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Mild-acid hydrolysis of a native polysulfated fraction from *Acanthophora muscoides* generates sulfated oligosaccharides displaying *in vitro* thrombin generation inhibition

José Ariévilo Gurgel Rodrigues[1*], Ismael Nilo Lino de Queiroz[1], Ana Luíza Gomes Quinderé[2], Norma Maria Barros Benevides[2], Ana Maria Freire Tovar[1] and Paulo Antônio de Souza Mourão[1*]

*[1]Laboratório de Tecido Conjuntivo, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Av. Brigadeiro Trompowsky, s/n.º, 21941-590, Ilha do Fundão, Rio de Janeiro, Brazil. [2]Laboratório de Carboidratos e Lectinas, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. *Author for correspondence. *E-mail: arieviloengpesca@yahoo.com.br; pmourao@hucff.ufrj.br*

ABSTRACT. *A. muscoides* (Rhodophyta) has three polysulfated fractions (-1, -2 and Am-3). Am-2 displayed anti-inflammation and serpin-independent anticoagulation effects; however, no effect of oligomers on thrombin-generation (TG) has been demonstrated. This study employed mild-acid hydrolysis to obtain low-molecular-size derivatives from Am-2 and compared *in vitro* inhibitory effects between intact Am-2 and its hydrolysates on a TG assay. The polysaccharidic extract was fractionated by DEAE-cellulose that revealed Am-2 eluted with 0.75-M NaCl containing sulfate (23%), hexoses (51%) and absence of proteins, and indicating, by one-dimension nuclear magnetic resonance, structure of galactan similar to that of the extract. The depolymerization with HCl (0.02 or 0.04-M, 60°C) for different times progressively reduced the charge density and the molecular-size of Am-2 based on electrophoresis in agarose and polyacrylamide gels, respectively, where at higher acid concentration and critical time up to 5h yielded fragment of ~14-kDa similar to that of unfractionated heparin (UHEP). Regarding the TG assay, intact Am-2 inhibited concentration-dependent intrinsic pathway, whereas its hydrolysates abolished it like UHEP, except the analog fragment (92.87% inhibition), when in 60-fold diluted human plasma using chromogenic method in a continuous system. The results reveal an alternative approach for the production of oligosaccharides from *A. muscoides* with TG inhibition.

Keywords: Rhodophyceae, polyanionics, depolymerization, thrombin.

Hidrólise ácida branda de uma fração polissulfatada de *Acanthophora muscoides* gera oligossacarídeos sulfatados mostrando inibição de geração de trombina *in vitro*

RESUMO. A rodofícea *A. muscoides* possui três frações polissulfatadas (-1, -2 e Am-3). Am-2 mostrou efeito anti-inflamação e anticoagulação independente de serpina. Entretanto, não se demonstrou efeito de oligômeros sobre ensaio de geração de trombina (GT). Este estudo empregou hidrólise ácida branda para obter derivados de tamanho molecular baixo de Am-2 e os efeitos inibitórios *in vitro* entre Am-2 intacta e hidrolisados comparados sobre um ensaio de GT. O extrato polissacarídico, fracionado por DEAE-celulose, revelou Am-2 eluída com NaCl-0,75M contendo sulfato (23%), hexoses (51%) e destituída de proteínas. E, ainda, por ressonância magnética nuclear-unidimensional, indicando galactana semelhante a do extrato. A depolimerização com HCl (0,02 ou 0,04-M; 60°C) reduziu, progressivamente durante tempos diferentes, a densidade de carga e o tamanho molecular de Am-2 baseada nas eletroforeses em géis de agarose e de poliacrilamida, respectivamente, em que, concentração ácida elevada e tempo crítico de até 5h renderam fragmento de ~14-kDa semelhante ao da heparina não fracionada (HEPNF). Já no ensaio de GT, Am-2 intacta, quando em plasma humano diluído 60 vezes, usando método cromogênico por meio de sistema contínuo, quem inibiu a via intrínseca dependente de concentração, ao passo que seus hidrolisados aboliram como HEPNF, exceto fragmento análogo (inibição 92,87%). Os resultados revelam uma abordagem alternativa para produzir oligossacarídeos de *A. muscoides* com inibição de GT.

Palavras-chave: Rhodophyceae, polianiônicos, depolimerização, trombina.

