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Structural analysis of a sulfated polysaccharidic fraction obtained from the coenocytic green seaweed *Caulerpa cupressoides* var. *lycopodium*

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ABSTRACT. Researches on structural chemistry of sulfated polysaccharides (SPs) have been mainly focused on red and brown algae. *Caulerpa cupressoides* var. *lycopodium* (Chlorophyta) contains three SPs fractions (Cc-SP_1, Cc-SP_2 and Cc-SP_3). Cc-SP_1 and Cc-SP_2 had anticoagulant (*in vitro*) and anti- and prothrombotic, antinociceptive and/or anti-inflammatory (*in vivo*) effects. However, their structural features have not yet been investigated. This study analyzed the chemical composition, elemental microanalysis and structural features by infrared (IR) and nuclear magnetic resonance (^{1}H NMR) spectroscopy of Cc-SPs. Fractionation of SPs by DEAE-cellulose yielded Cc-SP_1, Cc-SP_2 and CcSP_3 containing differences among the relative proportions of sulfate (14.67-26.72%), total sugars (34.92-49.73%) and uronic acid (7.15-7.22%). Carbon (21.76-29.62%), sulfate (2.16-4.55%), nitrogen (0.85-1.57%) and hydrogen (4.57-5.86%) contents were obtained using a CHN equipment. Data from IR indicated occurrence of sulfate ester, galactose-6-sulfate, uronic acid and glycoside linkages. For ^{1}H NMR spectrum of the soluble Cc-SP1 fraction, it was mainly found β-galactopyranose residues and CH_3 group. The results showed that Cc-SPs fractions have some structural features similar to others studied Caulerpaceae SPs.

Keywords: Chlorophyta, Caulerpaceae, sulfated polymers, chemical analysis, structural chemistry.

Análise estrutural de uma fração polissacarídica sulfatada obtida da alga marinha verde cenocítica *Caulerpa cupressoides* var. *lycopodium*

RESUMO. Pesquisas sobre química estrutural de polissacarídeos sulfatados (PSs) têm focado principalmente em algas vermelhas e pardas. A Chlorophyta *Caulerpa cupressoides* var. *lycopodium* contém três frações de PSs (Cc-PS_1; Cc-PS_2 e Cc-PS_3). As Cc-PS_1 e Cc-PS_2 apresentaram efeitos anticoagulante (*in vitro*), anti- e pró-trombótico, antinociceptivo e/ou anti-inflamatório (*in vivo*). Entretanto, ainda não foram investigadas suas características estruturais. Este estudo analisou de Cc-PSs a composição química, microanálise elementar e características estruturais por infravermelho (IV) e espectroscopia de ressonância magnética nuclear (RMN ^{1}H). O fracionamento dos PSs por DEAE-celulose rendeu Cc-PS_1; Cc-PS_2 e Cc-PS_3 contendo diferenças entre as proporções relativas de sulfato (14,67-26,72%), açúcares totais (34,92-49,73%) e ácido urônico (7,15-7,22%). Foram obtidos, usando um equipamento CHN, conteúdos de carbono (7,15-7,22%), sulfato (2,16-4,55%), nitrogênio (7,15-7,22%) e hidrogênio (7,15-7,22%). O IV forneceu dados sobre a ocorrência de éster sulfato, galactose-6-sulfato, ácido urônico e ligações glicosídicas. Foram encontrados principalmente para o espectro de RMN ^{1}H da fração solúvel Cc-PS_1, resíduos de β-galactopiranoses e de grupo CH_3. Os resultados mostraram frações de Cc-PSs detentoras de algumas características estruturais semelhantes aos de outros PSs de Caulerpaceae estudados.

Palavras-chave: Chlorophyta, Caulerpaceae, polímeros sulfatados, análise química, química estrutural.

Introduction

Natural products derived from aquatic organisms for functional food, biochemical research and other biotechnological applications hagve aroused special interest in recent years (POMIN; MOURÃO, 2008; RODRIGUES et al., 2011b; SMIT, 2004; VANDERLEI et al., 2010).

Amino acids, lipids, fatty acids, proteins, vitamins, β-carotene, tocopherol, minerals, and carbohydrates from seaweeds are the most important nutritional attributes for human and animal diets (MARINHO-SORIANO et al., 2006; MOHAMED et al., 2012; OLIVEIRA et al., 2009). Currently, some edible seaweed species have been marketed for consumption in European countries (DAWCZYNSKI et al., 2007).

