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Gurgel Rodrigues, José Ariévilo; Lino de Queiroz, Ismael Nilo; Façanha Bessa, Érika; Oliveira Coura, Chistiane; das Neves Amorim, Rodrigo César; Barros Benevides, Norma Maria
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Anticoagulant activity of sulfated polysaccharides fractions from an aqueous extract obtained from the red seaweed *Halymenia floresia* (Clemente) C. Agardh

José Ariévilo Gurgel Rodrigues[1], Ismael Nilo Lino de Queiroz[2], Érika Façanha Bessa[2], Chistiane Oliveira Coura[2], Rodrigo César das Neves Amorim[3] and Norma Maria Barros Benevides[3*]

*[1]Programa de Pós-graduação em Biotecnologia, Rede Nordeste de Biotecnologia, Universidade Estadual do Ceará, Fortaleza, Ceará, Brazil. [2]Programa de Pós-graduação em Bioquímica, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. [3]Laboratório de Carboidratos e Lectinas, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Av. Mister Hull, s/n, 60455-970, Fortaleza, Ceará, Brazil. *Author for correspondence. E-mail: nmbb@ufc.br*

ABSTRACT. Heparin (HEP) is known due to their side effects and the red seaweed *Halymenia floresia* (Hf) sulfated polysaccharides (SP) are heparinoids. In this study we purified the Hf-SP obtained from an aqueous extract and evaluated their anticoagulant activities. Hf-SP_1 (25°C), Hf-SP_2 (80°C) and Hf-SP_3 (80°C) were sequentially isolated. Hf-SP_3 had the highest sulfate content (37.45%). Hf-SP_3 was fractionated by ion exchange chromatography on a DEAE-cellulose column using a NaCl gradient. Fractions were lyophilized and submitted to 0.5% agarose gel electrophoresis. The anticoagulant activity was evaluated by the activated partial thromboplastin time using rabbits plasma and expressed in international units per mg of SP using standard HEP (193 IU mg^{-1}). The chromatographic procedure separated into four different SP fractions (F I, F II, F III and F IV) eluted at concentrations of 0.50, 0.75, 1.00 and 1.25 M of NaCl, respectively, reveling among them different marked on charge density, when compared by electrophoresis. F III had the highest anticoagulant activity (10.72 IU mg^{-1}), suggesting that the sulfate is important in this process. In conclusion, our results suggest that sequential extractions of Hf-SP are an important biotechnological tool for identification of novel anticoagulants and studies of structural characterization are already in progress.

Keywords: marine alga, sulfated macromolecules, purification, physical-chemical characterization, blood coagulation.

RESUMO. Atividade anticoagulante de frações polissacarídicas sulfatadas de um extrato aquoso obtido da alga marinha vermelha *Halymenia floresia* (Clemente) C. Agardh. A heparina (HEP) é conhecida devido aos seus efeitos colaterais e os polissacarídeos sulfatados (PS) da alga marinha vermelha *Halymenia floresia* (Hf) são heparinoides. Objetivou-se purificar os Hf-PS obtidos de um extrato aquoso e avaliar suas atividades anticoagulantes. Foram isolados seqüencialmente Hf-SP_1 (25°C); Hf-SP_2 (80°C) e Hf-SP_3 (80°C). Os Hf-SP_3 apresentaram o maior conteúdo de sulfato (37,45%), sendo fracionados por cromatografia de troca iônica em coluna de DEAE-celulose utilizando um gradiente de NaCl. As frações obtidas foram liofilizadas e submetidas à eletroforese em gel de agarose a 0,5%. A atividade anticoagulante foi avaliada por meio do tempo de tromboplastina parcial ativada usando plasma de coelho e HEP padrão (193 UI mg^{-1}). O procedimento cromatográfico separou em quatro diferentes frações de PS (F I, F II, F III e F IV), eluídas nas concentrações 0,50; 0,75; 1,00 e 1,25 M de NaCl, respectivamente, revelando diferenças marcantes na densidade de carga entre elas, quando comparadas por eletroforese. A maior atividade anticoagulante foi obtida na F III (10,72 UI mg^{-1}), sugerindo que o sulfato é importante nesse processo. Os resultados sugerem que extrações seqüenciais de Hf-SP são uma ferramenta biotecnológica importante para a identificação de novos anticoagulantes. Estudos relacionados à caracterização estrutural já estão em andamento.