Introduction

Over the past years, a number of marine bioproducts (e.g., carbohydrates, proteins and lipids) has been described by many scientific groups and corporate institutions of the world (Mayer, Rodríguez, Berlink, & Hamann, 2007; Queiroz et al., 2015; Rodrigues, Torres, Alencar, Sampaio, & Farias, 2009; Smit, 2004). As a result, these extensive

efforts at prospecting of diverse classes of unique compounds from distinct origins have generated a vast knowledge in marine biotechnology to develop novel therapeutics to control several physiological reactions, e.g. angiogenesis (Cardozo et al., 2007; Pomin, 2012), coagulation (Leite et al., 1998; Rodrigues et al., 2009; Rodrigues et al., 2013), inflammation (Rodrigues et al., 2012; Smit, 2004) and infections (Mayer et al., 2007; Smit, 2004). Among all chemicals, sulfated polysaccharides (SPs - also known as glycans) from seaweeds, due to their intrinsic abilities (e.g., gelling and stabilizing) to produce high added-value products (Cardozo et al., 2007; Smit, 2004), differential levels of health related-biomedical responses (Pomin, 2012; Rodrigues et al., 2009; Rodrigues et al., 2012) and in non-food industrial innovation fields, such as cosmetics and printing, have been vigorously studied, positively impacting the modern societies (Cardozo et al., 2007; Prajapati, Maheriya, Jani, & Solanki, 2014; Smit, 2004).

The extracellular-matrix of red seaweeds is naturally rich in sulfated galactans (Mourão, 2015; Pomin, 2012; Prajapati et al., 2014), a class of highly complex and heterogeneous SPs of hydrophilic character due to the occurrence of ester sulfate groups (S = O), which are mainly known in the enantiomeric forms "carrageenan" (D-) and "agaran" (L-) or hybrid form (D-/L-) on the same backbone constitution of alternating 4-linked α-galactopyranosyl and 3-linked β-galactopyranosyl units, with variable sulfation (Cardozo et al., 2007; Prajapati et al., 2014; Smit, 2004; Souza et al., 2015). The phyla Phaeophyta and Chlorophyta are the most common sources of fucan or fuicodan (Leite et al., 1998; Pomin, 2012) and sulfated heteropolysaccharides (Rodrigues et al., 2013, 2014; Zhang et al., 2008), respectively. Other organisms were also shown to have SPs (Aquino, Landeira-Fernandez, Valente, Andrade, & Mourão, 2005; Chang, Lur, Lu, & Cheng, 2013; Dantas-Santos et al., 2012). Overall, SPs have high sulfation patterns and high molecular weights (> 100 kDa), directly influencing *in vitro* and *in vivo* systems (Mayer et al., 2007; Pomin, 2012; Yao, Wu, Zhang, & Du, 2014).

Although they exhibit a versatility of applications, SPs present difficulties for the study of their properties due to their highly heterogeneous structures (e.g., sulfation pattern, side-chain substitutions, and the presence of methyl ethers or anhydro sugars) (Cardozo et al., 2007; Mourão, 2015; Pomin, 2012). Thus, some alternative methods (depolymerization) to obtain low molecular weight derivatives (called oligosaccharides or oligomers) have been described, such as enzymatic hydrolysis that cleave the SPs core through rapid decrease in the molecular masses (Athukorala, Jung, Vasanthan, & Jeon, 2006; Yao et al., 2014), H_2O_2 degradation that cleave on the glicosidic linkages of the SPs (Zhang et al., 2008), and mild-acid hydrolysis that specifically desulfate and depolymerize the SPs at determined sites (Melo & Mourão, 2008; Pomin, Valente, Pereira, & Mourão, 2005; Queiroz et al., 2015). In addition, the rate of decrease in the molecular masses of the SPs depends on the type of linkage and on the chain length (Pomin et al., 2005; Athukorala et al., 2006). Recently, Panagos, August, Jesson, and Uhrin (2016) reported that dermatan sulfate (a class of SP obtained from animal tissue), using Fenton type free radical depolymerization, yielded exclusively oligosaccharides with reducing end acetylgalactosaminic acid. Previous studies demonstrated that chemically produced-sulfated oligomers from unmodified SPs of seaweeds had anticoagulant (Athukorala et al., 2006), antithrombotic (Melo & Mourão, 2008), antioxidant (Vijayabaskar, Vaseela, & Thirumaran, 2012) and anti-angiogenic (Yao et al., 2014) effects.

Thrombin generation (TG) inhibition assays have became relevant tools to analyze the action of SPs as plasma alternative anticoagulants to unfractionated heparin (UHEP) (Glauser et al., 2009; Mourão et al., 2001; Nishino, Fukuda, Nagumo, Fujihara, & Kaji, 1999; Zhang et al., 2014), a drug commercially used in anticoagulant therapy (e.g. extracorporeal circulation and hemodialysis) with bleeding effects (Mourão, 2015; Nader et al., 2001). However, no study has described the *in vitro* inhibitory effects of TG in plasma using marine sulfated oligosaccharides.

