ActaFish (2017) 5(2): 33-41
DOI 10.2312/ActaFish.2017.5.2.33-41

ARTIGO ORIGINAL

Acta of Fisheries and Aquatic Resources

Physical and chemical characterization, structural analysis and anticoagulation of a polysulfated fraction from the seaweed *Acanthophora muscoides* (Linnaeus) Bory de Saint-Vicent

Caracterização fisico-química, análise estrutural e anticoagulação de uma fração polisulfatada da alga *Acanthophora muscoides* (Linnaeus) Bory de Saint-Vicent

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

[1] Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro - UFRJ

[2] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará - UFC

*Email: arieviloengpesca@yahoo.com.br

Recebido: 23 de outubro de 2016 / Aceito: 25 de março de 2017 / Publicado: 1 de maio de 2017

Abstract Red seaweeds contain sulfated polysaccharides (SPs) that frequently exhibit anticoagulation, as potential alternatives to unfractionated heparin (UHEP) which induces extensive bleeding. This investigation evaluated the physical and chemical characteristics and the *in vitro* effects on coagulation of the SPs isolated from *Acanthophora muscoides*. A structural analysis of a rich fraction was also conducted by nuclear-magnetic-resonance (^{1}H NMR) spectroscopy. Papain digestion performed in 100 mM sodium acetate buffer (pH 5) containing cysteine and EDTA (both 5 mM) yielded 16% crude SP, and the profile of DEAE-cellulose chromatography, using a NaCl stepwise, presented three SPs fractions (Am-1, Am-2 and Am-3 eluted with 0.5, 0.75 and 1 M of NaCl) containing differences among the relative proportions of sulfate (3.61-22.53%) and total sugars (18.04-47.39%). Agarose gel electrophoresis revealed different patterns on charge density among the fractions and compared to UHEP, whereas no electrophoretic mobility for glycosaminoglycans chondrotin-4 or 6-sulfate, UHEP and dextran sulfate was observed by polyacrylamide analysis which revealed fractions with distribution of their molecular masses of >100 kDa. For ^{1}H NMR spectrum of the soluble Am-2 fraction, it was mainly found 4-linked-α-galactopyranosyl, 3-linked-β-galactopyranosyl and 3,6-anhydrogalactose units, 2-*O*-CH_3 and CH_3 group, similat to extract. Regarding the activated partial thromboplastin time assay, the fractions had no virtually anticoagulation (1.80, 3.00 and 1.46 IU mg^{-1} for Am-1, Am-2 and Am-3) when compared with UHEP (193 IU mg^{-1}). Therefore, SPs from *A. muscoides* have less anticoagulant potency than UHEP, when evaluated by APTT coagulation model.

Keywords: Rhodophyta, sulfated polymers, physical-chemical analyses, clot.

Resumo As algas marinhas vermelhas contém polissacarídeos sulfatados (PSs) que exibem anticoagulação, como alternativas potenciais à heparina não-fracionada (HEPNF) a qual induz sangramento extensivo. Esta investigação avaliou as características físico-químicas e os efeitos *in vitro* sobre a coagulação dos PSs isolados de *Acanthophora muscoides*. Foi conduzida uma análise estrutural de uma fração rica por espectroscopia de ressonância-magnética-nuclear (RMN ^{1}H). Digestão com papaína desenvolvida em tampão acetato de sódio 100 mM (pH 5) contendo cisteína e EDTA (ambos 5 mM) rendeu 16% de PS bruto e o perfil cromatográfico em DEAE-celulose, usando um passo-a-passo de NaCl, apresentou três frações de PSs (Am-1; Am-2 e Am-3 eluídas com 0,5; 0,75 e 1 M de NaCl) contendo diferenças entre as relativas proporções de sulfato (3,61-22,53%) e açúcares totais (18,04-47,39%). A eletroforese em gel de agarose revelou diferenças na densidade de cargas entre as frações comparadas à HEPNF. Enquanto, na análise poliacrilamida, a qual revelou frações com distribuição de suas massas moleculares de >100 kDa, não observou-se mobilidade eletroforética para os glicosaminoglicanos condroitim-4 ou 6-sulfato, HEPNF e dextrana-sulfatada. Similar ao extrato, foram encontrados principalmente para o espectro de RMN ^{1}H da fração solúvel Am-2, unidades de α-galactopiranose 4- ligada, β-galactopiranose 3-ligada e 3,6-anidrogalactose, além de 2-*O*-CH_3 e de grupo CH_3. As frações praticamente não alteraram o tempo de tromboplastina parcial ativada (1,80; 3,00 e 1,46 UI mg^{-1} para Am-1, Am-2 e Am-3), comparadas à HEPNF (193 UI mg^{-1}). Portanto, PSs de *A. muscoides* possuem potência anticoagulante inferior à HEPNF, quando avaliados pelo modelo de coagulação do TTPA.

Palavras-chave: Rhodophyta, polímeros sulfatados, análises físico-químicas, coágulo.

This study was *funded by* Brazilian *funding* agencies (CAPES-PNPD, FAPERJ, CNPq, MS and MCT).

