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Structural features and inactivation of coagulation proteases of a sulfated polysaccharidic fraction from *Caulerpa cupressoides* var. *lycopodium* (Caulerpaceae, Chlorophyta)

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ABSTRACT. Studies on biopolymers from macroalgae suggested sulfated polysaccharides (SPs) as research agents to investigate events related to haemostasis. *Caulerpa cupressoides* var. *lycopodium* is a marine green alga containing three SPs fractions (SP_1, SP_2 and SP_3). SP_2 had anticoagulant (*in vitro*) and anti- and prothrombotic (*in vivo*) actions; however, its effect on the coagulation system is not fully understood. This study aimed to determine the infrared (IR) spectroscopy, chemical composition (CC), elemental microanalysis (EM), molecular weight (MW) and the effect on coagulation proteases of SP_2. The presence of sulfate ester, galactose-6-sulfate, uronic acid and glycoside linkages for IR spectrum; contents of sulfate (28%), total sugars (40%) and uronic acids (7.18%) for CC; and content of carbon (21.98%), sulfate (4.27%), nitrogen (1.3%) and hydrogen (4.86%) for EM were obtained. The average molecular weights of four different SPs (SP-1, SP-2, SP-3 and SP-4) subfractions from the SP_2 ranged from ~ 8 to >100 kDa. SP_2 was tested on coagulation proteases (thrombin and factor Xa) in the presence of antithrombin (AT) and heparin cofactor II (HCII) using human plasma, being both thrombin and factor Xa target proteases inhibited, but requiring a concentration of about 2.5-fold higher of HCII than the thrombin inactivation by AT.

Keywords: Caulerpaceae, polysulfated, chemical analysis, coagulation, modulator action.

Características estruturais e inativação de proteases da coagulação de uma fração polissacarídica sulfatada de *Caulerpa cupressoides* var. *lycopodium* (Caulerpaceae; Chlorophyta)

RESUMO. Estudos sobre biopolímeros de macroalgas sugeriram polissacarídeos sulfatados (PSs) como agentes de pesquisa para investigar eventos relacionados à hemostasia. A alga marinha verde *Caulerpa cupressoides* var. *lycopodium* contém três frações de PSs (PS_1; PS_2 e PS_3). A PS_2 apresentou ações anticoagulantes (*in vitro*), anti- e pró-trombótica (*in vivo*). Entretanto, carece de investigações de seus efeitos sobre o sistema de coagulação. Objetivou-se determinar de PS_2 a espectrocopia por infravermelho (IV), composição química (CC), microanálise elementar (ME), peso molecular (PM) e os efeitos sobre proteases da coagulação. Foi obtida a presença de éster sulfato, galactose-6-sulfato, ácido urônico e ligações glicosídicas para o espectro de IV; conteúdos de sulfato (28%), açúcares totais (40%) e ácidos urônicos (7,18%) para a CC; e conteúdos de carbono (21,98%), sulfato (4,27%), nitrogênio (1,3%) e hidrogênio (4,86%) para a ME. Os pesos moleculares médios das quatro diferentes subfrações de PSs (PS-1; PS-2; PS-3 e PS-4) de SP_2 variaram de ~ 8 a >100 kDa. A PS_2 foi testada sobre proteases da coagulação (trombina e fator Xa) na presença de antitrombina (AT) e cofator II da heparina (CIIH) usando plasma humano, sendo inibidas ambas as proteases alvo trombina e fator Xa. Entretanto, requerendo uma concentração de aproximadamente 2,5 vezes maior de CIIH do que para inativação de trombina por AT.

Palavras-chave: Caulerpaceae, polisulfatados, análises químicas, coagulação, ação moduladora.

Introduction

Marine algae have been commonly consumed in the Orient (China, Japan and South Korea) as part of the daily diet, and documented as marine products in traditional medicine (WANG et al., 2008; ZHU et al., 2010). In the last decades, the interest of scientists for natural products extracted from seaweed has significantly increased. These algal products include fatty acid, protein, carbohydrate and sterol (ARAÚJO et al., 2011; MAO et al., 2011; MARINHO-SORIANO et al., 2006; SCHEVCHENKO et al., 2009), and mineral in the form of cations associated with carbohydrates (CAMPO et al., 2009).