Seaweeds have revealed important therapeutic properties for health and disease management (e.g., anticancer, antiobesity, antidiabetic, antihypertensive, anticoagulant, anti-infective, anti-inflammatory and tissue healing properties), arousing thus growing interest by pharmaceutical companies (JIAO et al., 2011; MOHAMED et al., 2012; SMIT, 2004). They are an abundant source of sulfated polysaccharides (SPs) naturally occurring as structural components in the extracellular matrix (POMIN; MOURÃO, 2008). However, these biomaterials are not found only in marine macroalgae (ARAÚJO et al., 2011; ATHUKORALA et al., 2006; MAZUMDER et al., 2002; RODRIGUES et al., 2009), but also in seagrass (AQUINO et al., 2005), microalgae (MAJDOUB et al., 2009), vertebrate (called glycosaminoglycans) (RODRIGUES et al., 2011b), invertebrates (POMIN; MOURÃO, 2008), and, recently, in freshwater plants (DANTAS-SANTOS et al., 2012).

In seaweeds, sulfated galactans are the most common source of SPs (CAMPO et al., 2009; FONSECA et al., 2008; MURANO et al., 1997). SPs from Phaeophyceae are called fucan or fucoidan (BILAN et al., 2004; LEITE et al., 1998; POMIN; MOURÃO, 2008). Heteropolysaccharides containing xylose, rhamnose, galactose, arabinose, mannose, pyruvate, glucuronic acid and/or glucose are present in Chlorophyceae (BILAN et al., 2007; CIANCIA et al., 2012; FARIAS et al., 2008; SCHEVCHENKO et al., 2009). SPs vary among different organisms, but have features conserved among phyla (AMORIM et al., 2012; POMIN; MOURÃO, 2008; USOV, 1998). These polymers would represent a potential source of compounds for diverse clinical therapies due to their bioactivities (AMORIM et al., 2011; ANANTHI et al., 2010; CAMPO et al., 2009; CHOI et al., 2009; COURA et al., 2012; JIAO et al., 2011; QUINDERÉ et al., 2014).

The structural diversity of SPs found in seaweeds varies with species. For example, Bilan et al. (2004) obtained a fucoidan fraction made up of a regular structure comprising alternating 3-linked α-L-fucopyranose 2,4-disulfate and 4-linked α-L-fucopyranose 2-sulfate residues from the brown marine alga *Fucus distichus*. The structure of a highly pyruvylated galactan sulfate from the green seaweed *Codium yezoense* was reported by Bilan et al. (2007). A highly sulfated galactan from the red marine alga *Halymenia durvillei* was isolated by Fenoradosoa et al. (2009). Araújo et al. (2011) identified two SP fractions (κ- and ι-carrageenans, respectively) present in the crude extract from the red seaweed *Solieria filiformis*. Recently, crude SPs containing galactose-4-sulfate from *Gracilaria ornata* (Rhodophyta) were extracted by Amorim et al. (2012). However, there are very few studies about the chemical structures of SPs belonging to Chlorophyta species (CHATTOPADHYAY et al., 2007a and b; CIANCIA et al., 2012; FARIAS et al., 2008; POMIN; MOURÃO, 2008; QI et al., 2012; SCHEVCHENKO et al., 2009; ZHANG et al., 2008).

The genus *Caulerpa* Lamouroux (1809) includes species generally occurring in tropical and subtropical marine waters. Approximately one hundred species have been described and are important contributors to the algal biomass of coral reefs and lagoons (TRI, 2009). Polysaccharides from *Caulerpa* genus consisting of sulfate, galactose, glucose, arabinose and xylose, and small amounts of mannose and rhamnose and traces of fucose residues have been identified with medicinal importance (GHOSH et al., 2004; HAYAKAWA et al., 2000; JI et al., 2008; MAEDA et al., 2012). From the Brazilian species *Caulerpa cupressoides* var. *lycopodium*, three SPs fractions (Cc-SP_1, Cc-SP_2 and Cc-SP_3) have been recently isolated. Cc-SP_2 showed anticoagulant (*in vitro*), anti- and prothrombotic (*in vivo*) (RODRIGUES et al., 2011a), antinociceptive and anti-inflammatory (*in vivo*) (RODRIGUES et al., 2012) effects. Cc-SP_1 and Cc-SP_3 had no anticoagulant effect, but an antinociceptive action of Cc-SP_1 was recently investigated (RODRIGUES et al., 2013b). Nevertheless, only one study reported the structure features of its SPs (Cc-SP_2) (RODRIGUES et al., 2013a). In the present study, some structural properties of Cc-SP_1 were investigated.