ISSN: 2357-8068
Indexadores: Sumários (www.sumarios.org) - Diretórios: Diadorim (Diadorim.ibict.br) - Latindex (www.latindex.org)

Introduction

Current trends in biomaterials study from renewable sources have revealed seaweeds (Rodrigues, Torres, Alencar, Sampaio & Farias, 2009), fishes (Rodrigues et al., 2011), vascular plants (Silva et al., 2012) and sea cucumbers (Luo et al., 2013) as promising organisms with vast range of properties and characteristics that may justify their potential use within the nutritional, biomedical and food fields (Cardozo et al., 2007). Natural products (e.g., sulfated polysaccharides - SPs) have benefit effects for health and disease management, and in biotechnology due to their bioactivities (e.g., anticoagulation and anti-inflammation) (Yoon et al., 2007; Rodrigues et al., 2012) of biomedical relevance and intrinsic properties (e.g., firmness and adhesiveness) with potential applicability in food industry (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014).

The physical-chemical features and the structures of seaweeds SPs are highly complex and heterogeneous varying with algal species (Pomin & Mourão, 2008). Furthermore, their compositions and bioactivities depend on extraction procedure, harvest time, season, growth conditions and life cycle of the algae (Cardozo et al., 2007), usually exhibiting high molecular masses (> 100 kDa) (Pomin, 2012). Red and brown seaweeds have sulfated galactans (mainly agarans and carrageenans) and fucoidan or fucan, respectively. While heteropolysaccharides are the most found in green algae (Pomin & Mourão, 2008). SPs also occur in other living organisms (Dantas-Santos et al., 2012; Silva et al., 2012; Chang et al., 2013).

The increasing incidence of cardiovascular diseases in recent decades (mainly venous thrombosis) has been almost two times higher than cancer. On this scenario, the current treatment and prophylaxis of these diseases is frequently required unfractionated heparin (UHEP), a known commercially available anticoagulant obtained from animal tissues (porcine intestine or bovine lung). This SP has been widely used for the last 60 years as a medication as antithrombotic agent and other clinical practices, as in cardiovascular surgery and extracorporal circulation. However, as a consequence of its prolonged use lead to adverse effects, mainly hemorrhage; furthermore, the risk of contamined samples with pathogens. Therefore, there is a continuous effort for alternative sources of anticoagulants (Mourão & Pereira, 1999).

Diverse aquatic organisms have been identified as sources of heparinoids. For example, for the SPs fractions from the red seaweed *Halymenia pseudofloresia* Collins & M. Howe, Rodrigues, Torres, Alencar, Sampaio & Farias (2009) revealed significantly higher anticoagulant effects than UHEP. Rodrigues et al. (2011) and Luo et al. (2013), evaluating SPs from the freshwater fish *Oreochromis niloticus* Linnaeus, 1758 and three species of sea cucumbers (*Holothuria edulis* Lesson, 1930, *H. nobilis* Selenka, 1867 and *Apostichopus japonicas* Selenka, 1867), respectively, discovered anticoagulant effects. Silva et al. (2012) examined SPs isolated from the sea grass *Halodule wrightii* Asch, 1868 and observed anticoagulant effect 2.5-fold higher than that of UHEP. Queiroz et al. (2014) revealed significantly lower anticoagulation of SPs fractions isolated from *Gelidiella acerosa* (Forsskal) Feldman & Hamel (Rhodophyta) compared with that of UHEP.

Rhodomelaceae species, particularly from the genera *Acanthophora* and *Amansia*, have been very few investigated on their cell wall SPs, which are variably sulfated on C-2 of 3-linked β-D-galactose (A-unit) and have mainly 3,6-anhydro-α-L-galactose in B-unit, and their bioactivies, such as antiviral, analgesic, anti-inflammatory and antiangiogenesis effects (Duarte et al., 2004; Souza et al., 2012; Quinderé et al., 2013). Studies on the SPs found in *Acanthophora* species have been very limited in the literature.

Given the biotechnological importance of algae of the genus *Acanthophora*, the aim of this investigation was to analyze the physical and chemical characteristics, and to evaluate the *in vitro* effects on coagulation of a SPs fraction from the red seaweed *Acanthophora muscoides*, naturally distributed on the Ceará coast, Brazil.

Material and Methods

Extraction and physical-chemical and structural analyses of the *A. muscoides* SPs

In March 2012, Brazilian samples of *A. muscoides* (Linnaeus) Bory de Saint-Vincent (Ceramiales) were manually collected at Pacheco Beach (Ceará state, Caucaia) at low tide at mesolittoral zone. After collection, salt, macroscopic epiphytes and sand were removed from algae samples with tap water and, finally, carefully rinsed with distillated water and stored at 20°C until use. A voucher specimen (no. 46093) was deposited in the Herbarium Prisco Bezerra (EAC) in the Department of Biology, Federal University of Ceará, Brazil.

The crude SP was extracted from the dehydrated algal tissue (at room temperature) by papain digestion (60°C, 6 h) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine and EDTA (both 5 mM)

(Farias et al., 2000), and was then dissolved (12 mg) in 6 mL of 50 mM sodium acetate buffer (pH 5.0) and submitted to fractionation by anion-exchange chromatography on a DEAE-cellulose column (1 × 11 cm) equilibrated with the same solution. The column was developed using a NaCl stepwise (0→1.25 M, with 0.25 M of intervals) in the same solution. Fractions (2.5 mL min^{-1}) were collected and monitored by metachromatic assay containing dimethylmethylene blue (Fandarle, Buttle & Barrett, 1986) using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA) at 525 nm, and the total sugars was determined based on method in plate format (Masuko et al., 2005), using the same ELISA reader at 490nm.