According to Smit (2004), the market of macroalgae is estimated at a multi-billion dollars, being based on cultivated edible species or on the production of hydrocolloids (agar, carrageenan and alginate). New biomaterials for pharmaceutical, food and cosmetic industries have been identified and studied in wild and/or cultivated algae species (ARAÚJO et al., 2012; CAMPO et al., 2009; MAZUMDER et al., 2002; POMIN, 2012; PUSHPAMALI et al., 2008; RODRIGUES et al., 2011a, 2013). Of all seaweed chemicals, sulfated polysaccharides (SPs), an intriguing group of highly complex and heterogeneous polymers (PEREIRA et al., 2005) found at high concentrations in the extracellular matrix of marine algae (POMIN; MOURÃO, 2008), have gained attention in the modern society (CAMPO et al., 2009; SMIT, 2004). SPs also occur in sea grasses (AQUINO et al., 2005), animals (POMIN; MOURÃO, 2008), microalgae (MAJDOUB et al., 2009) and freshwater plants (DANTAS-SANTOS et al., 2012). They exhibit a wide structural diversity among different marine organisms (ANANTHI et al., 2010; AQUINO et al., 2005; CAMPO et al., 2009; MAJDOUB et al., 2009; POMIN; MOURÃO, 2008). Previous studies have suggested SPs for data at level of biology, taxonomic supplement and evolution of marine algae (POMIN; MOURÃO, 2008; USOV, 1998).

Species of the genus *Caulerpa* of green seaweeds (Bryopsidales) are usually found in water tropical and subtropical marine environments. They are considered invasive and usually resulting in ecological and economical problems. The family Caulerpaceae presents a great diversity of metabolites with chemotaxonomic significance (MAO et al., 2011; RODRIGUES et al., 2012). Polysaccharides from Caulerpaceae consisted mainly of galactose and sulfate, small amount of arabinose, xylose, manose and uronic acid (HAYAKAWA et al., 2000; GHOSH et al., 2004; CHATTOPADHYAY et al., 2007; JI et al., 2008; SCHEVCHENKO et al., 2009). Some biological activities of the *Caulerpa* SPs have been reported. Hayakawa et al. (2000) revealed that *C. okamurai* and *C. brachypus* had SPs that interacted with heparin cofactor II (HCII) to inhibit the thrombin activity.

A hot-water extract containing SPs from *C. racemosa* with *in vitro* anticoagulant and antiviral activities was described by Ghosh et al. (2004). In addition, Ji et al. (2008) reported that SPs fractions from *C. racemosa* had antitumoral properties (*in vitro* and *in vivo*) against K562 and H22 cell lines. More recently, it was demonstrated that *C. cupressoides* var. *lycopodium* contained three different SPs fractions (SP_1, SP_2 and SP_3), but only SP_2 had anticoagulant activity (*in vitro*) dependent on its high sulfate content and molecular mass distribution compared to those non-anticoagulant SP_1 and SP_3 (RODRIGUES et al., 2011d). In another study, SP_2 was tested in an assay with coagulation inhibitor using thrombin as the target protease, where an inhibitory effect mediated by antithrombin (AT) was observed (RODRIGUES et al., 2011b). This mechanism of interaction was suggested as being different when compared to other studied *Caulerpa* SPs (HAYAKAWA et al., 2000). Surprisingly, SP_2 also presented *in vivo* anti- and prothrombotic effects when assayed in a venous thrombosis model in Wistar rats (RODRIGUES et al., 2011b), similarly to red seaweeds SPs (FONSECA et al., 2008). In another study, Rodrigues et al. (2013) reported that SP_1 was lack of *in vitro* anti-clotting effect, but able to acts as an *in vivo* analgesic agent, without modify the locomotor behavior of mice. However, there are few studies concerning the structural and/or biological properties of SPs from Caulerpaceae, especially the effects on coagulation system.