Palavras-chave: alga marinha, macromoléculas sulfatadas, purificação, caracterização físico-química, coagulação sanguínea.

Introduction

Seaweed sulfated polysaccharides (SP) are widely used in pharmaceutical, cosmetic and food industries (MELO et al., 2002; CAMPO et al., 2009; SILVA et al., 2010), and aquatic sciences (BARROSO et al., 2007; ARAÚJO et al., 2008). These compounds have gained attention as biological macromolecules due to their potential as

anticoagulant and antithrombotic agents (ASSREUY et al., 2008; ATHUKORALA et al., 2006; AZEVEDO et al., 2009; FARIAS et al., 2000, 2001; FONSECA et al., 2008; MEDEIROS et al., 2008; MOURÃO, 2004; PEREIRA et al., 2005; PUSHPAMALI et al., 2008; RODRIGUES et al., 2009, 2010a, 2011a; YOON et al., 2007; ZHANG et al., 2008). In addition, the seaweed SP are polymers complex and heterogeneous found as constituents of extracellular matrix (KLOAREG; QUATRANO, 1988). No risk of contamination by viral particles or priors has also been reported (LEITE et al., 1998). Owing to several side effects of heparin (HEP) (NADER et al., 2001), a SP commercial anticoagulant widely used in prevention and treatment of thromboembolic disorders (MOURÃO; PEREIRA, 1999) and extracorporeal circulation (MELO et al., 2008), the development of new anticoagulant drugs is needed. Furthermore, HEP is extracted in low concentrations in pig intestine or bovine lungs (THOMAS, 1997).

Although seaweed SP have been described as anticoagulants for some time, difficulty of the isolation in pure form is still reported (ASSREUY et al., 2008; FARIAS et al., 2000, 2001; LEITE et al., 1998; PUSHPAMALI et al., 2008; RODRIGUES et al., 2009; SILVA et al., 2005). Their heterogeneity and polydispersity limit their structural study (AZEVEDO et al., 2009; FARIAS et al., 2000; MELO et al., 2002; MOURÃO, 2004; PEREIRA et al., 2005; RODRIGUES et al., 2009; YOON et al., 2007; ZHANG et al., 2008). In contrast, some researchers have been done for obtaining of these macromolecules more homogeneous (RODRIGUES et al., 2009, 2010b).

In this context, we also expanded our investigations to purification of SP from marine alga. In case of red seaweeds, we have observed that SP species of the *Halymenia* genus occur on distinct molecular characteristics into the algal tissue when obtained by successive extractions. These studies have also revealed as an important biotechnological tool for identification of novel anticoagulants (RODRIGUES et al., 2009, 2010b). More recently, we extended our studies to green alga *Caulerpa cupressoides* (RODRIGUES et al., 2011a). The *C. cupressoides* SP also occur on distinct molecular characteristics into the algal tissue. However, we observed macromolecules excessively poly-disperses along of the technique. Thus, the studies suggest that the molecular characteristics of seaweed SP vary among different species, when obtained by papain digestion.

The *Halymenia floresia* SP (Hf-SP) have been studied (AMORIM et al., 2011). We report now the purification and anticoagulant activity of Hf-SP fractions from an aqueous extract obtained from this species.