Total sugars content was measured by phenol-sulfuric acid analysis using D-galactose as standard (in plate format) (Masuko et al., 2005). After acid hydrolysis of the soluble polysaccharides (1 mL of HCl for 5 h at 100°C), the sulfate content was measured by the $BaCl_2$/gelatin method (Dogson & Price, 1962). The content of contaminant proteins (CPs) was measured by Bradford's method (Bradford, 1976) with bovine serum albumin as reference.

SPs fractions eluted from DEAE-cellulose column were analyzed by 0.5% agarose gel electrophoresis according to Dietrich & Dietrich (1976). Samples of each SPs fraction (25 µg) were applied to a gel and run for 1 h at 110 V in 0.05 M 1.3 diaminopropane-acetate buffer (pH 9.0). SPs on gel were fixed with 0.1% *N*-cetyl-*NN*-*N*-trimethyl-ammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue and discolored with an acetic acid:absolute ethanol:distilled water solution (0.1:0.45:0.45).

Polyacrylamide gel electrophoresis (PAGE) technique was carried out to estimate the molecular weights of the SPs fractions based on Yoon, Pyun, Hwang & Mourão (2007). Fractions (25 µg) were applied to a 6% 1-mm-thick polyacrylamide slab gel in 0.02 M sodium barbital, pH 8.6, and run for 30 min. at 100 V. Then, the gel was stained with 0.1% toluidin blue. The molecular masses of the SPs fractions were estimated by comparison with the electrophoretic mobility of standard glycosaminoglycans chondroitin-4-sulfate (C-4-S, ~ 40 kDa), chondroitin-6-sulfate (C-6-S, ~ 60 kDa), dextran sulfate (DexS, ~ 8 kDa) and UHEP (~ 14 kDa).

Structural Analysis By ^{1}H NMR Spectroscopy

One-dimension ^{1}H NMR spectra of the crude SPs extract and SPs fraction (Am-2) from the red seaweed *A. muscoides* were recorded using Bruker DRX 800 MHz apparatus with a triple resonance probe (5 mm). About 5 mg of each sample was dissolved in 0.6 mL 99.9% deuterium oxide (Cambridge Isotope Laboratory, Cambridge, MA). The spectra were recorded at 35°C with HOD suppression by presaturation and the chemical shift was obtained using 16 scans and inter-scan delay set to 1 s (Fenoradosoa et al., 2009).

In vitro Coagulation Assay

Blood Human Samples

Coagulation analyses were conducted using venous blood samples collected in citrated vacutainer tubes containing 3.2% sodium citrate from 10 different donors (University Hospital Clementino Fraga Filho, FURJ), followed by centrifugation at 2000 × *g* for 15 min prior to tests. Normal citrated human plasma aliquots of 1 mL were frozen and stored at - 70°C until use.

Activated Partial Thromboplastin Time (APTT) Test

APTT test was performed using normal citrated human plasma (10 different donors) according to the manufacturers' specifications. For this, a mixture of 100 µL of citrated normal human plasma and concentration of SPs (1 mg mL^{-1}) was incubated with 100 µL of APTT reagent (kaolin bovine phospholipid reagent). After 2 min of incubation at 37°C, 100 µL of 25 mM $CaCl_2$ was added to the mixtures, and the clotting time was recorded in a coagulometer (Amelung KC4A). Heparin with 193 international units per mg (IU mg^{-1}) of polysaccharide was used as the standard. All the tests were performed in triplicate and analysis of variance (Anova) was performed, followed by Tukey's test for unpaired data, with $p < 0.001$ or 0.01 as statistically significant.

Results and Discussion

Yield of the *A. muscoides* Matrix Extracellular Crude SPs-containing Extract

The dehydrated *A. muscoides* tissue was subjected to papain digestion (6 h, 60°C), followed by lyophilization procedure to obtain crude SPs extract and chemical determination, and the results are in Table 1. As can be seen, 16% of lyophilizated total yield were extracted containing 52.2% and 33%, total sugars and sulfate, respectively. These values were in conformity with the values previously found by

Quinderé et al. (2013).

Table 1 Yields and chemical analyses of crude SPs extracts among red seaweed species of the family Rhodomelaceae.

Species	Method	Chemical composition (%)				Reference
		Yield[a]	Total sugars[b]	Sulfate[c]	CPs[d]	
Acanthophora muscoides	Proteolytic digestion (papain)	16	52.2	33	-	This study
Acanthophora muscoides	Proteolytic digestion (papain)	11.6	54	31.8	-	Quinderé et al. (2013)
Acanthophora spicifera	Aqueous	3.6	57	12.8	1.2	Duarte et al. (2004)
Amansia multifida	Proteolytic digestion (maxatase)	nr	50.9	18	2.2	Souza et al. (2012)

a - Yield calculated as the percentage of dehydrated matter (nr: no reported); b - Dosage by Masuko et al.' method using D-galactose as standard; c - Dosage by Dodgson and Price' method using $NaSO_3$ as standard; d - Dosage by Bradford' method using bovine serum albumin (- not detected).