The *Acanthophora* genus J. V. F. Lamouroux (order Ceramiales) of red seaweeds is widely found along the tropical and subtropical regions of the world. Phytochemicals and pharmaceutics from this genus have been documented (Duarte et al., 2004; Muthuraman, Mani, Thangaraj, & Sivasubramanian, 2014). Regarding the species *A. muscoides* (Linnaeus) Bory de Saint-Vicent, three SPs fractions (-1, -2 and Am-3) have been previously isolated. Am-2 displayed analgesic, anti-inflammatory (Quinderé et al., 2013; Quinderé et al., 2015) and serpin-independent anticoagulant / antithrombotic (Quinderé et al., 2014) effects. Analyses by NMR revealed complex SP consisting of variable sulfation and methyl ether substitutions along with the occurrence of 3,6-anhydro-α-galactose (Quinderé

et al., 2014). The present study employed the mild-acid hydrolysis to obtain sulfated oligosaccharides from Am-2 and compared the *in vitro* inhibitory effects between unmodified Am-2 and its solution derivatives on TG in 60-fold diluted human plasma using chromogenic method in a continuous system.

Material and methods

Marine alga and preparation of Am-2

The red seaweed *A. muscoides*, from the family Rhodomelaceae, was carefully collected in September 2008 along the coastal zone of Pacheco, Northeastern region of Brazil. The macroscopic epiphytes attached to the material were removed and the algae was further washed with distilled water, dehydrated at room temperature, macerated in liquid nitrogen and stored at 20°C until use. A voucher specimen (no. 46093) was deposited in the Herbarium Prisco Bezerra in the Department of Biology, Universidade Federal do Ceará, Brazil. Crude SP extract was obtained by papain digestion (60°C, 6h) in 100 mM sodium acetate buffer (pH 5.0) containing EDTA and cysteine (both 5 mM). The structural features of the crude SPs extract were analyzed by one-dimension nuclear magnetic resonance (^{1}H NMR) experiment (10 mg, at 35°C) using a Bruker DRX 600 MHz apparatus with a triple resonance probe. Procedure of DEAE-cellulose column was developed by stepwise elution (0→1M, with 0.25 M of intervals) and the fractions -1, -2 and Am-3 were monitored by metachromasia using 1,9-dimethylmethylene blue (525 nm). SPs present in Am-2 were examined for sulfate [S], hexoses [HEXs] and contaminant proteins [CPs], and the degree of purity was verified by both electrophoresis in agarose gel and in polycrylamide gel (Page), respectively. All these techniques were employed as previously described (Quinderé et al., 2013, 2014).

Mild-acid hydrolysis for production of Am-2-derived sulfated oligosaccharides

This method was based on Pomin et al. (2005). Native Am-2 (10 mg) was dissolved in 1 mL of 0.02 or 0.04 M HCl and maintained at 60°C for different periods. After this depolymerization procedure, the pH was neutralized by the addition of 1 mL of ice-cold 0.02 or 0.04 M NaOH. The reduction of metachromatic property and the molecular masses of the different low molecular size fragments were analyzed by electrophoresis (agarose and Page) at 100 V for ~50 min., by comparison with the electrophoretic mobility of the standard low molecular-weight compounds dextran sulfate (~8 kDa), chondroitin-4-sulfate (~40 kDa), chondroitin-6-sulfate (~60 kDa) and/or UHEP (~14 kDa). After both electrophoresis, the SPs were stained with 0.1% toluidine blue in 1% acetic acid.

Activated partial thromboplastin time (APTT) test

Am-2 was assessed by *in vitro* APTT test, using normal citrated human plasma (different donors) according to the manufacturers' specifications, to confirm primarily the modest anti-clotting effect (Quinderé et al., 2014) in a coagulometer Amelung KC4A before the *in vitro* TG assay. UHEP (193 IU mg^{-1}) (fourth International Standard (85 502^{-1})) from the National Institute for Biological Standards and Control (Potters Bar, UK) (Nader el al., 2001) was used as reference.

TG assay of native Am-2 and its different low-molecular size fragments

TG assay was performed in a microplate format, containing: 10 μL of cephalin (contact-activator system) + 30 μL of 0.02 M Tris HCl/PEG-buffer (pH 7.4) + 10 µL of SPs (Am-2 [native]: 0, 4.1, 8.3, 41.6 or 83.3 μg well-plate^{-1} [hydrolysates^{-1} in 10 mL of solution], or UHEP: 2 μg well-plate^{-1}) + 60 µL of 20 mM $CaCl_2$ 0.33^{-1} mM chromogenic substrate S2238 (10:50 ratio, v v^{-1}). The *in vitro* reaction was triggered at 37°C by addition of plasma (diluted 60-fold well-plate^{-1}) (10 µL), and the absorbance (405 nm) was recorded for 70 min. (Plate reader Thermo-max, America Devices). The *in vitro* inhibitory response of TG by SPs was determined by peak thrombin (PTh), endogenous thrombin potential (ETP) and time to peak (TPeak).

