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Anticoagulant properties of a crude sulfated polysaccharide from the red marine alga *Halymenia floresia* (Clemente) C. Agardh

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ABSTRACT. Alternative sources of anticoagulants have arisen as a result of the increasing demand for safer anticoagulant clinical therapy, and the sulfated polysaccharides of seaweeds have gained attention in biomedicine. In this study, crude sulfated polysaccharide fractions (denominated Hf1, Hf2 and Hf3) were obtained from the red marine alga *Halymenia floresia* and the anticoagulant properties of a soluble crude polysaccharide fraction (Hf2s) were assayed. The three differential extractions yielded 38.6%. The polysaccharides are composed mainly of galactose, with small amounts of xylose and glucose. The anticoagulant properties of Hf2s containing 53.8% sulfate and 3% protein was also compared to those of heparin (193.0 IU mg^{-1}) by assays of activated partial thromboplastin time (APTT) and thrombin time (TT) using normal human plasma. Hf2s showed a higher anticoagulant activity (68.4 IU mg^{-1}) than those of Hf1s and Hf3s, whose activities were 37.6 and 36.6 IU mg^{-1}, respectively. The compound was less active than heparin, but its anticoagulant mechanism suggested that it is dependent on cofactor heparin II to inhibit thrombin activity, but not on cofactors VIII and IX. Therefore, the polysaccharide from *H. floresia* interfered on coagulation cascade.

Keywords: halymeniales, *Halymenia floresia*, sulfated galactans, anticoagulant activity.

RESUMO. Propriedades anticoagulantes de um polissacarídeo sulfatado bruto da alga marinha vermelha *Halymenia floresia* (Clemente) C. Agardh. O aumento da demanda por anticoagulantes para a terapia clínica tem motivado a busca por fontes alternativas de anticoagulantes mais seguros e os polissacarídeos sulfatados de algas marinhas têm ganhado atenção na biomedicina. Objetivou-se obter frações de polissacarídeos sulfatados brutos (denominadas Hf1; Hf2 e Hf3) da alga marinha vermelha *Halymenia floresia* e para avaliar as propriedades anticoagulantes de uma fração polissacarídica bruta solúvel anticoagulante (Hf2s). As três extrações diferenciais renderam 38,60%. Os polissacarídeos são principalmente compostos de galactose com pequenas quantidades de xilose e glucose. As propriedades anticoagulantes da Hf2s, contendo 53,80% de sulfato e 3% de proteínas, foram também comparadas com a heparina (193,00 UI mg^{-1}) pelo ensaio do tempo de tromboplastina parcial ativada (TTPA) e tempo de trombina (TT), usando plasma humano normal. A Hf2s apresentou maior atividade anticoagulante (68,40 UI mg^{-1}) que Hf1s a Hf3s, cujas atividades foram 37,60 e 36,60 UI mg^{-1}, respectivamente. O composto foi menos ativo que a heparina, mas sugere-se que o mecanismo anticoagulante seja dependente do cofator II da heparina para inibição da atividade da trombina, exceto pelos cofatores VIII a IX. Portanto, o polissacarídeo de *H. floresia* interferiu na cascata de coagulação.

Palavras-chave: halymeniales, *Halymenia floresia*, galactanas sulfatadas, atividade anticoagulante.

Introduction

Sulfated polysaccharides are complex and heterogeneous macromolecules found in diverse marine aquatic organisms, including animal (POMIN; MOURÃO, 2008; WU et al., 2010), marine algae (MELO et al., 2002; GHOSH et al., 2004; AZEVEDO et al., 2009; RODRIGUES et al., 2011) and sea grasses (AQUINO et al., 2005) tissues, and their biological activities have been recognized in marine algae (FARIAS et al., 2000; MELO et al., 2004; PEREIRA et al., 2005; FONSECA et al., 2008; RODRIGUES et al., 2009a;

LINS et al., 2009; RODRIGUES et al., 2010), fishes (RODRIGUES et al., 2009b) and marine invertebrates (WU et al., 2010).