However, these values contrasted along with results obtained by some other researchers from other red algae species of the family Rhodomelaceae, including *A. spicifera* (3.6% yield; 57% total sugars and 12.8% sulfate, respectively) and *Amansia multifida* (50.9% total hexose and 18% sulfate, respectively) when different extraction methods were employed. Herein, CPs were also not detected in analyzed sample, as previously reported (Quinderé et al., 2013). According to literature data, crude SPs extracts from the red seaweeds *A. spicifera* (Duarte et al., 2004) and *A. multifida* (Souza et al., 2012) had levels of 1.2 and 2.2% for proteins, respectively.

Based on these observations, the yield and chemical composition of SPs vary among protocols and algal species (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014). Enzymatic digestion of algae results high yields of bioactive compounds for screening studies on their bioactivities (Athukorala, Jung, Vasanthan & Jeon, 2006; Rodrigues, Torres, Alencar, Sampaio & Farias, 2009; Quinderé et al., 2013). According to Prajapati, Maheriya, Jani & Solanki (2014), there are current efforts based on the use of natural polymers than the synthetic materials because algae SPs are safe, biocompatible, biodegradable, non-toxic and low cost.

ANION-EXCHANGE CHROMATOGRAPHY (DEAE-CELLULOSE) AND COMPOSITION OF THE SPS FRACTIONS

The crude SPs extract, obtained from the red seaweed *A. muscoides*, was further submitted to ion-exchange chromatography on a DEAE-cellulose column, as shown in Figure 1. The profile of DEAE-cellulose chromatography showed three different SPs fractions (Am-1, Am-2 and Am-3), that were eluted with 0.5, 0.75 and 1 M of NaCl, respectively. The major peak eluted from the column with 0.75 M of salt (Am-2) contained highest dosage of total sugars when compared with its metachromasy (Athukorala, Jung, Vasanthan & Jeon, 2006; Queiroz et al., 2014), as measured by the phenol-H_2SO_4 reaction (Masuko et al., 2005). On the contrary to this finding, fractions Am-1 and Am-3 had a relatively low total sugars dosage. These observations led us to hypothesis that neutral sugars would be capable of interacting with the DEAE-cellulose column (Queiroz et al., 2014).

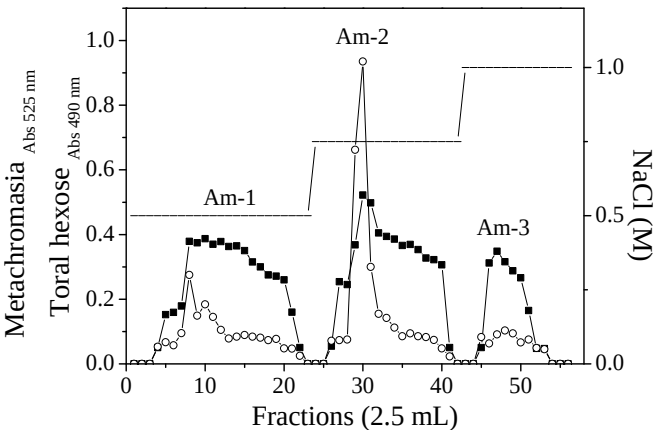

Figure 1 Anion-exchange chromatography (DEAE-cellulose) of the SPs from the red seaweed *Acanthophora muscoides*. Fractions were collected and checked by metachromasia using 1,9-dimethylmethylene blue (■—■) and phenol-H_2SO_4 (○—○). (-) NaCl concentration.

According to Duarte et al. (2004), SPs fractions from the red seaweed *A. spicifera*, when eluted with NaCl stepwise (DEAE-Sephadex column), presented a dispersion in the rates of 3,6-anhydrogalactose and/or xylose, with some them containing major amounts of xylose and glucose.

SPs from seaweeds, when fractionated by DEAE-cellulose, may reveal characteristics among different species, such as on *Ecklonia cava* (Athukorala, Jung, Vasanthan & Jeon, 2006), *H. pseudofloresia* (Rodrigues, Torres, Alencar, Sampaio & Farias, 2009) and *G. acerosa* (Queiroz et al., 2014).

Yields and compositional analyses of the SPs fractions from *A. muscoides* are listed in Table 2. The highest SPs yield was found in the fraction Am-2 (57%) compared with fractions Am-1 and Am-3.

Table 2 Yield and chemical analyses of the SPs fractions, obtained by DEAE-cellulose, from the red seaweed *Acanthophora muscoides*.

Fractions	Yield[a]	Chemical analyses (%)		
		Total sugars[b]	Sulfate[c]	CPs[d]
Am-1	17.10	39.16	19.26	-
Am-2	57.00	47.39	22.53	-
Am-3	0.80	18.04	3.61	-

[a]Yield calculated as the percentage from a sample of extract applied on DEAE-cellulose column; [b]Dosage by Masuko et al.' method using D-galactose as standard; [c]Dosage by Dodgson and Price' method using $NaSO_3$ as standard; [d]Dosage by Bradford' method using bovine serum albumin (- not detected).