Heparin is a SP member of a family of glycosaminoglycans widely used as anticlotting and antithrombotic drug, consisting of 1,4-linked residues of uronic acid and D-glucosamine. Commercial heparin is obtained from pig and bovine intestine, but at low concentrations. The continuous clinical use of heparin can be accompanied by various risk factors, such as frequent activated partial thromboplastin time monitoring, the inability to inhibit thrombin bound to the clot, hemorrhage and the occurrence of thrombocytopenia in some patients. Possibility of prions and viruses can also occur. It is also employed during extracorporeal circulation (vascular surgery and hemodialysis), exhibiting anticoagulant action mainly through potentiation of AT, a serine protease inhibitor (serpin) which controls the coagulation process, especially thrombin and factor Xa (NADER et al., 2001; QUINSEY et al., 2004).

Once the genus *Caulerpa* of green seaweeds has been little explored, and few descriptions of structural and biomedical properties of its SPs are still reported, we have now focused the studies with SP_2 fraction (*C. cupressoides* var. *lycopodium*) on coagulation proteases. Some molecular features of this fraction were also investigated.

Material and methods

Reagents

Antithrombin, heparin cofactor II, thrombin and factor Xa (Haematologic Technologies Inc., Essex

Junction, VT, USA); chromogenic substrates S-2238, S-2222 and S-2302 (Chromogenix AB, Mondal, Sweden); low molecular weight dextran sulfate (8 kDa) and chondroitin-4-sulfate (40 kDa) from whale cartilage; chondroitin-6-sulfate (60 kDa) from shark cartilage; and other reagents were commercially purchased.

Marine alga and isolation of SP2 fraction and chemical analyses

Specimens of the green alga *C. cupressoides* var. *lycopodium* (West) C. Agardh (Chlorophyta, Caulerpaceae) was collected on the seashore of Flecheiras Beach on the Atlantic coast of Ceará State, Brazil. The algal samples were taken to the Carbohydrate and Lectins Laboratory (CarboLec), Department of Biochemistry and Molecular Biology, Federal University of Ceará, Brazil, and then cleaned of epiphytes, washed with distilled water and store at -20°C until use. A voucher specimen (4977) was classified and archived by Ana Cecília Fortes Xavier at the Prisco Bezerra Herbarium, Federal University of Ceará. The crude SP was extracted by papain digestion (6h, 60°C), and then separated into three fractions of SPs (SP_1, SP_2 and SP_3) by anion-exchange chromatography on a DEAE-cellulose column, and eluted using a saline gradient (0.5-1 M of NaCl). The purity of SP_2 was checked by agarose gel electrophoresis procedure. The chemical composition of total sugars, sulfate, and contaminant proteins contents of the SP_2 was also determined. These procedures were performed based on Rodrigues et al. (2011d). Chemical analysis of uronic acids content of SP_2 was carried out by carbazole-sulfuric acid method using spectrophotometer (AMERSHAM BIOSCIENCES ULTROSPEC 1100) at 525 nm, using glucuronic acid as standard (DISCHE, 1962).

Infrared (IR) spectroscopy

To investigate the chemical structure, IR spectrum of SP_2 fraction was determined. Fourier transform IR spectrum (FT-IR) was recorded with a SHIMADZU IR spectrophotometer (model 8300) between 4000 and 500 cm^{-1}. The sample was evaluated as KBr pellet.

Elemental microanalysis

Nitrogen, carbon, hydrogen and sulfate of *C. cupressoides* var. *lycopodium* SP_2 fraction were also determined (PERKIN-ELMER CHN 2400).

Polyacrylamide gel electrophoresis (PAGE)

This procedure was performed to determine the molecular weights of the SP_2 fraction based on Yoon et al. (2007). In this experiment, SP2 (10 μg) was 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% acetic acid. The molecular masses of the SP_2 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) and dextran sulfate (Dex, ~ 8 kDa).