Sulfated galactans (a form of sulfated polysaccharide) occur in high concentrations in marine algae (CAMPO et al., 2009; RODRIGUES et al., 2009a, 2011). In recent years, several crude sulfate polysaccharide algae have been used as immunostimulant agents to prevent stress in fish and shrimp cultures (CAMPA-CÓRDOVA et al., 2002; ARAÚJO et al., 2008), arousing great interest, not only in the medical sciences, but also in the biotechnology of aquatic organisms.

Heparin is a compound that has been widely used as an anticoagulant and antithrombotic agent for more than 50 years. This drug is also employed during extracorporeal circulation (NADER et al., 2001). However, several adverse effects of heparin have been identified, such as the development of thrombocytopenia, hemorrhage and low platelet count (THOMAS, 1997). Thus, new compounds with similar properties to heparin are needed, and the sulfated polysaccharides of marine resources, which have anticoagulant and antithrombotic actions, are an attractive alternative (PEREIRA et al., 2005).

Anticoagulant activity is one of the most important biological properties of sulfated polysaccharides. A purified sulfated galactan was isolated from *Botryocladia occidentalis*, with anticoagulant activity similar to that of heparin (FARIAS et al., 2000). Pereira et al. (2005) showed anticoagulant properties of a sulfated galactan isolated from the alga *Gelidium crinale*. Athukorala et al. (2006) reported an anticoagulant polysaccharide from an enzymatic hydrolysate of *Ecklonia cava* (Phaeophyceae). A soluble sulfated polysaccharide extracted from the green marine alga *Monostroma latissimum* showed different anticoagulant activities when fragmented into various molecular weights (ZHANG et al., 2008). More recently, Rodrigues et al. (2009a) isolated several sulfated polysaccharide anticoagulant fractions from *Halymenia pseudofloresia* (Rhodophyceae) by successive papain digestion procedures, which were more active when compared to heparin.

We have identified a number of algae species with anticoagulant properties and report here on the anticoagulant activity of a crude sulfated galactan extracted from the red marine alga *Halymenia floresia* collected on the Atlantic coast of the state of Ceará, Brazil.

Material and methods

Collection of alga and extraction of sulfated polysaccharides

H. floresia (Clemente) C. Agardh (registered under number 0622 at the Herbarium of the Tropical Marine Sciences Institute, Federal University of Ceará – Labomar) was collected in March 2004 on the Northeast coast of Brazil (Pedra Rachada Beach, state of Ceará). Initially, the sulfated polysaccharides were extracted from the red seaweed H. floresia by mechanical stirring for 24h at room temperature in water at 1.5% (w v^{-1}). The residue was removed by centrifugation (5.000 × g for 15 min. at 4ºC). The supernatant was precipitated with absolute EtOH (1:3; v v^{-1}), centrifuged, re-dissolved in distilled water, dialyzed against water, freeze-dried and denominated Hf1. The algal residue was re-extracted but this time at 80°C for 4h, followed by centrifugation under the same conditions. The hot extraction was repeated once more, using the second extraction residue. The supernatants were precipitated with absolute EtOH (1:3; v v^{-1}), and denominated Hf2 and Hf3 for the second and third extractions, respectively.

Mass of 0.2 g of each of the Hf1, Hf2 and Hf3 fractions was dissolved in hot distilled water (60ºC), and centrifuged. The clear supernatant was then freeze-dried, giving rise to originating the soluble fractions Hf1s, Hf2s and Hf3s. These fractions were used in the subsequent studies.

Composition analysis

The total sugar content of the soluble crude fractions was estimated by phenol-sulfuric acid analysis using D-galactose as standard (DUBOIS et al., 1956). Soluble protein content was measured by Bradford's (1976) method, using bovine serum albumin to construct the standard curve. Sulfate content was determined according to the method of Dodgson and Price (1962) using sodium sulfate ($NaSO_3$) as standard.