In fact, the observed differences in the relative amounts of SPs among the fractions justified the metachromasia and dosage of total hexose from DEAE-cellulose chromatogram (Figure 1) (Athukorala, Jung, Vasanthan & Jeon, 2006; Queiroz et al., 2014). The metachromatic property depends on largely upon the charge density of the whole molecule (Athukorala, Jung, Vasanthan & Jeon, 2006). Thus, fractions also showed, by quantitative analysis, concordance to that found for SPs isolated from Rhodomelaceae species (30-68% total sugars and 4-28% sulfate, respectively) (Duarte et al., 2004; Souza et al., 2012). All the analyzed fractions had no CPs (Queiroz et al., 2014). This result was important because algae SPs are usually bound to a large number of proteins (Pomin & Mourão, 2008; Souza et al., 2012). Results indicated heterogeneity in the composition of the SPs present in *A. muscoides* (Farias et al., 2000; Yoon et al., 2007; Queiroz et al., 2014).

In order to analyze the physical and chemical characteristics of the isolated SPs fractions from *A. muscoides*, the agarose gel electrophoresis and PAGE techniques were employed as a property of charge and molecular mass, respectively.

The electrophoretic profile on agarose gel revealed single, homogeneous and coincident metachromatic bands for Am-1 and Am-2, while Am-3 exhibited very weak charge density (Yoon, Pyun, Hwang & Mourão, 2007). All the fractions also presented a distinct mobility when compared with UHEP (Figure 2A). As the complex formed between SPs and diamine is similar to that observed for glycosaminoglycans from animals (Dietrich & Dietrich, 1976), our results were in concordance to the sulfate content of the isolated fractions (Table 1) (Queiroz et al., 2014), as quantified by the Dogson and Price et al.' method. In fact, those fractions containing higher SPs yields and sulfate contents (Table 2) exhibited strong metachromatic property (Figures 1 and 2) (Athukorala, Jung, Vasanthan & Jeon, 2006; Queiroz et al., 2014).

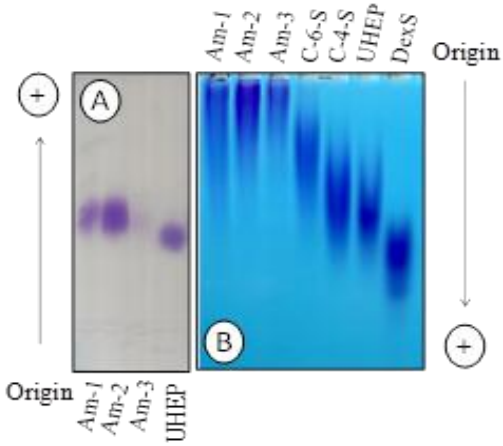

Figure 2 (A) Agarose gel electrophoresis and (B) polycrylamide gel electrophoresis (PAGE). SPs from the red seaweed *Acanthophora muscoides* (Am-1, Am-2 and Am-3) and standards unfractionated heparin (UHEP, 14 kDa), chondroitin-4-sulfate (C-4-S, 40 kDa), chondroitin-6-sulfate (C-6-S, 60 kDa) and/or dextran sulfate (DexS, 8 kDa) present on gel were stained with 0.1% toluidine blue.

Regarding the PAGE procedure, it was observed that the SPs fractions (*A. muscoides*) had no electrophoretic mobility for any standard glycosaminoglycan (Figure 2B) (Athukorala, Jung, Vasanthan & Jeon, 2006), as a consequence of their high molecular masses (> 100 kDa), typical for algae SPs (Athukorala, Jung, Vasanthan & Jeon, 2006; Yoon, Pyun, Hwang & Mourão, 2007). Interestingly, increasing molarities of NaCl resulted in fractions presenting differences in their polydispersive character. This finding would

support Am-2 to be high-molecular-weight since that it contained significant levels of total hexose, sulfate and yield within all the analyzed fractions (Table 2) (Duarte et al., 2004). Further, it was conducted a comparative structural analysis of the crude SP extract and its fraction Am-2.

STRUCTURAL ANALYSIS OF THE EXTRACT *VERSUS* FRACTION AM-2

The ^{1}H NMR spectra of the crude SPs extract and its native Am-2 fraction (obtained from DEAE-cellulose chromatography), which showed to be soluble in D_2O solution, are illustrated in Figure 3. The ^{1}H NMR technique, which comprises the most useful and employed analytical technique for structural information of SPs (Prajapati, Maheriya, Jani & Solanki, 2014), revealed a very complex spectrum due to the numerous unresolved anomeric signals from the analyzed polymer sample, as a consequence of structural heterogeneity commonly found for algae-derived SPs (Duarte et al., 2004; Yoon, Pyun, Hwang & Mourão, 2007; Fenoradosoa et al., 2009). Based on our findings, the structural analyses were not fully carried out because contained a mixture of signals of protons in the spectra (Yoon, Pyun, Hwang & Mourão, 2007; Fenoradosoa et al., 2009).

Anomeric resonances were attributed to be both down- and high-field regions (Fenoradosoa et al., 2009), with α- (from δ_H ~ 3.39 to 5.32 ppm) and β- (from δ_H ~ 3.75 to 4.79 ppm) anomeric hydrogens belonging to the 4-linked, 3-linked galactopyranoses and anhydrogalactopyranose units of the sugar residues, respectively (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014). In addition to our spectral findings, H-1 (δ_H~5.2 ppm) would correspond to the 4-linked α-D-galactopyranose units and/or the presence of glucose (floridean starch) (Prajapati, Maheriya, Jani & Solanki, 2014). The ^{1}H-signals displayed at δ_H~3.2 and 1.2 ppm indicated 2-*O*-CH_3 and CH_3 group (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014), respectively, which were related to the protons of the methyl-esterified on C-2 (Duarte et al., 2004).