Results and discussion

In the present study, the red seaweed *A. muscoides* was submitted to dehydration at 25°C for 48h prior to papain digestion (60°C, 6h), and then the structural features of a sample of the crude SP extract were analyzed by a solution ^{1}H NMR experiment, an analytic approach widely used to provide valuable information of complex mixtures and identification of molecular features of seaweeds SPs (Cardozo et al., 2007; Mourão, 2015; Pomin et al., 2005; Rodrigues et al., 2014), as shown in the chemical shift of Figure 1.

^{1}H spectral signals of the native extract were recorded in the δ_H ~5.6-1.6 ppm region, showing diverse complex unresolved peaks for a highly heterogeneous structure of sulfated galactan, as typically found for SPs from Rhodophyceae species (Mourão, 2015; Pomin, 2012). Both α- and β-^{1}H-anomeric signals of the spectrum resonating from δ_H

~4.6 to 5.6 ppm were ascribed to be down- and high-field regions belonging to the 4-linked units and 3-linked galactopyranoses and anhydrogalactopyranose units, respectively (Cardozo et al., 2007; Prajapati et al., 2014; Smit, 2004). The intensity of the proton-resonances at $\delta_H \sim 3.5$ and 1.7 ppm corresponded to the 2-*O*-CH_3 (Cardozo et al., 2007) and CH_3 groups (Duarte et al., 2004; Rodrigues et al., 2014), respectively. These combined results were attributed to the occurrence of the methyl-esterified on C2 (Duarte et al., 2004), similarly to the fraction Am-2 isolated from this same algal species, previously investigated in another study (Quinderé et al., 2014).

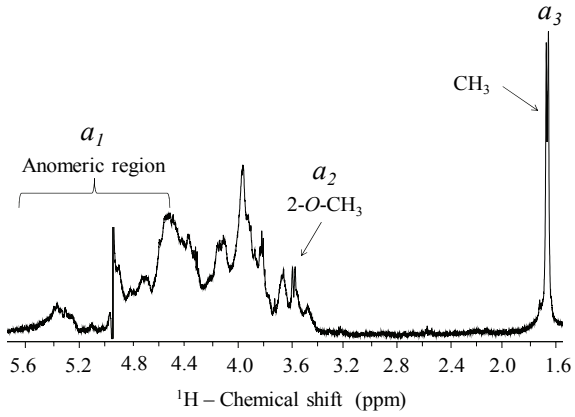

Figure 1. ^{1}H NMR spectrum of the crude SPs extract from the red seaweed *A. muscoides*. Down- and high-fields reveal numerous unresolved anomeric signals. (a_1) Signals reasoned to 1,3-linked β-galactopyranoses and 1,4-linked α-galactopyranoses and anhydro galactopyranose, with two singlets related to 2-*O*-methyl groups (a_2), respectively; (a_3) Signal assigned to the hydrogens of the methyl group of the sugar residues.

The DEAE-cellulose chromatography separated the raw material into three major SPs fractions (-1, -2 and Am-3), when eluted at 0.5, 0.75 and 1 M NaCl, respectively. The fraction Am-2 eluted at intermediate salt concentration showed high contents of both sulfate (23%) and HEXs (51%) within all the analyzed fractions, as well as absence of CPs according to the quantitative chemical determination. Additionally, both physical and chemical analyses in agarose gel and Page revealed Am-2 migrating as a homogenous band in charge density when compared to the crude SP extract and fractions -1 and Am-3, which were also essentially free of CPs (Quinderé et al., 2013); and showing behavior as a heterogeneous system of > 100 kDa, respectively (data not shown) (Quinderé et al., 2014). When Am-2 was used to measure APTT, it had only a discrete anticoagulation effect at high concentration (1 mg mL^{-1}) since UHEP altered drastically the APTT *in vitro* (data not shown), as previously observed by Quinderé et al. (2014).

The spectral analysis by ^{1}H NMR demonstrated that the composition of *A. muscoides*' cell wall SPs had a highly heterogeneous nature (Figure 1). Although employing alkali-treatment to allow a better accuracy by NMR spectroscopy, which displays 6-sulfated α-galactopyranosyl units into their 3,6-anhydro-α-galactose-derivatives, resulting in a more homogeneous saccharide chain with basis in α-units (Cardozo et al., 2007; Prajapati et al., 2014; Smit, 2004), Quinderé et al. (2014) had already demonstrated that both desulfated and alkali-derivatives of the native polymer (named by fraction Am-2) still had very complex NMR spectra, showing structural heterogeneity in terms of sulfation and/or methyl ether substitutions, with 3,6-anhydro-α-galactosyl units, as well as pyruvate. Based on these methodology limitations, fraction Am-2 was submitted to an alternative strategy, based on mild acid hydrolysis to depolymerize the native SPs for different times, and the *in vitro* inhibitory effects on TG in 60-fold diluted human plasma were examined using the chromogenic method by a continuous detection system.