Material and methods

Marine algae

The red seaweed *H. floresia* (Clemente) C. Agardh was collected in March, 2004 on the Northeast coast of Brazil (Pedra Rachada Beach, Ceará State). After collection, the material was washed with distilled water, and stored at –20°C at Carbohydrate and Lectins Laboratory (CarboLec), Department of Biochemistry and Molecular Biology, Federal University of Ceará, Brazil. Hf-SP were extracted as previously described (AMORIM et al., 2011). Briefly (Figure 1), the algae were submitted to 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 4°C). 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 Hf-SP_1. The algal residue was reextracted 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 Hf-SP_2 and Hf-SP_3 for the second and third extractions, respectively.

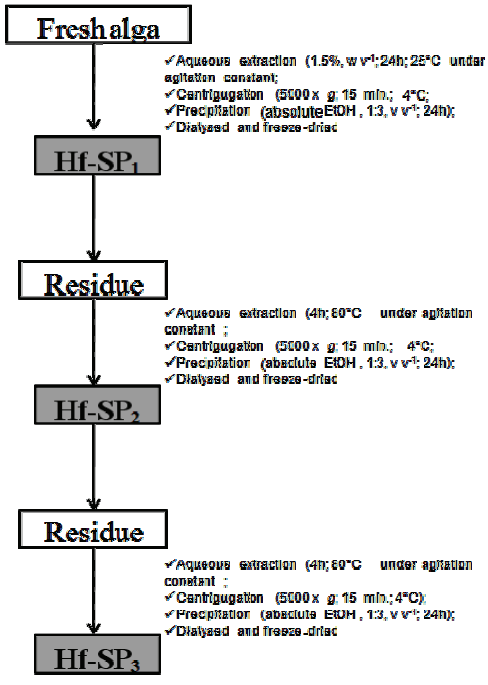

Figure 1. Protocol of obtaining of crude sulfated polysaccharides (Hf-SP_1, Hf-SP_2 and Hf-SP_3) from the red seaweed *Halymenia floresia*.

Chemical composition of Hf-SP

The total sugars (TS) content was estimated by phenol-sulfuric acid analysis using D-galactose as the standard (DUBOIS et al., 1956) at 490 nm. After acid hydrolysis of the soluble polysaccharides (1 mL of HCl for 5h at 100°C), free sulfate (FS) was measured by the $BaCl_2$/gelatin method (DODGSON; PRICE, 1962). The contaminant proteins (CP) content was measured by the method of Bradford (1976), using bovine serum albumin to construct the standard curve.

Ion-exchange chromatography

Hf-SP_3 (16 mg) were dissolved in 0.05 M sodium acetate buffer (pH 5.0) (Vetec Química) (2 mg mL^{-1}) and submitted to an ion exchange chromatography on a DEAE-cellulose column (1.5 × 12 cm) (Sigma Chemical) equilibrated and washed with this same buffer. Hf-SP_3 adsorbed on gel were eluted at different concentrations of NaCl (0.50, 0.75, 1.00 and 1.25 M). Fractions of 3 mL were collected in a collector FRAC-920 (90 mL h^{-1}) and monitored by metachromasia with the 1,9 dimethylmethylene blue (Sigma-Aldrich) at 525 nm (AMERSHAM BIOSCIENCES ULTROSPEC 1100) (FARNDALE et al., 1986). Then, the metachromatic fractions were exhaustively dialyzed against distillate water and freeze-dried for posterior use.

Total sugar (TS) content of metachromatic fractions obtained by ion-exchange chromatography (DEAE-cellulose)

The TS content was estimated by phenol-sulfuric acid analysis using D-galactose as the standard (DUBOIS et al., 1956) a plate (MASUKO et al., 2005), using an Elisa raider (AMERSHAM BIOSCIENCES, BIOTRAK II) at 492 nm. The presence of sulfate in the obtained fractions by ion exchange chromatography (DEAE-cellulose) was also estimated by the metachromatic integrated area using the 7.0 ORIGIN program.