Inhibition of thrombin or factor Xa by antithrombin (AT) and heparin cofactor II (HCII) in the presence of the *C. cupressoides* var. *lycopodium* SP2 fraction

These assays were carried out based on Fonseca et al. (2008) using incubations in 96-well plates. The final concentrations of reactants included AT (10 nM) or HCII (15 nM), thrombin or factor Xa (both 2 nM) and SP_2 (0-1,000 μg) in 40 μL of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg mL^{-1} polyethylene glycol 8,000, pH 7.4). Thrombin or factor Xa was added last to initiate the reaction. After 60 s at 37°C, 25 μL chromogenic substrate (S-2238 for thrombin or S-2222 for factor Xa) was added, and the absorbance at 405 nm was recorded for 120 s. The rate of change of absorbance was proportional to the thrombin or factor Xa activity remaining in the incubation. No inhibition occurred in control experiments in which thrombin or factor Xa was incubated with AT or HCII in the absence of SP_2. Also, no inhibition was detected when thrombin or factor Xa was incubated with SP_2 alone over the range of concentration tested.

Results and discussion

The green seaweed *C. cupressoides* var. *lycopodium* SPs were extracted by papain digestion (6h, 60°C), and then fractionated by ion-exchange chromatography (DEAE-cellulose), resulting into three different SPs fractions (SP_1, SP_2 and SP_3 eluted at concentrations of 0.5, 0.75 and 1 M of NaCl, respectively). Fraction SP_2 presented highest charge density by agarose gel electrophoresis procedure compared with SP_1 and SP_3, being corroborated by its highest sulfate (28%) and total sugars (40%) contents. These values were similar to those found by Rodrigues et al. (2011b, 2011d, 2013), but highest compared with those isolated *C. racemosa* SPs (CHATTOPADHYAY et al., 2007; GHOSH et al., 2004; JI et al., 2008). No contaminant proteins were detected. In addition, only SP_2 has changed the *in vitro* normal coagulation time (APTT test, data not shown), confirming thus previous investigations (RODRIGUES et al. 2011b and d). From these previous data, it was investigated the structural

features of SP_2 from *C. cupressoides* var. *lycopodium*, as well as its role on coagulation proteases *in vitro*.

IR

To provide information on the occurrence of sulfate, a sample of SP_2 fraction from the green seaweed *C. cupressoides* var. *lycopodium* was analyzed by FT-IR technique, as seen in Figure 1.

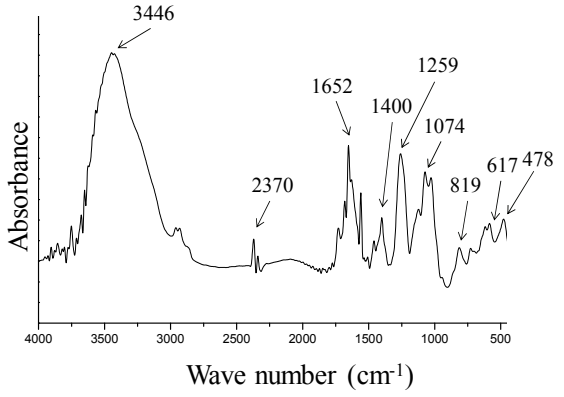

Figure 1. The IR spectrum of the SP_2 fraction from the green alga *Caulerpa cupressoides* var. *lycopodium* at 4000 and 500 cm^{-1} in kBr pellets.

The characteristic signals of sulfate (at 1259 cm^{-1}, S = O) (ARAÚJO et al. 2011; CHATTOPADHYAY et al., 2007; GHOSH et al., 2004; MAZUMDER et al., 2002; SCHEVCHENKO et al., 2009), uronic acid (at 1652 cm^{-1}, COO^- or O-H) (ANANTHI et al., 2010; ZHANG et al., 2008) and/or proteins (amide band) (ESTEVEZ et al., 2009) were identified in the FT-IR spectrum of SP_2. The original band at 819 cm^{-1} was attributed to the bending vibration of galactose-6-sulfate, suggesting that sulfate radical occurred at position C-6 of galactose (CHATTOPADHYAY et al., 2007; GHOSH et al., 2004; MAZUMDER et al., 2002). The IR spectrum of SP_2 also showed a characteristic absorption band at 1074 cm^{-1}, possibly associated with arabinogalactan sulfate backbone (ESTEVEZ et al., 2009). Ghosh et al. (2004) deduced the occurrence of O-3 of arabinose in a hot-water extract containing SPs obtained from the green seaweed *C. racemosa*. 3446 (O-H) (ANANTHI et al., 2010; ZHANG et al., 2008), 617 and 478 cm^{-1} (O = S = O) were also detected. The obtained signal at 1400 cm^{-1} would correspond to the carboxyl group of the pyruvic acid based on Estevez et al. (2009).