Material and methods

Marine alga and SPs and chemical analyses

The green seaweed *C. cupressoides* var. *lycopodium* (Vahl) C. Agardh (Caulerpaceae, Bryopsidales) was collected on the seashore from Flecheiras beach, Ceará State, Brazil. A voucher of this specimen has been deposited in the Prisco Bezerra Herbarium (Department of Biology, Federal University of Ceará, Brazil). The crude SP was extracted from the dehydrated algal tissue (room temperature) by papain digestion (60°C, 6h), and then subjected to fractionation by anion-exchange chromatography on a DEAE-cellulose column using a NaCl gradient (0→1 M, with 0.25 M of intervals). Fractions (Cc-SP_1, Cc-SP_2 and Cc-SP_3 eluted with 0.5, 0.75, and 1

M of NaCl, respectively) were obtained, as described by Rodrigues et al. (2011a). Quantitative determination of sulfate, total sugars and contaminant proteins of the SPs fractions were carried out. The uronic acid content of the SP fractions was determined by carbazole-sulfuric acid method using spectrophotometric analysis (AMERSHAM BIOSCIENCES ULTROSPEC 1100) at 525 nm, using glucuronic acid as standard. These experimental protocols were performed, as previously described (RODRIGUES et al., 2011a). Nitrogen, carbon, hydrogen and sulfate contents were determined by elemental microanalysis using a CHN equipment Perkin Elmer model 2400 based on Maciel et al. (2008).

Structural analysis

Infrared (IR) spectroscopy

To study structural features, Fourier Transform IR (FT-IR) spectra of the SP fractions were determined using a SHIMADZU IR spectrophotometer (model 8300) between 4000 and 500 cm^{-1}. The samples (5 mg) were pressed on KBR pellets.

Nuclear magnetic resonance (NMR) spectroscopy

One dimension ^1H NMR spectrum of a SP fraction from *C. cupressoides* var. *lycopodium* (Cc-SP_1) was recorded using a Bruker DRX 800 MHz apparatus with a triple resonance (5 mm). About 3 mg of sample was dissolved in 0.5 mL 99.9% deuterium oxide. The spectrum was recorded at 60°C with HOD suppression by presaturation. Chemical shifts were given relative to external trimethylsilyl-propionic acid standard at 0 ppm (FARIAS et al., 2008).

Results and discussion

Chemical analyses

The fractionation of the crude SP obtained from *C. cupressoides* var. *lycopodium* performed on a DEAE-cellulose column resulted in different chemical proportions of sulfate and total sugars contents among the SPs fractions, while the uronic acid content was almost equal among them (Table 1).

Differences between the relative proportions of sulfate and sugars may occur when comparing fractions eluted at different molarities of salt (ARAÚJO et al., 2011; CHATTOPADHYAY et al., 2007a; LEITE et al., 1998). Cc-SP_1 exhibited the lowest content of sulfate (14.67%) and total sugars (34.92%) in comparison with others fractions. The sulfate and total sugars contents of this species were higher than the amount found for *C. racemosa* (CHATTOPADHYAY et al., 2007a; GHOSH et al., 2004). Enzymatic extraction of algae results in high bioactive yield and shows enhanced biological activity in comparison with water and organic extracts (ATHUKORALA et al., 2006). Fractions from *C. cupressoides* var. *lycopodium* were enriched with uronic acid. Differences among the relative proportions of uronic acid were found in Chlorophyta *C. racemosa* (4-7.9%), (GHOSH et al., 2004; JI et al., 2008), *C. lentillifera* (4.3%) (MAEDA et al., 2012), *Monostroma latissimum* (10.77-14.58%) (ZHANG et al., 2008) and *Bryopsis plumosa* (5.6%) (CIANCIA et al., 2012). As expected, proteins were removed in the extraction process (ARAÚJO et al., 2011; RODRIGUES et al., 2011a, 2013b).

Table 1. Chemical analyses of SPs fractions obtained by anion-exchange chromatography (DEAE-cellulose) from the green seaweed *Caulerpa cupressoides* var. *lycopodium*.