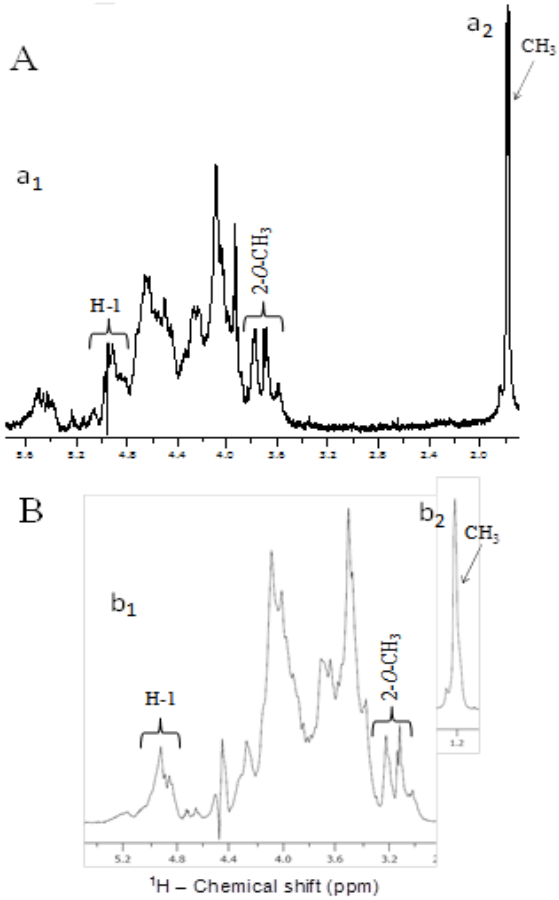

Figure 3 ^{1}H NMR spectra of the crude SPs extract (A) and its Am-2 fraction (B) obtained by anion-exchange chromatography (DEAE-cellulose) from the red seaweed *Acanthophora muscoides*. Down- and high-field regions revealing numerous unresolved anomeric signals. (a_1 and b_1) Signals assigned to 1,3-linked β-galactopyranoses and 1,4-linked α-galactopyranoses and anydrogalactopyranose, with two singlets related to 2-*O*-methyl groups, respectively; (a_2 and b_2) Signal attributed to the proton of methyl group of the sugar residues.

Thus, more detailed studies of these SPs focusing on their structural features should be performed by complementary NMR experiments (Farias et al., 2000).

EVALUATION OF THE *IN VITRO* ANTICOAGULANT EFFECT

Anticoagulant potency of the SPs fractions isolated from the red seaweed *A. muscoides* was evaluated by the APTT assay, a standard-coagulation test that stimulates the intrinsic and/or common pathway thought contact activators and phospholipids (Mourão & Pereira, 1999), as shown in Table 3.

Anticoagulant potential of SPs has been described for various aquatic organisms (Rodrigues, Torres, Alencar, Sampaio & Farias, 2009, 2011; Dantas-Santos et al., 2012; Souza et al., 2012; Luo et al., 2013). According to our findings, *A. muscoides* contained SPs fractions exhibiting minimal actions on APTT (Table 3) since that the values were only duplicated when a high concentration of Am-2 (1 mg mL^{-1}, 66.67 ± 0.57s), eluted with 0.75 M of NaCl, was added to normal plasma when compared with the control sample without

Table 3 *In vitro* anticoagulant effect of the SPs fractions, obtained by anion-exchange chromatography (DEAE-cellulose), from the red seaweed *Acanthophora muscoides* compared to UHEP.

		APTT assay (s)*		
Fraction	NaCl (M)	1 mg mL^{-1}**	T_1 T_0^{-1}***	IU mg^{-1}****
Am-1	0.50	38.87 ± 0.14 $^{\Delta}$	1.21	1.80
Am-2	0.75	66.67 ± 0.57 $^{\Delta\Delta}$	2.08	3.00
Am-3	1.00	32.07 ± 0.03	1.00	1.46

NaCl – Sodium Chloride; * Activated partial thromboplastin time (APTT); ** SP concentration to prolong the APTT in seconds; *** Anticoagulant effect expressed as T_1 T_0^{-1}; **** Anticoagulant effect expressed in international units (IU) per mg of SP (IU mg^{-1}); UHEP (193.00 IU mg^{-1}; 0.010 mg mL^{-1}; APTT: 41.83 ± 0.75 s $^{\Delta\Delta}$); Control: 31.9 ± 0.23 s (n = 3, $p < 0.001$ $^{\Delta}$ or 0.01 $^{\Delta\Delta}$ *vs* control).

SPs (31.9 ± 0.23 s) ($p < 0.01$). Furthermore, all the fractions had a lower potency (1.46-3.00 IU mg^{-1}) in comparison with UHEP (0.010 mg mL^{-1}, 41.83 ± 0.75 s, 193 IU mg $^{-1}$). Herein, the anticoagulant profile of SPs from the red seaweed *A. muscoides* was comparable to that observed for *G. acerosa* (Rhodophyta) SPs that affected virtually the intrinsic and/or common pathways of the coagulation system (Queiroz et al., 2014).