Few data have been described for SPs from *Caulerpa* species (CHATTOPADHYAY et al., 2007; GHOSH et al., 2004; HAYAKAWA et al., 2000; JI et al., 2008; RODRIGUES et al., 2011d, 2012; SCHEVCHENKO et al., 2009). In this study, the presence of galactose-6-sulfate (819 cm^{-1}) in the chemical structure of SP_2 (*C. cupressoides* var. *lycopodium*) is in accordance with SPs found in *Caulerpa* species (CHATTOPADHYAY et al., 2007; GHOSH et al., 2004). It has been suggested that SPs from Caulerpaceae are preponderantly made up by galactose monosaccharide (HAYAKAWA et al., 2000). Furthermore, the occurrence of arabinogalactan sulfate backbone (1074 cm^{-1}) in the IR spectrum of SP_2 could also support the hypothesis of molecular markers (RODRIGUES et al., 2012) based on their structural studies as auxiliary tools in studies on morphology, anatomy and life history of marine algae (USOV, 1998). Pomin and Mourão (2008) reported that the sulfated galactans (glycosidic linkage β(1→3)) are structural sugars highly conserved in some taxonomic groups, including Rhodophyta, Chlorophyta, Angiosperms, Echinoderms and Mollusks. They occur among these phyla differing mainly in sulfation sites, with a tendency towards 4-sulfation in algae and marine angiosperms, 2-sulfation in invertebrates, and 6-sulfation, which is dispersed in minor amounts throughout phylogeny.

Also, the presence of galactose-6-sulfate (a biological precursor to transform in 3,6-anhydrogalactose) in the chemical structure of SP_2 (Figure 1) could be of interest for obtaining of commercially important SPs. Mazumder et al. (2002) observed that SPs extracted with water from *Gracilaria corticata* (Rhodophyta) presented galactose-4-sulfate and galactose-6-sulfate. SPs obtained by alkaline treatment exhibited a signal more intense at 930 cm^{-1} (characteristic of 3,6-anhydrogalactose). Probably the occurrence of galactose-6-sulfate may be chemically converted to 3,6-anhydrogalactose after alkaline treatment. According to Campo et al. (2009), the alkaline extraction or treatment of red seaweeds SPs has a positive effect on the functional properties of the gel formation as thickening, gelling and stabilizing agents for food application.

Chemical analyses

The content of uronic acid of the SP_2 fraction from the green seaweed *C. cupressoides* var. *lycopodium* was 7.18%. This value is similar to those SPs extracted from the green seaweed *C. racemosa* (4-7.9%) (GHOSH et al., 2004; JI et al., 2008), but exhibited the lowest uronic acid content compared with those SPs from the Chlorophyta *Monostroma latissimum* (10.77-14.58%) (ZHANG et al., 2008). Uronic acid was also obtained in SPs extracted from the brown seaweed *Lobophora variegata* by Medeiros et al. (2008), whereas for a sulfated galactan from the marine green seaweed *Codium cylindricum* it was not detected (MATSUBARA et al., 2001).

In respect to elemental microanalysis, the analytical data for *C. cupressoides* var. *lycopodium* SP_2 fraction showed a nitrogen content (1.30%) similar to that of cold extract fraction of SPs (1.22%) from the red seaweed *Gracilaria birdiae* by Maciel et al. (2008). The presence of nitrogen in the SP_2 fraction could be attributed to amino acids of the proteins (GHOSH et al., 2004; JI et al., 2008) in the chemical structure of the residual polysaccharide of *C. cupressoides* var. *lycopodium*, when extracted by papain digestion, confirming perhaps the absorption signal (amide band) previously indentified (Figure 1). Marinho-Soriano et al. (2006) reported that the protein levels in marine macroalgae could be correlated with the nitrogen content. Ji et al. (2008) suggested that amino acids could be related with the bioactivity (antitumor) of *C. racemosa* SPs, especially aspartic acid, an amino acid that has an important role in cell energy and nitrogen metabolism. Probably, the fraction SP_2 (*C. cupressoides*) is a polysaccharide-protein complex (proteoglycan), contrasting with a mistaken conclusion reported by Rodrigues et al. (2011d).