			Chemical analyses (%)						
Fraction	NaCl (M)	Sulfate[a]	Total[b] sugars	Uronic[c] acid	Protein[d] contaminant	S[e]	C[f]	H[g]	N[h]
Cc-SP_1	0.5	14.67	34.92	7.22	-	2.16	29.62	5.86	1.57
Cc-SP_2	0.75	26.72	49.73	7.15	-	4.33	21.76	4.57	1.32
Cc-SP_3	1	23.34	46.67	7.19	-	4.55	22.5	4.75	0.85

a – Dosage by Dodgson and Price' method using NaSO3 as standard; b – Dosage by Dubois et al.' method using D-galactose as standard; c – Dosage by Dische' method using glucuronic acid as standard; d – Dosage by Bradford' method using bovine serum albumin (- not detected); e – sulfate, f – carbon, g – hydrogen and h – nitrogen were determined by elemental microanalysis using a CHN equipment Perkin Elmer model 2400 (MACIEL et al., 2008).

The sulfate profile of the SPs fractions eluted with different NaCl molarities corroborated the chemical analyses (Table 1). In addition, the sulfate content was about 2-fold higher in comparison with the cold extract containing SPs from *Gracilaria birdiae* (Rhodophyta) (MACIEL et al., 2008), suggesting the ability of enzymes (papain) to extract pharmaceutically important biomaterials (ARAÚJO et al., 2011; RODRIGUES et al., 2011a, 2013b). This comparison between sulfate content by the two different methods was good. Also, as the sulfate of Cc-SP1 becomes lower in comparison with others fractions; the 1259 cm^{-1} (ester sulfate groups) band intensity decreases (Figure 1A). Although investigations by IR are progressively less accurate, the values determined by elemental microanalysis could be appropriate (MELO et al., 2002).

Although proteins have not been detected, analyses revealed the presence of nitrogen in SP fractions (Table 1). Values ranged from 0.85 to 1.57%. These data suggested the presence of amino acids of the proteins in the SPs fractions, indicating the hypothesis that *C. cupressoides* var. *lycopodium* may be capable of biosynthesizing proteoglycans as described for *Caulerpa* species (GHOSH et al., 2004; JI et al., 2008) and in animals (RODRIGUES et al., 2011b). It could indicate a correlation between proteins levels and nitrogen content (MARINHO-SORIANO et al., 2006). The nitrogen content of SPs fractions (*C. cupressoides* var. *lycopodium*) was similar to that described for *G. birdiae* (1.22%) by Maciel et al. (2008), but higher than obtained for the red seaweed *G. cornea* (0.41-0.47%) (MELO et al., 2002). Fractions Cc-SP_1, Cc-SP_2 and Cc-SP_3 also showed levels of 29.62, 21.76 and 22.5% for carbon; and 5.86, 4.57 and 4.75% for hydrogen, respectively (Table 1). Maciel et al. (2008) found 40% carbon content in the cold extract for *G. birdiae*. Further investigations should be conducted to infer the effect of these molecules on the bacterial growth (AMORIM et al., 2012).

FT-IR

The FT-IR spectra of SPs fractions of *C. cupressoides* var. *lycopodium* are shown in Figure 1. Typical absorption bands related to the presence of ester sulfate groups (S=O stretching) were observed (ANANTHI et al., 2010; CHATTOPADHYAY et al., 2007a and b; FENORADOSOA et al., 2009; MAZUMDER et al., 2002). Furthermore, the intensity of the signals has decreased from Cc-SP_2 and Cc-SP_3 fractions to the Cc-SP_1 (1263→1259 cm^{-1}) and it was corroborated by the sulfate content (Table 1) (AMORIM et al., 2012; ARAÚJO et al., 2011; SILVA et al., 2010). Bands absorption from 815 to 819 cm^{-1} suggested the occurrence of galactose-6-sulfate structural feature in the fractions (CHATTOPADHYAY et al., 2007a; DANTAS-SANTOS et al., 2012; GHOSH et al., 2004; MACIEL et al., 2008), but with the lowest intensity of this signal verified in Cc-SP_1 (Figure 1A). In previous studies, Cc-SP2 had *in vitro* anti-clotting effect. Cc-SP1 and Cc-SP3 had no anticoagulant effect (RODRIGUES et al., 2011a, 2013b). Here, differences among the relative intensity of galactose-6-sulfate by IR were noted between fractions (Figure 1), suggesting the importance of this sulfated residue for anticoagulant action of some molecules (DANTAS-SANTOS et al., 2012; MESTECHKINA; SHCHERBUKHIN, 2010). 3446 cm^{-1} (OH stretching), 2931 cm^{-1} (CH stretching) (DANTAS-SANTOS et al., 2012; ZHANG et al., 2008), 1652 cm^{-1} (COO^- or O-H stretching) (ZHANG et al., 2008), 1400-1404 cm^{-1} (carboxyl group of the pyruvic acid stretching) and 1076 cm^{-1} (arabinogalactan sulfate backbone stretching) (ESTEVEZ et al., 2009) were also observed in the IR spectra.