Agarose gel electrophoresis

The fractions obtained by ion-exchange chromatography (DEAE-cellulose) were analyzed by 0.5% agarose gel electroforesis according to Dietrich and Dietrich (1976). Sample of 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: distillated water solution (0.1:0.45:0.45).

Evaluation of SP fractions by the Activated Partial Thromboplastin Time (APTT) test

The assay was carried out using citrated rabbit plasma according to the manufacturers' specifications. 50 μL of rabbit plasma was mixed with 10 μL of a solution of different amounts of polysaccharide before addition of 50 μL of APTT reagent. The mixture was then incubated at 37°C for 3 min. Then, 50 μL of 0.025 M of calcium chloride reagent was added to the mixture to trigger the coagulation cascade. The clotting time was recorded in a coagulometer (DRAKE QUICK TIMER). HEP (National Institute for Biological Standards and Control (Potters Bar, UK)) with 193 international units per mg of polysaccharide (IU mg^{-1}) was used as the standard. All tests were performed in triplicate.

Results and discussion

We have previously reported that the red seaweed *H. floresia* is composed by three crude SP (Hf-SP_1, Hf-SP_2 and Hf-SP_3). These polysaccharides showed *in vitro* anticoagulant activity (APTT test) dependent of the sulfate content. The Hf-SP accelerates thrombin inhibition by heparin cofactor II. The chemical composition also showed that the Hf-SP is composed of 6-*O*-metylgalactose and 3,6-anidrogalactose (AMORIM et al., 2011). Here, to further evaluate other characteristics of Hf-SP, we extended our investigation to an aqueous extract (80°C) (denomined Hf-SP_3) obtained and fractionated by ion-exchange chromatography on a DEAE-cellulose column and analyzed by agarose gel electrophoresis procedure. Initially, the different obtained aqueous extracts (Hf-SP_1 (25°C), Hf-SP_2 (80°C) and Hf-SP_3 (80°C)) were obtained. Among them, the crude Hf-SP_3 had the highest FS content (37.45%), TS (85.88%) and the low CP content (1.63%), as shown in Table 1, and was used on subsequent studies. The high CP found in these crude SP may perhaps be the presence of amino acids (GHOSH et al., 2004) and/or polysaccharides-protein complex forms (MELO et al., 2002; PUSHPAMALI et al., 2008). Thus, a more detailed study of these macromolecules is suggested.

Table 1. Chemical composition of aqueous extracts obtained from the red seaweed *Halymenia floresia*.

Polysaccharides	°C [a]	TS [b] (%)	FS [c] (%)	CP [d] (%)
Hf-SP_1	25	62.40	20.60	8.20
Hf-SP_2	80	74.29	32.20	2.50
Hf-SP_3	80	85.88	37.45	1.63

a - Polysaccharides obtained by aqueous extractions at 25 and 80 °C; b – Dosage by Dubois et al. (1956)' method using D-galactose as standard; c – Dosage by Dodgson and Price (1962)' method using $NaSO_3$ as standard; d – Dosage by Bradford (1976)' method using bovine serum albumin.

In this study, the obtaining of distinguish isolated SP at different temperatures from the *H. floresia* tissue support the hypothesis of Percival and McDowell (1967), suggesting that the use of consecutive extractions result in the obtaining of different macromolecules in chemical composition. This fact further justifies the occurrence of distinct SP into tissue of the studied species. Therefore, such disproportions in chemical composition also justify the complexity and heterogeneity of these polymers (FARIAS et al., 2000; GHOSH et al., 2004; SILVA et al., 2005; RODRIGUES et al., 2010a, 2011b). From these data (Table 1), we chose the Hf-SP_3 (80°C) which was submitted to ion-exchange chromatography on a DEAE-cellulose column, an important technique for separation of these compounds.