Thrombin plays a crucial role in hemostasis that result in formation of clot. The factors associated with intrinsic and/or common pathway interact with calcium and phospholipids surface to make platelet aggregation and stop bleeding for tissue repair (Mourão & Pereira, 1999). Calcium chloride was added to display the APTT assay in measuring the anticoagulant potency and any influence in the evaluation of this test was observed since the plasma without SPs exhibited normal coagulation values. Additionally, *A. muscoides* samples digested with papain resulted in high sulfate content (Table) and helped in the solubility of its SPs (Queiroz et al., 2014).

The present report showed an attempt of correlation between the clotting formation delay and the sulfate content among the fractions (Table 2) (Pomin, 2012), but not with their respective molecular masses (Figure 2A). Interestingly, fractions Am-1 and Am-2, when analyzed by sugar/sulfate ratio, had similar values (2.03 and 2.10, respectively) (Table 2), as well as their similar physical and chemical characteristics by agarose electrophoresis (Figure 2A), no displaying strong anticoagulation *in vitro*. Possibly, a peculiar chemical nature (e.g., sulfation and glycosylation sites, anomericity and/or conformational preference) could perhaps occur between these SPs fractions concerning their modest actions on APTT (Table 3) (Pomin, 2012). Therefore, the discretely prolonged APTT of Am-2 lack of more details on its ^{1}H NMR analysis that suggested intense signals related to the presence of 3,6-anhydrogalactose (Figure 3), which can be enzymatically generated from biological precursor galactose-6-sulfate present in some Rhodophyta species (Pomin & Mourão, 2008). In the food industry, alkaline extraction has also been widely used for red algae SPs increase the functional ability of the gel as thickening, gelling and stabilizing to produce high added-value products (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014).

Less amounts of galactose-6-sulfate and other charged chemical components in Am-2 perhaps support the suggestion of its lacking anticoagulant action, as observed for agarans extracted from the red seaweed *A. spicifera* (Duarte et al., 2004). Agar is a complex SP made up of a neutral fraction (agarose) and an anionic fraction called agaropectin, with low sulfate content (Cardozo et al., 2007). Importantly, the modest-anticoagulant actions observed in the present study for SPs fractions from *A. muscoides* (Table 3) seemed to be dissociated from their antinociceptive and anti-inflammatory effects (Pomin, 2012) because the SPs concentration was close to that recorded in another study (Quinderé et al., 2013). This observation would lead to the view for the further novel therapeutically important biomaterials development within other health-related economical fields (Cardozo et al., 2007; Prajapati, Maheriya, Jani & Solanki, 2014), although lacking of function-structure relationship data (Pomin, 2012). However, additional investigations should be conducted to infer the effect of these molecules on more refined coagulation models (Yoon, Pyun, Hwang & Mourão, 2007; Pomin, 2012; Queiroz et al., 2014).

Conclusion

Brazilian samples of *Acanthophora muscoides* (Rhodophyta) contains, when employed anion-exchange chromatography (DEAE-cellulose) procedure, three sulfated polysaccharidic fractions that differ on their physical and chemical characteristics in terms of charge density and molecular mass, as revealed by agarose and polycrylamide gel electrophoreses, respectively. When a soluble fraction was analyzed by ^{1}H NMR technique, the polysaccharide reveals high structural heterogeneity, similar to the family Rhodomelaceae. However, with significantly lower *in vitro* anticoagulant effects than unfractioned heparin, when measured by APTT assay.

References

Athukorala, Y., Jung, W.K., Vasanthan, T. & Jeon, Y.J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*. *Carbohydr. Polym.*, 66(2): 184-191.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72(1-2): 248-254.

Cardozo, K.H.M., Guaratini, T., Barros, M.P., Falcão, V.R., Tonon, A.P., Lopes, N.P., Campos, S., Torres, M.A., Souza, A.O. & Pinto, E. (2007). Metabolites from algae with economical impact. *Comp. Biochem. Physiol. Part C - Toxicol. Pharm.*, 146(102): 60-78.

Chang, C.W., Lur, H.S., Lu, M.K. & Cheng, J.J. (2013). Sulfated polysaccharides of *Amillariella mellea* and their anti-inflammatory activities via NF-κB suppression. *Food Res. Inter.*, 54(1): 239-245.

Dantas-Santos, N., Gomes, D.L., Costa, L.S., Cordeiro, S.L., Costa, M.S.S.P., Trindade, E.S., ... & Rocha, H.A.O. (2012). Freschwater plants synthesize sulfated polysaccharides: Heterogalactans from water hycinth (*Eicchornia crassipes*). *Inter. J. Mol. Sci.*, 13(1): 961-976.

Dietrich, C.P. & Dietrich, S.M. C. (1976). Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. *Anal. Biochem.*, 70(2): 645-647.

Dodgson, K.S. & Price, R.G. (1962). A note on the determination of the ester sulfato content of sulfated polysaccharides. *Biochem. J.*., 84(1): 106-110.

Duarte, M.E.R., Cauduro, J.P., Noseda, D.G., Noseda, M.D., Gonçalves, A.G., Pujol, C.A., Damonte, E.B. & Cerezo, A.S. (2004). The structure of the agaran sulfate from *Acanthophora spicifera* (Rhodomelaceae, Ceramiales) and its antiviral activity. Relation between structure and antiviral activity in agarans. *Carbohydr. Res.*, 339(2): 335-347.