Monosaccharide composition

The monosaccharide composition was estimated by gas-chromatography-mass-spectrometry (GC-MS) of alditol acetate derivates using the reductive hydrolysis procedure (STEVENSON; FURNEAUX, 1991). A Varian 3300 chromatograph and Finnigan Mat ITD spectrometer were used and helium was the carrier gas (1.0 mL min^{-1}).

Clotting assays

All coagulation assays were performed with a coagulometer (Amelung KC4A) according to

Anderson et al. (1976). Activated partial thromboplastin time (APTT) clotting assays were carried out using human plasma samples (90 μL) mixed with 10 μL of polysaccharide (2.5-100 μg) solution and incubated for 1 min. at 37°C. Then, 100 μL of activated partial thromboplastin time reagent (Celite Biolab) was added to the mixture and incubated for 2 min. at 37°C. Next, 100 μL of 25 mM $CaCl_2$ was added and the clotting time recorded on a coagulometer. The activity was expressed as international units per mg of polysaccharide (IU mg^{-1}) using a parallel standard curve based on the 4th International Heparin Standard (193 IU mg^{-1}) from the National Institute for Biological Standard and Control, Potters Bar, Herts, UK. The APTT assays were also carried out using deficient human plasma from cofactors VII and IX.

Agarose gel electrophoresis

The Hf2s fraction, which showed the highest anticoagulant activity, was analyzed by 0.5% agarose gel electrophoresis according to Dietrich and Dietrich (1976). A sample of Hf2s (25 μg) was applied to a gel and run for 1h at 110 V in 0.05 M 1.3 diaminopropane-acetate buffer (pH 9.0). SP on gel were fixed with 0.1% *N*-cetyl-*N*-*N*-*N*-trimethylammonium bromide solution. After 12h, the gel was dried and stained with 0.1% toluidine blue and discolored with an acetic acid:absolute ethanol:distilled waster solution (0.1:0.45:0.45).

Effect of Hf2s on thrombin inactivation by heparin cofactor II

This assay was based on the amidolytic thrombin assay using chromogenic substrate as described by Farias et al. (2000). Hf2s solution (0-100 μg) was mixed with 5 μL of 1.5 μM heparin cofactor II (100 μg mL^{-1}) from Diagnostic Stago, Asnièris, France, in 85 μL of TS/PEG buffer (0.05 M Tris-HCl, 0.15 M NaCl and 1 mg mL^{-1} polyethylene glycol 8000, pH 7.4). Next, 15 μL of purified human thrombin was added to initiate the reaction. After 60 s incubation at 25°C, 500 μL of 24 mM chromogenic substrate S-2238 from Chromogenix AB (Molndal, Sweden) was added, and the remaining thrombin activity recorded for 120 s at 405 nm. Later, the rate of absorbance change was proportional to the thrombin activity remaining in the incubation.

Results and discussion

The total yield of the water-soluble polysaccharide extracted from the red alga *H. floresia* at room temperature (25°C) and sequentially at 80°C (twice) was 38.6%. However, the yield of Hf2s (20.6%) was greater than that of Hf1s (4.0%) and Hf3s (14.0%), respectively (Figure 1).

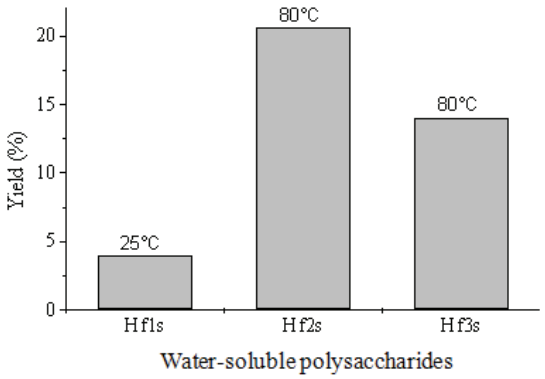

Figure 1. Yields of water-soluble polysaccharides obtained by different extraction conditions (25 and 80°C) from the red seaweed *H. floresia*.