The sulfate content for SP_2 (4.27%), when determined by elemental microanalysis, was also about 2-fold higher compared with aqueous SPs extract from *G. birdiae* (Rhodophyta) (MACIEL et al., 2008), suggesting the hypothesis of the use of enzymes to extract pharmaceutically important materials (ARAÚJO et al., 2012; RODRIGUES et al., 2011a). Fraction SP_2 had carbon and hydrogen contents of 21.98 and 4.86%, respectively. Maciel et al. (2008) found 40% of carbon content in the cold extract fraction in red seaweed *G. birdiae*.

PAGE

In order to estimate the average molecular size of the *C. cupressoides* var. *lycopodium* polysaccharide, the SP_2 fraction was submitted to a PAGE procedure (Figure 2).

Interestingly, it was observed that SP_2 fraction contained various polysaccharides (named SP-1, SP-2, SP-3 and SP-4) of different molecular weights. SP-1 and SP-2 remained close to the origin of the gel, indicating polysaccharides of high molecular masses (> 100 and ~ 100 kDa, respectively). In respect to polysaccharides SP-3 and SP-4, the electrophoretic profile revealed a mobility similar to standards chondroitin-4-sulfate (C-4-S, ~ 40 kDa) and dextran sulfate (DexS, ~ 8 kDa), respectively (YOON et al., 2007).

In red seaweeds, SPs constitute the intercellular matrix and nonfibrillar cell walls (POMIN; MOURÃO, 2008; USOV, 1998). In this study, SPs present in SP_2 (*C. cupressoides* var. *lycopodium*) presented a dispersive migration due to a heterogeneous molecular weight, typical of these polymers (PUSHPAMALI et al., 2008; YOON et al., 2007). However, fraction SP_2 showed at least four polysaccharides of different molecular weights (Figure 2). This contrasts with the results from the seaweeds *Laminaria cichorioides* (Phaeophyta) (YOON et al., 2007) and *Lomentaria catenata* (Rhodophyta) (PUSHPAMALI et al., 2008) where a unique polysaccharide was observed.

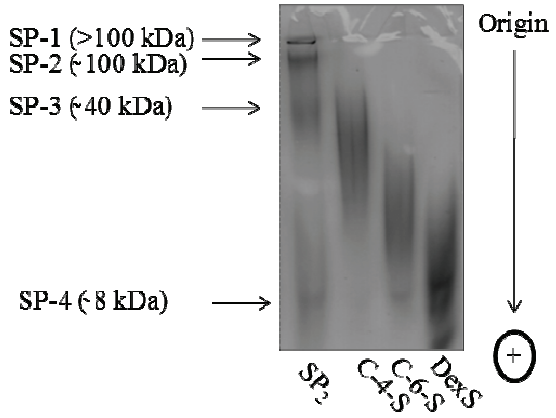

Figure 2. Polyacrylamide gel electrophoresis (PAGE). SP_2 from the green alga *Caulerpa cupressoides* var. *lycopodium* (Subfractions SP-1, SP-2, SP-3 and SP-4) and standards chondroitin-4-sulfate (C-4-S, 40 kDa), chondroitin-6-sulfate (C-6-S, 60 kDa) and dextran sulfate (DexS, 8 kDa) present on gel were stained with 0.1% toluidine blue.

The revealed characteristics of SP_2 present in *C. cupressoides* var. *lycopodium* could perhaps be explained considering the morphology and/or coenocytes tissue structure of this species (RODRIGUES et al., 2012). Rodrigues et al. (2011c) observed SPs extracted from *C. cupressoides* var. *lycopodium* occurred as a distinct resolution degree in different portions of its tissue, when analyzed by agarose gel electrophoresis procedure. Probably, the chemical characteristics of these compounds could not be only based on their polyanionic characters, but also in molecular masses (Figure 2). Further investigations should be conducted to better understand the biological role of these compounds in species of the genus *Caulerpa*.

