A sulfated polysaccharide, fucans, isolated from brown algae *Sargassum vulgare* with anticoagulant, antithrombotic, antioxidant and anti-inflammatory effects

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ABSTRACT

Fucan (SV1) sulfated polysaccharides from the brown algae *Sargassum vulgare* were extracted, fractionated in acetone and examined with respect to chemical composition, anticoagulant, anti-inflammatory, antithrombotic effects and cellular proliferation. These polysaccharides contain low levels of protein, high level of carbohydrate and sulfate. Monosaccharides analysis revealed that SV1 was composed of fucose, galactose, xylose, glucuronic acid and mannose. SV1 polysaccharide prolonged activated partial thromboplastin time (aPTT) and exhibited high antithrombotic action *in vivo*, with a concentration ten times higher than heparin activity. PSV1, a purified form in gel filtration showed very low biological activities. SV1 stimulated the enzymatic activity of FXa. Its action on DPPH radical scavenging activity was 22%. This polymer has no cytotoxic action (hemolytic) on ABO and Rh blood types in different erythrocyte groups. It displays strong anti-inflammatory action at all concentrations tested in the carrageenan-induced paw edema model, demonstrated by reduced edema and cellular infiltration.

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1. Introduction

Seaweeds have been the focus of growing interest in the biomedical area, mainly due to their bioactive substances, which show great potential for anti-inflammatory, antimicrobial, antiviral, and anti-tumoral drugs (Bluden, 1993). Sulfated fucans, polysaccharides that contain neutral sugars, substantial percentages of L-fucose and sulfated ester groups, are constituents of brown algae and some marine invertebrates (Berteau & Mulloy, 2003). Their structure varies according to algal species and extraction procedure. Although several studies have attempted to determine the fine structure of fucans, only a few examples of regularity were found. The linkages, branching, sulfate positions and composition of monosaccharides differ significantly, and the relationship between structure and biological activity has yet to be established (Holtkamp, Kelly, Ulber, & Lang, 2009).

Sargassum (Phaeophyceae) is an algal genus with an extensive geographical range (Duarte, Cardoso, Noseda, & Cerezo, 2001). Early studies of fucans from the *Sargassum* genus indicate they are generally composed of glucuronic acid, mannose, and galactose residues, with partially sulfated side-chains consisting of galactose,

xylose, and fucose. Fucans from *Sargassum* are widely studied owing to their broad therapeutic applications (Zhang, Hu, Liu, & Shuai, 2011).

Most thromboembolic processes require anticoagulant therapy. This explains current efforts to develop specific and potent anticoagulant and antithrombotic agents (Cumashi et al., 2007). Since the 1940s, heparin, sulfated polysaccharides, has been the predominant drug for treatment and prevention of venous thrombosis and thromboembolism. However, an obvious side effect of heparin administration is hemorrhagic. It is well-documented that marine brown algae are an abundant source of anticoagulant polysaccharides containing a variety of sulfated L-fucans with anticoagulant activity (Olson & Björk, 1993; Zhu et al., 2009). The proposed mechanism of anticoagulant action for fucoidan was predominantly related to *in vitro* potential ion of natural inhibitors of activated factor II (thrombin) and activated factor X (Ananthi et al., 2010).

Oxidative stress has been defined as a disturbance in the equilibrium between pro-oxidant and antioxidant systems in favor of pro-oxidation, due to intracellular signaling and defense against microorganisms (Ananthi et al., 2010). Other symptoms may result from ROS production associated with activation of the immune system. Cell membrane lipids and proteins are also sites of free radical reactions. Several investigations have been conducted to verify and to demonstrate the antioxidant properties in algae (Yuan et al., 2005).

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We previously demonstrated that *Sargassum vulgare* synthesizes alginic acid and heterofucans (Dietrich et al., 1995). In the present study, a fucan (SV1) was obtained from *S. vulgare* and its anticoagulant, antithrombotic, anti-hemolytic, antioxidant and anti-inflammatory properties were assessed using several *in vitro* and *in vivo* tests.

2. Experimental

2.1. Materials

Coomassie Brilliant Blue; KBr, agarose gel 1,3-diaminopropane; bovine thrombin; bovine serum albumin, toluidine blue, Heparin, *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, *N*-benzoyl-Phe-Val-Arg-γ-nitroanilide hydrochloride, D_2O, *N*-cetyl-*N,N,N*-trimethylammonium bromide,5-diphenyltetrazolium bromide, Folin Ciocalteau, glucose, galactose, arabinose, fucose, mannose, glucuronic acid, xylose, and rhamnose were purchased from Sigma (St. Louis, EUA) alkaline protease from *Esporobacillus* (BioBrás, Montes Claros, MG, Brazil), aPTT and PT commercial kits were purchased from Labtest (São Paulo, SP).

2.2. Animals

Experiments were conducted using male Wistar rats weighing 250–300 g, which were anesthetized with a mixture of ketamine (100 mg/kg intramuscularly) and xylasine (16 mg/kg intramuscularly). Experiments were in accordance with Brazilian National Law for the scientific management of animals.