Formation of sulfated oligosaccharides of different molecular size from Am-2 occurs drastically in longer periods of hydrolysis

Figure 2 shows the result of depolymerization fraction Am-2 after mild acid hydrolysis with HCl (0.02 or 0.04 M) at 60°C for different times to obtain distinct molecular size sulfated oligomers.

The use of both acid concentrations progressively reduced the molecular weight of Am-2 with the increasing time of hydrolysis according to the electrophoretic profiles in agarose gel and Page (Melo & Mourão, 2008; Pomin et al., 2005), which revealed a significant decrease not only at level of charge density (Figure A and B) (Leite et al., 1998), but also in molecular dispersion (Figure 2 C and D) (Melo & Mourão, 2008; Pomin et al., 2005), respectively, of the intact Am-2 fraction analyzed. In fact, there was a progressive loss of metachromasy, notably dependent on the molecular mass of Am-2 (Queiroz et al., 2015), since SPs have highly polyanionic character (ester sulfate groups) displaying complex binding property (metachromasy) (Athukorala et al., 2006; Quinderé et al., 2013, 2014; Souza et al., 2015) of the analyzed fragments (Pomin et al., 2005). Thus, for both 0.02 and 0.04 M HCl-treatments, Am-2 heated at 60°C for 1h was already susceptible to alterations in its structure, compared with standards (Figure 2C and D) (Pomin et al., 2005).

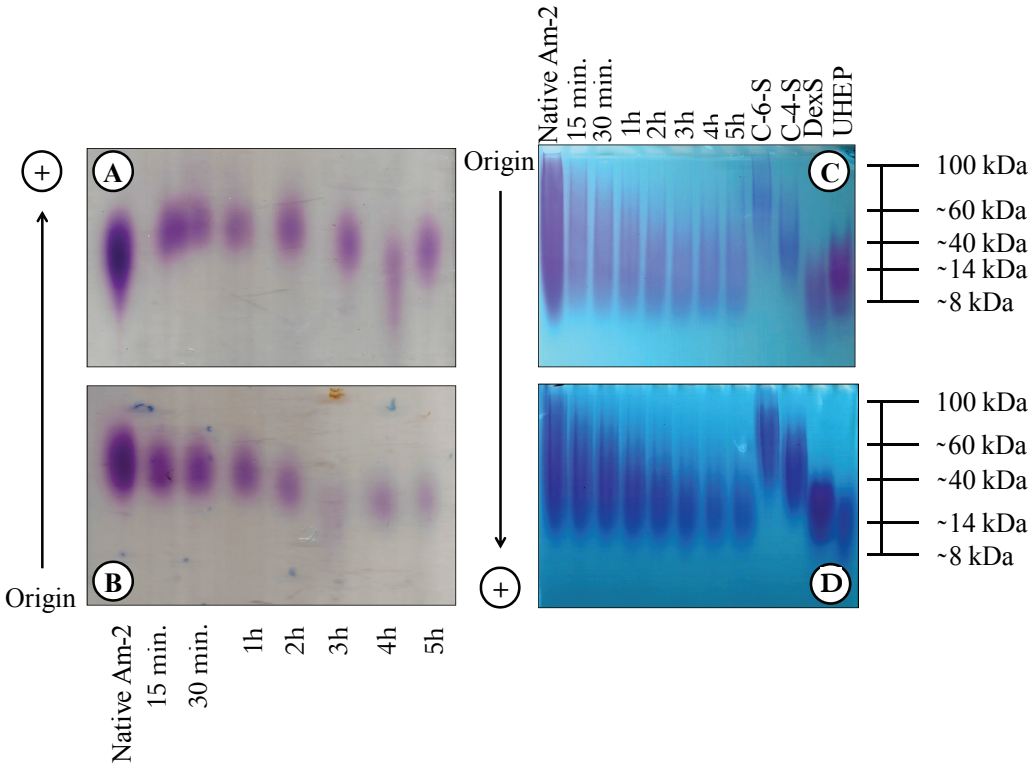

Figure 2. Electrophoresis in agarose gel (A and B) and in polyacrylamide gel (C and D) after the depolymerization procedure with 0.02 (A and C) or 0.04 (B and D) M HCl of Am-2, obtained by DEAE-cellulose, from the red seaweed *A. muscoides*. Low molecular weight dextran sulfate (~8 kDa, DexS), unfractionated HEP (~14 kDa, UHEP), chondroitin-4-sulfate (~40 kDa, C-4-S) and chondroitin-6-sulfate (~60 kDa, C-6-S) were used as standards. SPs present on gels were stained with 0.1% toluidine blue.