In enzymatic digestion using papain, Farias et al. (2000) obtained a yield of 4.0% (w w^{-1}% dried) from the red alga *B. occidentalis* and Pereira et al. (2005), using the same method, extracted the crude polysaccharides of *G. crinale* (2.6%). Polysaccharide yields of 21.4 and 36.2% were also obtained with papain digestion, respectively, from red seaweeds *G. cornea* (MELO et al., 2002) and *Champia feldmannii* (ASSREUY et al., 2008). The fucoidan from the brown alga *Sargassum polycystum* was extracted (CHOTIGEAT et al., 2004) using HCl as solvent, and yielded 22.3%. Athukorala et al. (2006), evaluating seven species of seaweed, showed that the brown alga *E. cava* had the highest yield in total polysaccharides, with 41.52%.

Thus, several methods can be used to extract sulfated polysaccharides. However, the yield can vary depending on the technique used, algae used and extraction conditions (CHOTIGEAT et al., 2004; ATHUKORALA et al., 2006; RODRIGUES et al., 2011). More recently, Rodrigues et al. (2009a) reported that the employment of successive extractions to obtain polysaccharides can be a valuable tool for discovery of new macromolecules possessing anticoagulant activity. According to the authors, the yield of polysaccharides was greater on first (40.5%) when compared to second (4.9%) and third (1.74%) extractions, respectively. These different extracts of polysaccharides were oven-dried (60°C; 24h). In this study, we performed three differential successive extractions and noticed that at high temperatures (80°C), 34.6% polysaccharides were obtained (Figure 1).

Chemical composition

Chemical composition analysis of soluble fractions showed mainly the presence of galactose, small amounts of xylose, glucose and traces of other sugars. All polysaccharide fractions showed high contents of 6-*O*-metylgalactose e 3,6-anhydrogalactose (Table 1). Table 2 shows that sulfate content was high in all the fractions, mainly in Hf2s, with about 53.8% as shown in table 2. The results also demonstrated that the polysaccharide from *H. floresia* had high protein content in Hf2s (3.0%). According to Pushpamali et al. (2008), the authors reported that the polysaccharide from red alga *Lomentaria catenata* was mainly composed of galactose and had high sulfate content (21.76%). Furthermore, this same polysaccharide showed a protein content of 9.42%, suggesting that it is a proteoglycan. In our case, we suggest that the high protein content obtained in Hf2s cannot be considered a complex form (proteoglycan), and more detailed studies must be done.

Table 1. Monosaccharide composition of soluble crude sulfated polysaccharide fractions from *H. floresia*.

	Neutral monosaccharides content (mol %)					
	AG	Xyl	6Gal	2Gal	Gal	Glc
Hf1s	9.2	2.7	14.4	1.7	66.9	5.1
Hf2s	11.0	n.d.	9.3	n.d.	79.7	n.d.
Hf3s	8.3	n.d.	10.9	n.d.	80.5	n.d.

Note: AG = 3,6-anhydrogalactose; Xyl = xylose; 6Gal = 6-*O*-metylgalactose; 2Gal = 2-*O*-metylgalactose; Gal = galactose; Glc = glucose; n.d. – not detected.

Table 2. Chemical analysis of soluble crude sulfated polysaccharide fractions from *H. floresia*.

Fractions	Sulfate (%)	Total sugar (%)	Protein (%)
Hf1s	28.9	81.5	1.8
Hf2s	53.8	88.5	3.0
Hf3s	38.6	87.7	0.9

Anticoagulant activity of crude sulfated polysaccharides fractions from *H. floresia*

The anticoagulant activity of the crude sulfated polysaccharide fractions was determined by APTT and TT assays. The APTT assays indicated that the soluble sulfated polysaccharide fractions (Hf1s, Hf2s and Hf3s) from *H. floresia* had anticoagulant activity but were less potent than heparin (Figure 2). A longer coagulation time was observed in fraction Hf2s (68.4 IU mg^{-1}), which became excessively saturated at a concentration of 100 μg mL^{-1}. The prolongation of APTT usually suggests inhibition of the intrinsic and/or common pathway on the coagulation cascade (ATHUKORALA et al., 2006, 2007; ZHANG et al., 2008; AZEVEDO et al., 2009; RODRIGUES et al., 2009a, 2010).