Therefore, low values were recorded in Cc-SP_1 when compared to those data found in Cc-SP_2 and Cc-SP_3 (Table 1 and Figure 1), revealing thus the occurrence of distinct SPs and that the employment of different NaCl molarities was important for *C. cupressoides* var. *lycopodium* SPs separation (DEAE-cellulose) (ARAÚJO et al., 2011).

Some SPs possessing highly complex and heterogeneous structures have been isolated from aquatic organisms. Bilan et al. (2004) investigated the presence of a highly regular fucoidan composed of alternating 3-linked α-L-fucopyranose 2,4-disulfate and 4-linked α-L-fucopyranose 2-sulfate residues from *F. distichus* (Phaeophyta). Marine angiosperms (*Ruppia maritima*, *Halodule wrightii* and *Halophila decipiens*) were described to have sulfated galactans.

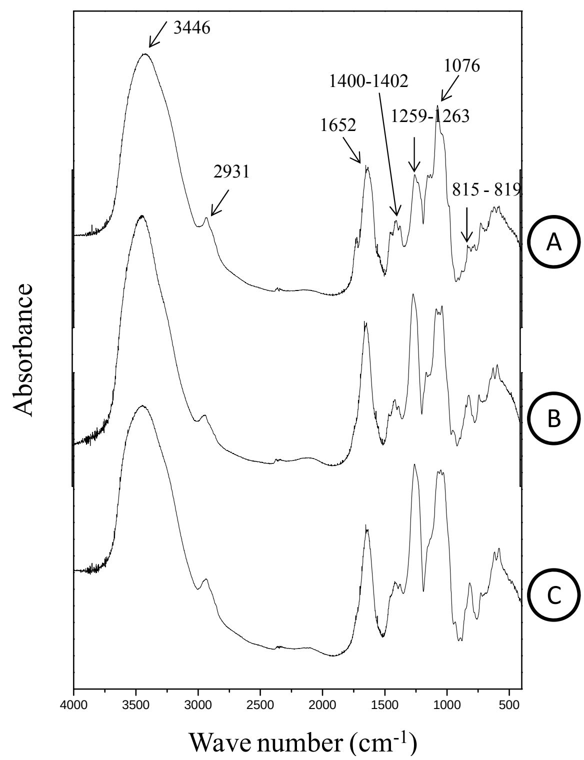

Figure 1. FT-IR spectra of Cc-SP_1 (A), Cc-SP_2 (B) and Cc-SP_3 (C) fractions obtained by anion-exchange chromatography (DEAE-cellulose) from the green seaweed *Caulerpa cupressoides* var. *lycopodium*.

Those found in *R. maritima* were constituted by a regular tetrasaccharide repeating unit that appeared to have an intermediate chemical structure compared to SPs obtained from marine invertebrates and red seaweeds (AQUINO et al., 2005). More recently, SPs obtained from the microalgae *Arthrospira platensis* were demonstrated to have a preponderance of rhamnose present in their chemical structures (MAJDOUB et al., 2009).

The present study, together with literature data, point out galactose as a highly conserved structural sugar in Caulerpaceae (CHATTOPADHYAY et al., 2007a; GHOSH et al., 2004) and could be of taxonomic significance (AMORIM et al., 2012;

AQUINO et al., 2005; DANTAS-SANTOS et al., 2012; POMIN; MOURÃO, 2008; USOV, 1998). Murano et al. (1997) investigated SPs (carrageenans) extracted from *S. filifomris* and *Agardhiella subulata* (Rhodophyta) from Mar Piccolo, Italy. The authors separately analyzed different crude SPs extracts by IR and NMR spectroscopic analysis showing similar polysaccharide structure backbone among them, but some irregularities notably were attributed to 6-sulfated 4-linked precursor units (galactose-6-sulfate). Differences in the precursor content could be derived from variations of season, growth conditions and life cycle of these macroalgae species. Galactose-6-sulfate is a natural biological precursor which can be converted to 3,6-anhydrogalactose (CAMPO et al., 2009), which may be found in SPs from some red seaweeds species with commercial interests (MACIEL et al., 2008; SILVA et al., 2010). According to Campo et al. (2009), the use of alkaline extraction for red seaweeds SPs increases the functional ability of the gel as thickening, gelling and stabilizing agents for biotechnological applications.