2.3. Polysaccharide extraction

The brown seaweed *S. vulgare* was collected at Búzios beach on the south coast of Rio Grande do Norte state, Brazil. Seaweeds were stored in our laboratory and dried at 50 °C under ventilation in an oven, ground in a blender and incubated with acetone to eliminate lipids and pigments. About 50 g of powdered algae was suspended with five volumes of 0.25 M NaCl and pH was adjusted to 8.0 with NaOH. Ten milligrams of maxataze, an alkaline protease from *Esporobacillus* (BioBrás, Montes Claros, MG, Brazil), was then added to the mixture for proteolytic digestion. After incubation for 24 h at 60 °C under agitation, and periodic pH adjustments, the mixture was filtered through cheesecloth and precipitated with increasing amounts of ice-cold acetone (0.3, 0.5, 1.0 and 1.5 v) under gentle agitation at 4 °C (Silva et al., 2005). Precipitates formed were collected by centrifugation at 10,000 × g for 20 min and dried under vacuum. The volume fraction obtained with 1.0 v, denominated SV1, was chosen for analysis because it exhibits a higher yield.

2.4. Chemical composition, monosaccharide composition analysis by HPLC and characterization by electrophoresis

Total sugars were determined using a phenol–H_2SO_4 reaction, with D-fucose as standard, as previously described (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Sulfate content was measured after acid hydrolysis (HCl 6 N, 6 h, 100 °C) using the turbidimetric method (Dodgson & Price, 1962). Protein content was quantified with Coomassie Brilliant Blue reagent and bovine serum albumin as standard (Bradford, 1976). Phenolic compounds were measured by the Folin Ciocalteau method (Swain & Hills, 1959), with some modifications.

2.5. Monosaccharide composition

After hydrolysis (2 M HCl, 100 °C, 2 h), the fraction of total monosaccharide composition was determined by high performance liquid chromatography (HPLC), with a refractive index detector and LiChroCART® 250-4 column. Glucose, galactose, arabinose, fucose, mannose, glucuronic acid, xylose, and rhamnose were used as the standard for analysis.

2.6. Characterization of polysaccharides fractionated in acetone by electrophoresis and purified by gel filtration

Several fractions (0.3, 0.5, 1.0 and 1.5 v) of sulfated polysaccharides from *S. vulgare* (∼15 μg) was applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane/acetate (pH 9.0). These sulfated fucans were fixed in the gel with 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide solution (Leite et al., 1998). After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v). The polysaccharides (SV1) had high yielding and was subjected to gel-permeation chromatography on Sepharose CL-4B (140 cm × 1.8 cm) using 0.2 M acetic acid as eluent. The elution was monitored for total sugar (Dubois et al., 1956). To estimate the MW of the polysaccharide, we used dextrans of different sizes as standards (Pharmacia). The eluted polysaccharide was dialyzed against water, freeze-dried and used in the assays.

2.7. Infrared and NMR spectroscopies of polysaccharides

For infrared analysis a sample of 5 mg of each polysaccharides SV1 and PSV1 were mixed thoroughly with dry potassium bromide (100 mg). Pellets was prepared and scanned on a Nicolet 5PC Fourier transform infrared spectrophotometer.

NMR spectroscopy of ^1H and ^{13}C spectra spectra was recorded at 500 MHz at using a Varian Unity 500 spectrometer. The sulfated fucan sample (PSV1) (∼10 mg) was converted to sodium salt by passage through a 10 cm × 1 cm column of Dowex 50-X8 Na+ form, and all samples were dissolved in approximately 0.7 mL of 99.8% D_2O.

2.8. Anticoagulant activity

Blood was collected by venous punction and mixed carefully with 3.2% sodium citrate at a proportion of 9:1. Next, the blood was centrifuged at 1000 × g for 10 min at ambient temperature. After centrifugation, the supernatant was removed and stored in plastic siliconized tubes, representing the citrated pool of plasma. Activated partial thromboplastin time (aPTT) and prothrombin time (PT) of the plasma pool mixed with SV1 in different concentrations were determined using commercial kits from Labtest (São Paulo, SP). The aPTT test evaluates the influence of compounds under intrinsic and common coagulation pathways, while the PT test assesses their influence under extrinsic and common coagulation pathways (Silva et al., 2005).

2.9. Enzymatic activity of coagulation factors (chromogenic substrate assay)

2.9.1. Assay of thrombin activity

The chromogenic substrate assay of thrombin was carried out in a hemolysis tube with a final volume of 1 mL, according to a modified method developed by Gaspar, Crause, and Neitz (1995), using 8 NIH/mL of bovine thrombin in 50 mM Tris–HCl + 0.1 M NaCl buffer (pH 8.0). The substrate *N*-benzoyl-Phe-Val-Arg-γ-nitroanilide hydrochloride was dissolved in buffer and methanol at a final concentration of 3 mM. In order to perform the assay, 30 μL of 8 NIH/mL thrombin, 815 μL of buffer, and 10 μL of SV1 (12.5, 25 and 50 μg) were incubated for 10 min at 37 °C, and 25 μL of thrombin chromogenic substrate at 3 mM concentration was then added. The mixture was incubated for 20 min at 37 °C. After incubation, 120 μL