Inhibitory effects of thrombin or factor Xa activities are mediated by AT and HCII in the presence of SP2 fraction

To initially confirm the anticoagulant mechanism of thrombin (factor IIa) inactivation by AT as previously described by Rodrigues et al. (2011b), SP_2 fraction was measured. As expected, the *C. cupressoides* var. *lycopodium* SP_2 fraction interfered with the coagulation system, by inhibiting the thrombin activity mediated by AT (IC_{50} ~ 18 μg mL^{-1}) against IC_{50} ~ 1 μg mL^{-1} for

inactivation by unfractionated heparin. From this previous experiment and owing that each new SP purified from marine alga could be a compound with unique structure and, consequently, forming a particular complex with plasma inhibitor and the target protease (MELO; MOURÃO, 2008; PEREIRA et al., 2005; YOON et al., 2007), it was also performed two more inhibition thrombin (factor IIa) or factor Xa by AT or HCII assays *in vitro*. In this regard, the Figure 3A shows an inhibition thrombin (factor IIa) mediated by HCII assay. Surprisingly, the fraction SP_2 had ability on thrombin inhibition through HCII-dependent pathway ($IC_{50} \sim 46$ μg mL^{-1}) similar to other studied *Caulerpa* SPs (*C. okamurai* and *C. brachypus*) that also exhibited interaction with HCII and its thrombin target protease (HAYAKAWA et al., 2000).

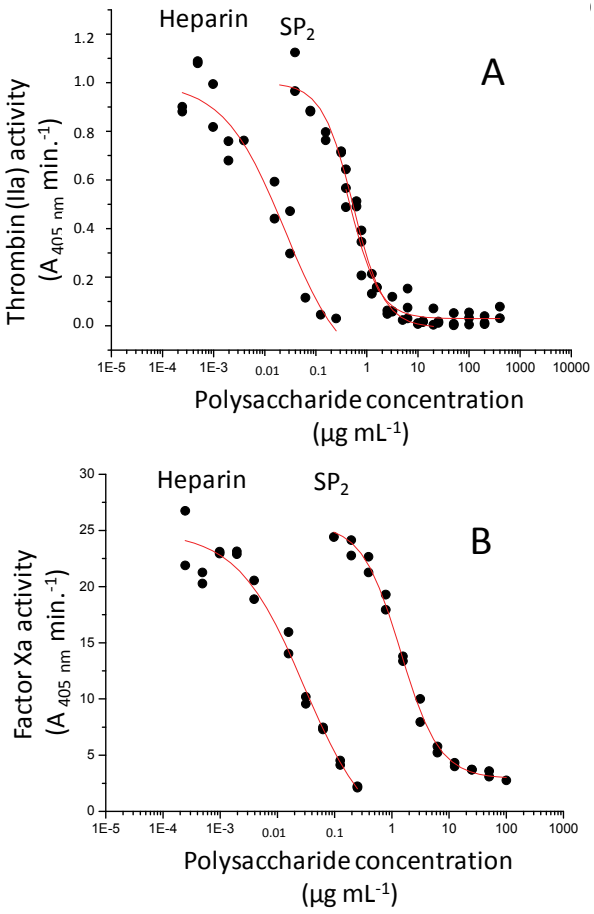

Figure 3. Dependence on the concentration of SP_2 fraction for inactivation of thrombin (factor IIa) (A) or factor Xa (B) by heparin cofactor II (HCII) or AT. HCII or AT (10 nM) was incubated with factor IIa or factor Xa (15 nM) under various concentrations of polysaccharide (0-1000 μg mL^{-1}, n = 3) from the green alga *Caulerpa cupressoides* var. *lycopodium* (SP_2) and heparin. Factor IIa or factor Xa activity was determined with a chromogenic substrate ($\Delta A_{405\ nm}$ $min.^{-1}$).