Ion-exchange chromatography

The DEAE-cellulose chromatography profile is shown in Figure 1. The chromatographic profile indicated the separation into four different fractions of SP (F I, F II, F III, and F IV) eluted at concentrations of 0.50, 0.75, 1.00, and 1.25 M of NaCl, respectively. F II had the highest metachromatic peak compared to other obtained fractions. The highest yield of SP and TS and FS contents were also obtained in F II, eluted with 0.75 M of NaCl, compared to F I, F III and F IV (Table 2).

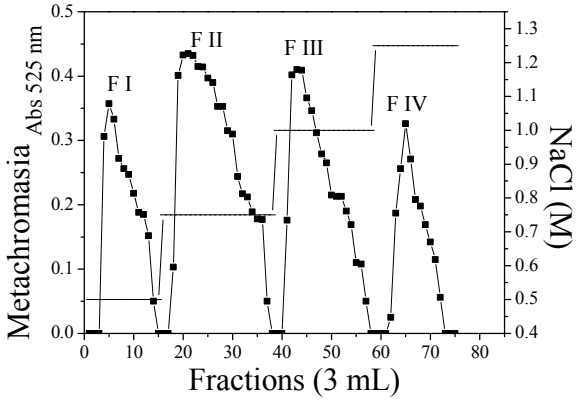

Figure 1. Purification of Hf-SP_3 from red seaweed *Halymenia floresia* by DEAE-cellulose. Fractions were collected and checked by metachromasia using 1,9-dimethymethylene blue (■—■). Vertical steps represent the NaCl concentration (—).

Table 2. Yield and chemical composition of sulfated polysaccharides fractions obtained by ion exchange chromatography (DEAE-cellulose) from the red seaweed *Halymenia floresia*.

Fraction [a]	NaCl (M)	Yield (%) [b]	TS (%) [c]	FS (%) [d]
F I	0.50	21.87	68.37	17.36
F II	0.75	24.38	87.64	40.75
F III	1.00	19.38	77.33	28.66
F IV	1.25	14.37	56.00	13.23

a – Fractions obtained on DEAE-cellulose column; b – Yields from a sample of SP applied on DEAE-cellulose column; c – Expressed by Dubois et al. (1956)' method in plate by Masuko et al. (2005)' method using D-galactose as standard; d – Sulfate expressed by metachromatic integrated area from the chromatographic profile (DEAE-cellulose).

The employment of DEAE-cellulose as a matrix has been widely reported for separation of SP, to reveal the characteristics of different algal species, such as on *Gelidium crinale* (PEREIRA et al., 2005), *Ecklonia cava* (ATHUKORALA et al., 2006), *Champia feldmannii* (ASSREUY et al., 2008), *Lomentaria catenata* (PUSHPAMALI et al., 2008), *Halymenia pseudofloresia* (RODRIGUES et al., 2009), *C. cupressoides*, *C. racemosa* (RODRIGUES et al., 2010a), *Halymenia* sp. (RODRIGUES et al., 2010b) and *Hypnea musciformis* (RODRIGUES et al., 2011b).

Agarose gel electrophoresis

The electrophoretic profile is shown in Figure 2. The agarose gel electrophoresis procedure showed marked differences in charge density among the isolated fractions. However, this was not corroborated by the higher presence of sulfate (Table 2). Thus, fractions F I, F II and F III were not observed on agarose gel, suggesting little sulfated groups in their chemical structures. On the other hand, F III, eluted with 1.00 M of salt, had a strong metachromatic band on gel, showing a similar migration to glycosaminoglycan condroitin sulfate (CS), while the Hf-SP_3 showed a similar charge density when compared to glycosaminoglycan dermatan sulfate (DS), both glycosaminoglycans obtained from animal tissues. Curiously, F III was also a homogeneous SP when compared to Hf-SP_3. This suggests that the native crude compound (Hf-SP_3) is homogeneous molecule (Figure 2) and the ion-exchange chromatography procedure is efficient for separation of Hf-SP (Figure 1). Therefore, the isolation of these compounds could be a useful tool for posterior structural characterization studies (FARIAS et al., 2000; PEREIRA et al., 2005; RODRIGUES et al., 2009, 2010a and b; SILVA et al., 2005).