Farias, W.R. L., Valente, A.P., Pereira, M.S. & Mourão, P.A.S. (2000). Structure and anticoagulant activity of sulfated galactans. Isolation of a unique sulfated galactan from the red alga *Botryocladia occidentalis* and comparison of its anticoagulant action with that of sulfated galactans from invertebrates. *Jour. Biol. Chem.*, 275(38): 29299-29307.

Farndale, R.W., Buttle, D.J. & Barrett, A.J. (1986). Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta*, 883(2): 173-177.

Fenoradosoa, T.A., Delattre, C., Laroche, C., Wadouachi, A., Dulong, V., Picton, L., Andriamadio, P. & Michaud, P. (2009). Highly sulphated galactan from *Halymenia durvillei* (Halymeniales, Rhodophyta), a red seaweed of Madagascar marine coasts. *Inter. J. Biol. Macromol.* 45(2): 140-145.

Luo, L., Wu, M., Xu, L., Lian, W., Xiang, J., Lu, F., Gao, N., Xiao, C., Wang, S. & Zhao, J. (2013). Comparison of physicochemical characteristics and anticoagulant activity of polysaccharides from three sea cucumbers. *Mar. Drugs* 11(2): 399-417.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.I. & Lee, Y.C. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal. Biochem.*, 339(1): 69-72.

Mourão, P.A.S. & Pereira, M.S. (1999). Searching for alternatives to heparin: sulfated fucans from marine invertebrates. *Tren. Cardiov. Med.* 9(8): 225-232.

Prajapati, V.D., Maheriya, P.M., Jani, G.K. & Solanki, H.K. (2014). Carrageenan: A natural seaweed polysaccharide and its applications. *Carbohydr. Polym.*, 105(25): 97-112.

Pomin, V. H. & Mourão, P. A. S. (2008). Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiol.*, 18(12): 1016-1027.

Pomin, V.H. (2012). Fucanomis and galactanomics: Current status in drug discovery, mechanisms of action and role of the well-defined structures. *Biochim.Biophys. Acta*, 1820(12): 1971-1979.

Quinderé, A.L.G., Fontes, B.P., Vanderlei, E.S.O., Queiroz, I.N.L., Rodrigues, J.A.G., Araújo, I.W.F., Jorge, R.J.B., Menezes, D.B., Silva, A.A.R., Chaves, H.V., Evangelista, J.S.A.M., Bezerra, M.M. & Benevides, N.M.B. (2013). Peripheral antinociception and anti-edematogenic effect of a sulfated polysaccharide from *Acanthophora muscoides*. *Pharmacol. Rep.* 65(3): 600-613.

Queiroz, I.N.L., Rodrigues, J.A.G., Quinderé, A.L.G., Holanda, M.L., Pereira, M.G. & Benevides, N.M.B. (2014). Inhibition of coagulation proteases and thrombosis and sub-chronic toxicological study of a sulfated polysaccharidic fraction from the red alga *Gelidiella acerosa*. *Acta Sci. Biol. Sci.*, 36(4): 393-401.

Rodrigues, J.A.G., Torres, V.M., Alencar, D.B., Sampaio, A.H. & Farias, W.R.L. (2009). Extração e atividade anticoagulante dos polissacarídeos sulfatados da alga marinha vermelha *Halymenia pseudofloresia*. *Rev. Ciên. Agron.*, 40(2): 224-231.

Rodrigues, J.A.G., Quinderé, A.L.G., Queiroz, I.N.L., Coura, C.O., Araújo, G.S. & Benevides, N.M.B. (2011). Purificação, caracterização físico-química e atividade anticoagulante de glicosaminoglicanos isolados da pele de tilápia do Nilo (*Oreochromis niloticus*). *Acta Sci. Technol.*, 33(3): 233-241.

Rodrigues, J.A.G., Vanderlei, E.S.O., Silva, L.M.C.M., Araújo, I.W.F., Queiroz, I.N.L., Paula, G.A., Abreu, T.M., Ribeiro, N.A., Bezerra, M.M., Chaves, H.V., Lima, V., Jorge, R.J.B., Monteiro, H.S.A., Leite, E.L. & Benevides, N.M.B. (2012). Antinociceptive and anti-inflammatory activities of a sulfated polysaccharide isolated from the green seaweed *Caulerpa cupressoides*. *Pharmacol. Rep.*, 64(2): 282-292.

Silva, J.M.C., Dantas-Santos, N., Gomes, D.L., Costa, L.S., Cordeiro, S.L., Costa, M.S.S.P., Silva, N.B., Freitas, M.L., Scortecci, K.C., Leite, E.L. & Rocha, H.A.O. (2012). Biological activities of the sulfated polysaccharides from the vascular plant *Halodule wrightii*. *Braz. J. Pharm.*, 22(1): 94-101.

Souza, L.A.R., Dore, C.M.P.G., Castro, A.J.G., Azevedo, T.C.G., Oliveira, M.T.B., Moura, M.F.V., Benevides, N.M.B. & Leite, E.L. (2012). Galactanas from the red seaweed *Amansia multifida* and their effect on inflammation, angiogenesis, coagulation and cell viability. *Biom. & Prev. Nutr.*, 2(3): 154-162.

Yoon, S.J., Pyun, Y.R., Hwang, J.K., Mourão, P.A.S. (2007). A sulfated fucan from the brown alga *Laminaria cichorioides* has mainly heparin cofactor II-dependent anticoagulant activity. *Carbohydr. Res.* 342(15): 2326- 2330.