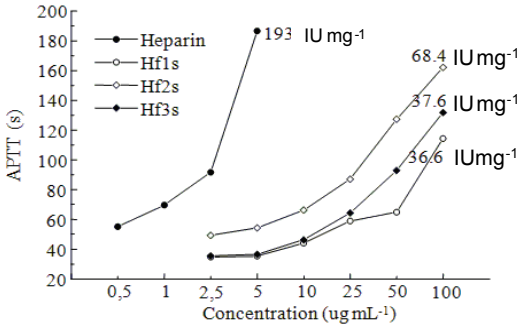

Figure 2. APTT clotting assays versus concentration of crude sulfated polysaccharides fractions. The assays were performed using normal human plasma at different polysaccharide concentrations. The activity was expressed as IU mg^{1} using the parallel standard curve with heparin.

The Hf1s, Hf2s and Hf3s fractions showed less potent anticoagulant activity when compared to the red seaweed *B. occidentalis* polysaccharides (150 IU mg^{-1}) by Farias et al. (2000), but similar to sulfated galactan from *G. crinale* (65 IU mg^{-1}) (PEREIRA et al., 2005). Pushpamali et al. (2008) also isolated an interesting purified anticoagulant from *L. catenata* (Rhodophyceae). The anticoagulant at a concentration of 40 μg mL^{-1} (> 1000 s) was capable of prolonging the APTT more than heparin (62.5 μg L^{-1}; > 1000 s; 183 IU mg^{-1}. The anticoagulant activity of Hf2s was higher than that of Hf1s and Hf3s, respectively, possibly due to the high sulfate content of polysaccharides as shown in Table 2. This finding confirms the hypothesis that an addition of sulfate ester in a single unit of α-galactose and the molecular weight of the galactan have an amplifying effect on the prolongation of clotting time, a finding also reported for the alga *B. occidentalis* (FARIAS et al., 2000). The desulfated Hf2s also showed no anticoagulant activity (data not shown), suggesting the importance of sulfate in this process.

The fraction Hf2s, which is a homogeneous crude sulfated polysaccharide (Figure 3A) and exhibited the highest anticoagulant activity (Figure 2), was also capable of prolonging the TT, almost completely inhibiting (91%) the activity of this enzyme at a concentration of 30 μg mL^{-1} (Figure 3B). Athukorala et al. (2006) and Zhang et al. (2008) suggested that a high inhibition of TT indicates an inhibition of fibrin polymerization. The inhibitory mechanism of the anticoagulant activity of *H. floresia* showed that this same polysaccharide was able to inhibit the action of thrombin by heparin cofactor II (Figure 3C). The prolongation of clotting time (APTT) was also evaluated in the presence of cofactor VIII and IX deficient plasma. The results showed that these two factors are not important to the inhibitory effects of Hf2s (Table 3).