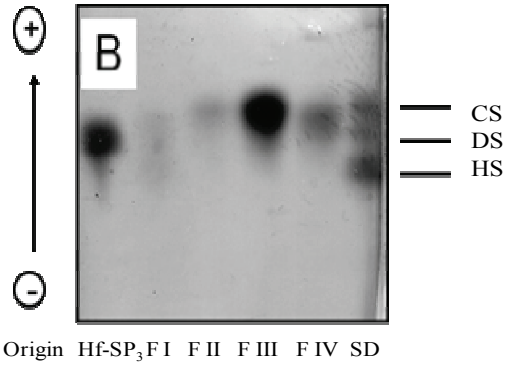

Figure 2. Agarose gel electrophorese of sulfated polysaccharides isolated from *Halymenia floresia*. Hf-SP_3, fractions (F I, F II, F III and F IV) and standards (SD) (condroitim sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS)) presents on gel were stained with 0.1% toluidine blue.

In this study, it was observed more homogeneous molecules in third aqueous extraction (Figure 2) compared to first (25°C) and second (80°C) ones, respectively (data not shown). According some researchers involving the obtaining of SP by successive extractions from other *Halymenia* species (RODRIGUES et al., 2009, 2010b). It seems that their molecular characteristics are common on this genus, but not show the same profile when compared to polysaccharides isolated from green seaweed *C. cupressoides* (RODRIGUES et al., 2011a). Therefore, this technique could also be a valuable tool for identification of molecular characteristics among different algal species and biological agents. However, the chemical composition of these compounds can vary due to, for example, temperature, light and water nutrients, as well as the place and season of the year (MARINHO-SORIANO; BOURRET, 2003; PERCIVAL; McDOWELL, 1967). In this connection, a more detailed study of these macromolecules is indicated. Our study also involved to evaluate the potential anticoagulant from their isolated fractions. In this line, the homogeneous SP (F III) shown by the electrophoretic technique led us to conduct anticoagulant assays.

Anticoagulant activity

We have been recently described that Hf-SP are heparinoids. The anticoagulant activity determined by APTT test for crude fractions Hf-SP_1, Hf-SP_2 and Hf-SP_3 about 37, 68 and 36 IU mg^{-1}, respectively. Sulfate content is important in this process (AMORIM et al., 2011). Although showing the lowest activity, Hf-SP_3 was explored because of its more homogeneous form (Figure 2). Thus, the anticoagulant assays (APTT) showed fractions of SP capable of modifying the normal coagulation time (Table 3). Fraction F III (1.00 M of NaCl), at a concentration of 0.25 mg mL^{-1} of SP, prolonged the APTT of normal rabbit plasma (20.45 s), whose activity was 10.72 IU mg^{-1}, comparing to standard HEP (193.00 IU mg^{-1}). Fractions F I, F II and F IV, eluted with 0.50, 0.75 and 1.25 M of NaCl, respectively, no practically extended the APTT, when 1.00 mg mL^{-1} of SP was measured. Therefore, Hf-SP_3 fractions had a low anticoagulant potential.

Based on the popular use of marine algae for disorders such as cardiovascular diseases and cancer, studies with SP from algae have intensified. In this context, the discovery of new anticoagulant compounds is needed (MOURÃO; PEREIRA, 1999), and seaweed SP are a potentially attractive source of macromolecules to investigate. These anticoagulant SP include fucoidans, sulfated galactans, ulvans and rhamnose (ASSREUY et al., 2008; ATHUKORALA et al., 2006; FONSECA et al., 2008; MOURÃO, 2004; PEREIRA et al., 2002; PUSHPAMALI et al., 2008; RODRIGUES et al., 2009, 2010a, 2011b; SILVA et al., 2005; MEDEIROS et al., 2008; YOON et al., 2007; ZHANG et al., 2008). It has been believed that the sulfate is important in this process (AZEVEDO et al., 2009; FARIAS et al., 2000; NISHINO et al., 1991; RODRIGUES et al., 2011a).