The higher concentration of HCl (0.04 M) affected the molecular size more drastically, since a higher extent of time (up to 5 h) yielded a fragment of ~ 14 kDa from the native polymer, similarly to that of UHEP, as indicated by the electrophoretic behavior (Figure 2D) (Melo & Mourão, 2008). Leite et al. (1998) isolated a sulfated xylofucoglucuronan (21 kDa) from the brown seaweed *S. shröederi* and then it was subjected to acid hydrolysis for different times. The hydrolysate formed up to 8h at 0.5 and 1.0 N HCl concentrations showed the presence of xylose, fucose and small amounts of glucuronic acid. When this product was enzymatically degraded by an assay of glycosidase activity, a loss in its chemical proportions of fucose (37.5%), xylose (57.5%) and sulfate (30%) was observed in the electrophoresis, and the further treatment of this same hydrolysate with 0.5 N HCl generated an oligosaccharide presenting an average molecular weight of 4.5 kDa, as revealed by a Sephadex G-25 column.

For the SPs from seven brown algae (*Ecklonia cava*, *Ishige okamurae*, *Sargassum fulvellum*, *S. horneri*, *S coreanum*, *S. thunbergii* and *Scytosiphon lomentaria*), Athukorala et al. (2006), using five carbohydrases (Viscozyme, Celluclast, AMG, Termamyl and Ultraflo), discovered that those of *E. cava*, when hydrolyzed by AMG, yielded fragments ranging from < 5 to > 30 kDa. It was demonstrated by Zhang et al. (2008) that the depolymerization by H_2O_2 degradation of an acidic SP, when extracted by hot water from the green seaweed *M. latissimum*, resulted into five sulfated products with molecular weights ranging from 10.6 to 216.4 kDa, respectively, when analyzed by high performance gel permeation chromatography. SPs isolated from the red seaweed *Botryocladia occidentalis* (Melo & Mourão, 2008) and from the sea urchins *Lytechinus variegatus*, *Strongylocentrotus pallidus* and *S. franciscanus* (Pomin et al., 2005) were examined by the method of mild acid hydrolysis, respectively. After 1, 2 or 6h of hydrolysis, Page demonstrated that their susceptibilities under acid conditions depended on the type of linkage and on chain length. A subsequent study from Queiroz et al. (2015) also revealed that the main sulfated oligosaccharides (named octa-, dodeca- and hexadecasaccharides) produced from *L. variegatus*, when obtained by mild acid hydrolysis, led to an impact of sulfation pattern on the conformation and dynamics of these sulfated fucan-derived oligosaccharides, as revealed by NMR and MD analyses. Recently, Panagos et al. (2016) applied photochemical depolymerization for

producing dermatan sulfate oligosaccharides and observed, for the first time, the presence of reducing end iduronic acid based on NMR analysis. Therefore, the rate of decrease in the molecular size of marine glycans depends on their origin and the approach used.

Since the end product of the acid reaction was similar in size to UHEP (Figure 2D), a glycosaminoglycan that has the highest negative charge density of any known natural biomaterial found in vertebrate tissues thus far (Mourão, 2015), it was speculated that the depolymerization process of Am-2 would, perhaps, lead to the elimination of sulfate of the structure in detriment of the reduced molecular size; therefore, possibly differing among the fragments in the pattern of sulfation (Leite et al., 1998; Queiroz et al., 2015). This hypothesis could be supported because the complex formed between SPs and diamine is similar to that observed for sulfated glycosaminoglycans derived from animal tissues. As a result, different SPs would show distinct mobility on the agarose gel in accordance to their structures (Figure 2 A and B) (Dietrich & Dietrich, 1976). Thus, the production of various derivates from unmodified Am-2 required higher acid concentration and critical time to yield sulfated oligosaccharides from the red seaweed *A. muscoides* (Pomin et al., 2005). Studies on the hydrolysates derived from intact Am-2 focusing on their structures should be performed, inferring the employment of HCl-treatment, including composition and NMR analyses.

Native Am-2 and its hydrolysates modify a continuous system of TG in diluted plasma

TG in cephalin-stimulated 60-fold diluted human plasma was measured in an experimental approach initiated by a mixture of contact activators and phospholipids (Mourão et al., 2001; Glauser et al., 2008; Zhang et al., 2014), to examine the *in vitro* anticoagulant efficacy of Am-2 and its hydrolysates in solution from the red seaweed *A. muscoides*, using the chromogenic method by a continuous detection system parallel with UHEP testing as a function of its *in vitro* inhibitory reference, as shown in Figure 3.