The antithrombotic action of algae SPs in animal experimentation (RODRIGUES et al., 2011b) requires the anti-factor Xa activity for a thrombosis-preventing effect (FONSECA et al., 2008). In this study, it was also evaluated the influence of the SP_2 fraction from *C. cupressoides* var. *lycopodium* on factor Xa inactivation by AT. The inhibitory effect of the factor Xa in the presence of SP_2 by AT was about 24 μg mL^{-1} (IC_{50}). The same property was also noted for heparin ($IC_{50} \sim 1.7$ μg mL^{-1}) (Figure 3B).

These evidences found herein suggested that the interaction of SP_2 (*C. cupressoides* var. *lycopodium*) with the coagulation inhibitors could involves specific structural requirements for its anticoagulant action. Matsubara et al. (2001) isolated a sulfated galactan from the green alga *Codium cylindricum*. This polymer had effect on the normal coagulation time by the activated partial thromboplastin time (APTT) and thrombin time (TT) tests using human plasma, except for prothrombin time (TP) test. To elucidate the inhibitory mechanism of the anticoagulant, the authors observed the lack of effect of this sulfated galactan on thrombin and factor Xa in the presence of AT or HCII, seeming that its anticoagulant effect is due to inhibition of fibrin polymerization or the intrinsic pathway without potentiating plasma inhibitors (AT). Pereira et al. (2005) compared SPs from the red seaweeds *Botryocladia occidentalis* and *Gelidium crinale* using specific proteases and coagulation inhibitors tests. The authors found that the proportions and/or the distribution of 2,3-di-sulfated α-units in galactan backbone may represent a specific requirement for anticoagulant action. Based on their heterogeneities, distribution of sulfate groups and the monosaccharide composition of SPs may also be depend on for their interactions on coagulation inhibitors and the target proteases.

Our study evidenced that both thrombin and factor Xa target proteases were inhibited by SP_2 (*C. cupressoides* var. *lycopodium*), but required a concentration of about 2.5-fold higher of HCII than for thrombin inactivation by AT (Figure 3). The identification of the structural signal corresponding to presence of galactose-6-sulfate in SP_2 fraction (Figure 1) could be useful to the understanding of its structure-activity relationship in further studies. Mestechkina and Shcherbukhin (2010) reported that chemically SPs ((1→4)-β-xylan; amylase, an (1→4)-α-glucan; cellulose, an (1→4)-β-glucan; curdlan, an (1→3)-β-glucan; and (1→3)-β-galactan) at the free hydroxyl groups were assayed for anticoagulant activity. The results revealed that the activity could be independent of the anomeric bond configuration, but the position of glycoside bond was important for the activity. In order to elucidate if the location of sulfate groups could be

significant for anticoagulant action, the polysaccharides were desulfated, demonstrating thus that the desulfation process specifically at position C-6 in the (1→3)-linked polysaccharide the target activity was lost. When polysaccharides formed by the presence or absence of sulfate at position C-6 in the (1→4) glycoside bond were investigated, they did not exhibit anticoagulant action.

Previous studies also pointed out that the molecular size of SPs could influence their anticoagulant actions (MESTECHKINA; SHCHERBUKHIN, 2010; POMIN, 2012). High molecular weight SPs preparations (216.4-61-9 kDa) had an important effect on the anticoagulant activity of the SPs obtained from green alga *M. latissimum* (ZHANG et al., 2008). Melo and Mourão (2008) reported that a red algal sulfated galactan (*B. occidentalis*), when fragmented for about 5 kDa, inhibited the venous thrombosis with discrete *in vivo* anticoagulant and hemorrhagic effects. More recently, a greater specificity of the activity of low molecular weight fucoidan for thrombin-induced platelet aggregation was found in *L. japonica* (Phaeophyta) by Zhu et al. (2010). Therefore, the fraction SP_2 from *C. cupressoides* var. *lycopodium* requires a more in-depth study with basis on the anticoagulant actions. Further investigations are in progress by our group.

Conclusion

A fraction containing sulfated polysaccharides from the green seaweed *Caulerpa cupressoides* var. *lycopodium* has chemical molecular features contrasting to other studied algal sulfated polysaccharides. The inactivation of thrombin and factor Xa may be mediated by heparin cofactor II and/or antithrombin.

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