Table 3. Anticoagulant activity of sulfated polysaccharides fractions (Hf-SP_3) obtained by ion-exchange chromatography (DEAE-cellulose) from *Halymenia floresia* compared to HEP.

Fractions	NaCl (M)	APTT test[a]				
		1.00[b]	0.75[b]	0.50[b]	0.25[b]	IU mg^{-1} [c]
F I	0.50	26.10	-	-	-	1.25
F II	0.75	25.70	-	-	-	1.23
F III	1.00	101.00	80.30	66.15	55.80	10.72
F IV	1.25	58.30	-	-	-	2.65

a - APTT in seconds; b - Concentration of SP (mg mL^{-1}) for prolonging the APTT; c - Activity express in international units (IU) per mg of SP; - Without activity; HEP (193.00 IU mg^{-1}; 0.01 mg mL^{-1}; APTT: 40.15 s);

In the present study, we extended to explore of SP fractions from *H. floresia* (Hf-SP_3) obtained by ion-exchange chromatography (DEAE-cellulose). All the SP fractions were capable of prolonging the APTT (Table 3). Fraction F III had the highest activity when compared to F I, F II and F IV. As expected, the difference in the activities of these fractions was dependent of the charge density observed by electrophoresis procedure, showing clearly that the presence of sulfate groups is also important in this process. This point of view was observed in a previously investigation, using a dessulfated crude SP (AMORIM et al., 2011). On the another hand, the effect of SP on coagulation system do not occur merely as function of charge density, but also of chemical composition, position of sulfate groups and the occurrence of dessulfated units (MOURÃO, 2004). Each polysaccharide has a structural requirement for interaction with coagulation cofactors and their target proteases are stereospecific (FARIAS et al., 2000; PEREIRA et al., 2002, 2005). Therefore, the chemical characteristics are also prerequisites for understanding of these polymers as their structure/biological function relationships (AZEVEDO et al., 2009; ZHANG et al., 2008). Animals model of thrombosis have been important tools in these investigations (MOURÃO; PEREIRA, 1999; FARIAS et al., 2001; FONSECA et al., 2008).

Our study suggest the hypothesis of Farias et al. (2000) 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 algae *Botryocladia occidentalis*. The inhibitory mechanism of the anticoagulant activity previously reported for *H. floresia* showed that the crude polysaccharide (Hf-SP_3) is able to inhibit the action of thrombin by heparin cofactor II. The clotting time (APTT) is also considerably prolonged in the presence of cofactor VIII and IX deficient plasma. However, the results showed that these two factors are not important to the inhibitory effects of polysaccharide (AMORIM et al., 2011). Thus, the obtained polysaccharide (Figure 2) could also be very important to evaluate and 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 (MOURÃO; PEREIRA, 1999).

Overall, our study reported a homogeneous heparinoid from *H. floresia*. Although showing a low anticoagulant potential, its posterior investigation may help to determine a close relationship between the structure and anticoagulant activity of SP, as has already been reported for heparin, arousing thus a great interesting for our group. Structural analysis of this SP fraction by infrared and NMR spectroscopies can help to this end, including animal studies.

Conclusion

The anticoagulant activity of sulfated polysaccharides fractions obtained from an aqueous extracted from the red alga *Halymenia floresia* were fewer actives than heparin. However, the used technique for isolation of these molecules showed to be an important tool in the identification of more homogeneous polysaccharides. Structural analysis of polysaccharide by infrared and NMR spectroscopies and its mechanism of action in posterior studies can help to better understanding its particular biological action using animal models of thrombosis.

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