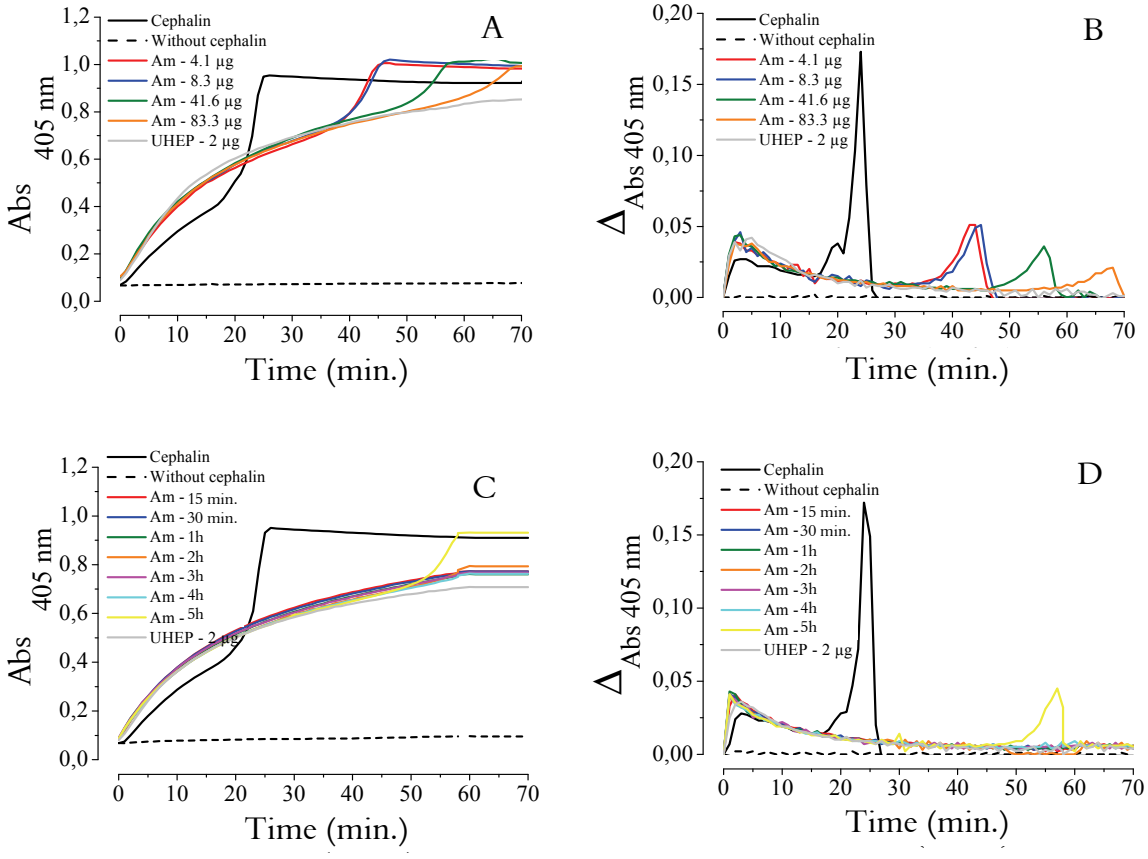

Figure 3. Effects of different concentrations of intact Am-2 (A and B), obtained by DEAE-cellulose, and its hydrolysates in solution for different times (C and D) from the red seaweed *A. muscoides* on cephalin-triggered TG in 60-fold diluted human plasma, using chromogenic method in a continuous detection system (405 nm) for 70 min at 37°C.

SPs from seaweeds frequently exhibit anticoagulation effects by inhibiting both the formation of thrombin and the subsequent conversion of fibrinogen to fibrin (Athukorala et al., 2006; Mourão, 2015; Rodrigues et al., 2013). As a rule, their actions are positively correlated with their sulfate content (Mourão, 2015; Pomin, 2012; Zhang et al., 2014) and molecular size (Athukorala et al. 2006; Melo & Mourão, 2008; Zhang et al., 2008), and classical coagulation tests, like APTT, have been widely used (Duarte et al., 2004; Leite et al., 1998; Zhang et al., 2014). Previous study of Quinderé et al. (2014) showed that the fraction Am-2 discretely affected the normal APTT, as in the present study (data not shown); however, it had an independent effect of serpin (antithrombin and heparin cofactor II) for *in vivo* thrombosis inhibition. In fact, due to the distinct structures varying with algal species (Duarte et al., 2004; Rodrigues et al., 2013; Souza et al., 2015; Zhang et al., 2008), SPs may have different mechanisms of action (Mourão, 2015) and the routine APTT test would not reflect an overall anticoagulation function because it only probes the initiation phase of coagulation (Castoldi & Rosing, 2011). In addition, the evident difference in molecular size between different classes of SPs makes comparison with UHEP difficult (Melo & Mourão, 2008; Mourão et al., 2015). TG assays may measure several feedback reactions in order to evaluate the anticoagulant dynamic (Glauser et al., 2009; Mourão et al., 2001; Zhang et al., 2014).