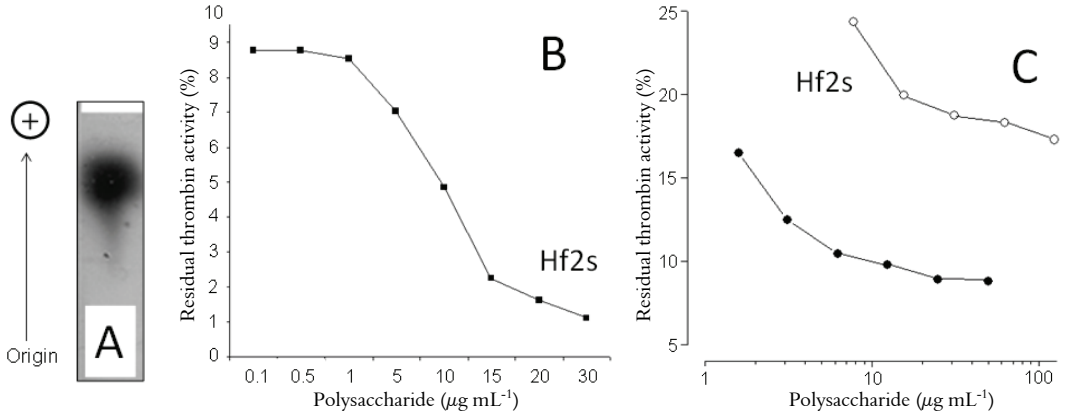

Figure 3. Agarose gel electrophoresis of Hf2s from *H. floresia* present on gel stained with 0.1% toluidine blue (A). Effect of Hf2s on thrombin inactivation (B) and by heparin cofactor II (C). Heparin cofactor II (30 nM) was incubated with thrombin (2 nM) in the presence of various concentrations of Hf2s. After 60 s of incubation at 37°C, the remaining thrombin activity was determined with a chromogenic substrate (A_{405} nm $min.^{-1}$). —○— in the absence of heparin cofactor II; —●— in the presence of heparin cofactor II.

Table 3. APTT clotting assays of fraction Hf2s from the alga *H. floresia* performed using deficient human plasma of cofactors VIII and IX.

	Cofactors	
	VIII	IX
Control plasma	32.9 s	
Deficient plasma	122.3 s	105.1 s
Control plasma + Hf2s	> 180.0 s	> 180.0 s
Control plasma + deficient plasma	37.6 s	38.5 s
Deficient plasma + Hf2s*	> 180.0 s	151.4 s
Deficient plasma + control plasma + Hf2s*	79.6 s	75.1 s

*At a polysaccharide concentration of 0.1 mg mL^{-1}.

Matsubara et al. (2001) reported an anticoagulant isolated from the green alga *Codium cylindricum* on thrombin activity in the absence of antithrombin III and heparin cofactor II using chromogenic substrate. The polysaccharide acting directly on thrombin. The results indicate that the anticoagulant from *H. floresia* acts on heparin cofactor II by inhibiting thrombin activity. These different anticoagulant mechanisms between the two species may depend on the polysaccharide structures that affect the interaction of polysaccharides and coagulation factors. The conformational activation of antithrombin with the consequent formation of the complex with thrombin seems to be less important than it is for heparin. Each type of polysaccharide may form a particular complex with plasmatic inhibitor and protease. The structural basis of this interaction is complex because it involves naturally heterogeneous polysaccharides, but depends on the distribution of sulfate groups and on monosaccharide composition (MELO et al., 2004). Thus, the chemical and structural characteristics of sulfated polysaccharides are also considered a prerequisite for understanding their biological activity (FARIAS et al., 2000; PEREIRA et al., 2005; FONSECA et al., 2008; ZHANG et al., 2008).

The anticoagulant action of Hf2s was dose-dependent, suggesting further studies of antithrombotic activities in rats (Figure 3). Fonseca et al (2008) suggested that the sulfated polysaccharides must be tested in different experimental animal models. In addition, it is also very important to compare not only the doses required to achieve thrombosis inhibition, but also the persistence of the effect, circulating plasma levels, the correlation between the anticoagulant action and antithrombotic effect, as well as bioavailability and absorption when administered by different routes. Thus, these studies may also help to determine a close relationship between the structure and anticoagulant activity of sulfated polysaccharides, as has already been reported for heparin, thus arousing a great interest for our group. Therefore, the structural analysis of this polysaccharide by infrared and NMR spectroscopies are already being conducted to elucidate its mechanism of action, including animal studies.

Conclusion

Soluble crude sulfated polysaccharide fractions obtained from *Halymenia floresia* had anticoagulant activity, but their effects were less potent than those of heparin. The anticoagulant mechanism of a crude fraction tested also suggests be dependent on cofactor heparin II to inhibit thrombin activity, but not on cofactors VIII and IX. Therefore, this polysaccharide interfered on the coagulation cascade and it can be a valuable tool for further studies of biological activities.

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