. The fractions eluted with 1.0 mol/L NaCl and 3.0 mol/L NaCl were, respectively, further purified by a Sephacryl S-400/HR column with 0.2 mol/L sodium acetate. The major fractions were pooled, concentrated, desalted and freeze-dried. Two sulfated polysaccharides were obtained and named as WF1 and WF3, respectively.

2.3. Composition analysis

Total sugar content was estimated by the phenol-sulfuric acid assay using rhamnose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Sulfate ester 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 the standard (Bitter & Muir, 1962). Optical rotation value was measured at 20 °C with digital polarimeter at 589 nm.

The composition of neutral monosaccharide was determined by gas chromatography (GC) 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. TFA was 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 of hydroxylammonium and 3 mg of inositol (as internal reference), the mixture was dissolved in 0.5 ml of 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. GC was performed on an HP5890II instrument.

2.4. Measurement of molecular weight

Molecular weight was 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 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 (M_w: 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).

2.5. Desulfation of the sulfated polysaccharide

The sulfated polysaccharide (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 pyridium salt. The salt was dissolved in 10 ml of dimethyl sulfoxide (DMSO) containing 10% (v/v) of anhydrous methanol and 1% pyridine, and then the solution was then shaken at 100 °C for 4 h. After the reaction was completed, the reaction 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 solution was then dialyzed and the non-dialyzable portion was lyophilized. Decreased amounts of sulfate ester were calculated from sulfate ester analysis. Desulfation was confirmed by disappearance of sulfate ester peaks in its IR spectrum.

2.6. Methylation analysis

Each sample was treated 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. CH_3I then 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 trifluoroacetic acid 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 gas chromatography–mass spectrometric (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. The peaks on the chromatogram were identified from their retention times. GC–MS was performed on an HP6890II instrument.

2.7. IR 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.

2.8. Anticoagulant activities of the sulfated polysaccharides

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 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 polysaccharide and incubated at 37 °C for 60 s. Then, 200 μl of