Under the conditions used, TG by the intrinsic pathway in 60-fold diluted human plasma was triggered at 37°C by adding cephalin in the presence of intact Am-2 and its hydrolysates in solution, and showed inhibition patterns in accordance with the TG parameters (Figure 3 and Table 1).

Table 1. Inhibition (%) of TG parameters in plasma (peak thrombin (PTh), endogenous thrombin potential (ETP) and time to peak (TPeak)) in the presence of different concentrations of intact Am-2 (obtained by DEAE-cellulose), and its hydrolysate in solution at 5h time, from the red seaweed *A. muscoides* compared to UHEP.

	TG parameters		
Intact Am-2 (well-plate^{-1})	PTh (%)	ETP (%)	TPeak (min.)
4.1 µg	71.67	68.28	44
8.3 µg	70.52	67.10	45
41.6 µg	79.19	84.68	56
83.3 µg	87.86	92.75	68
Hydrolysate (time)	PTh (%)	ETP (%)	TPeak (min.)
5 h	73.83	92.87	57
UHEP (2 µg well-plate^{-1})	100	100	-

For the native compound, no complete reduction was observed in all the Am-2 concentrations tested *in vitro* or had effects on the activity of thrombin generated in the intrinsic pathway dependently on the concentration of SP (Figure 3A and B) (Glauser et al., 2008; Mourão et al., 2001; Nishino et al., 1999). The almost inhibitory efficacy of TG by a cephalin-activated system was observed at 83.3 μg well-plate^{-1} (~ 92%), although requiring a concentration of SP 41.65-fold higher than UHEP, since it abolished TG on this system (Table 1), based on the amydolytic activity of thrombin that decayed rapidly until a plateau was reached (~25 min.) (Figure B) (Mourão et al., 2001).

Interestingly, hydrolysates in solution (10 μL) (Figure 3C and D) added to diluted plasma abolished TG, since maximum absorbance of active thrombin formed was decreased; therefore, higher effects on the intrinsic pathway were observed than those of intact Am-2, except at 5 h time (~ 14 kDa), which still presented a similar level of inhibitory action at 57 min. (92.87%) (Table 1). Melo & Mourão (2008) reported that a SP fragment of ~ 5 kDa (red seaweed *B. occidentalis*) was devoid of effect on the factor XII activation and maintained the same ability as UHEP in the inhibition of thrombus formation in rats. Our results were also in accordance with other published studies, where the samples with lower molecular weight were biologically active (Vijayabaskar et al., 2012; Yao et al., 2014; Zhang et al., 2008), when obtained by depolymerization. Therefore, intact Am-2 and its hydrolysates in solution after 0.04 M HCl hydrolysis prevented the event of TG in the intrinsic pathway in diluted plasma (Glauser et al., 2009; Nishino et al., 1999).

In summary, *A. muscoides* features a polysulfated fraction (Am-2) and its hydrolysates in solution with anticoagulant action, using a TG inhibition assay, could be useful tools to a more detailed study with basis on their structure-function relationships. Since SPs present a great versatility of biotechnological applications in the hydrocolloids industry (Smit, 2004), the introduction of TG assays (Castoldi & Rosing, 2011) associated with the physical and chemical properties (Cardozo et al., 2007) and bioactivities of these marine glycans (Mourão et al., 2015; Quinderé et al., 2013; Quinderé et al., 2015; Vijayabaskar et al., 2012) could also reveal new insight in biomedical research in future studies, including *in vitro* and *in vivo* approaches.

Conclusion

Analysis by one-dimension nuclear magnetic resonance of a polysulfated fraction isolated from the red seaweed *A. muscoides* reveals structure of galactan similar to that of the extract. When

submitted to depolymerization with HCl (0.02 or 0.04 M, 60°C), a progressive reduction in both charge density and molecular size of the fraction is observed by electrophoresis. Hydrolysis at 0.04 M HCl and critical time up to 5h yields a fragment of ~ 14 kDa similar to that of unfractionated heparin. Fragments display more potent *in vitro* effects on thrombin generation than the intact fraction; although similar to those of heparin, when in 60-fold diluted human plasma using chromogenic method in a continuous system.

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