NMR

For a more detailed structural investigation of Cc-SP1, which showed to be soluble in D_2O solution, ^1H NMR spectroscopy was carried out (Figure 2). This method gives valuable structural information of polysaccharides (CAMPO et al., 2009). The experiment was performed at high temperature (60 °C) to increase the solubility of the Cc-SP_1 solution (CAMPO et al., 2009; SILVA et al., 2010). However, its structural analysis was very difficult and not fully examined. Chemical shifts of Cc-SP_1 showed an evident anomeric proton signal at δ_H 4.65 ppm assigned to H-1 of the sugar residues (CHATTOPADHYAY et al., 2007b), with value of coupling constant of ~ 8.34 Hz. H-1 would correspond to the β-configuration of galactopyranoses (FARIAS et al., 2008), and glucose and/or xylose residues could be linked with the same molecule (CHATTOPADHYAY et al., 2007b). In contrast, glucan showing α-configuration was isolated from *C. racemosa* (Chlorophyta) by Chattopadhyay et al. (2007a). Anomeric signals located at the region ranging from δ_H~3.35 to 4.38 ppm could be attributed to protons of the C-2-C-5 of the sugar residues (QI et al., 2012); of uronic acid at δ_H~ 3.78 (COO^-) (LI et al., 2012); and discrete peaks assigned from δ_H ~ 4.5 to 4.8 ppm would indicate sulfated sugar residues (ROBIC et al., 2009) and/or of uronic acid in polymeric blocks (SINHA et al., 2010). The absence of low-field signals ($\delta_H > 5$) could confirm that the polymer consisted of β-pyranoses based on Bilan et al. (2007) and Chattopadhyay et al. (2007a). The signal at δ_H 1.42 ppm suggested the presence of CH_3 group in rhamnopyranose residues of the sample (BILAN et al., 2007; FARIAS et al., 2008; QI et al., 2012).

Although significant progresses in research on the structural chemistry of algae SPs had occurred in recent years, the structural heterogeneity of these compounds is still considered the major limitation to determine their precise chemical features (JIAO et al., 2011). There is also a lack of analytical methods to elucidate fine structures of these polymers (CAMPO et al., 2009). Each algal species could be a potential source of SPs exhibiting novel structures (ARAÚJO et al., 2011; BILAN et al., 2004, 2007; CIANCIA et al., 2012; FENORADOSOA et al., 2009; MAZUMDER et al., 2002; POMIN; MOURÃO, 2008). The study of the chemical structures and their molecular targets are essential steps to the design of new biomaterials for food and pharmacological uses (AMORIM et al., 2011; CAMPO et al., 2009; FONSECA et al., 2008; LEITE et al., 1998; LI et al., 2012; QUINDERÉ et al., 2014; SMIT, 2004).

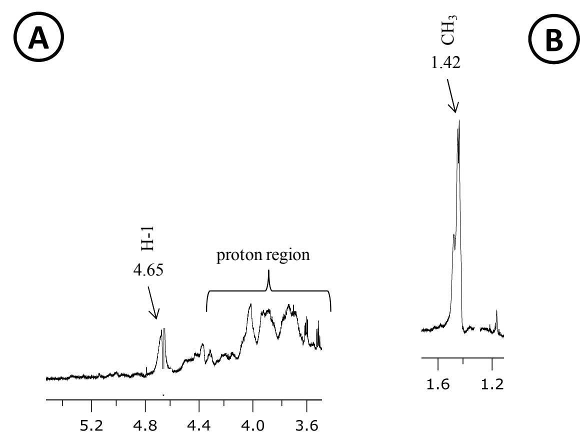

Figure 2. ^1H NMR spectrum of the Cc-SP_1 fraction obtained by anion-exchange chromatography (DEAE-cellulose) from the green seaweed *Caulerpa cupressoides* var. *lycopodium*. (A) Signal assigned to the β-configuration of galactopyranoses; (B) Signal corresponding to the proton of methyl group of the sugar residues.

In the present study, the employment of high temperature (60°C) to increase the solubility of the solution containing SPs from *C. cupressoides* var. *lycopodium*(Cc-SP_1) could affect the proton directly linked to carbons involved in glycosidic linkages. Possibly, it justified the absence of low-field signals (δ_H > 5) in the ^1H NMR spectrum of Cc-SP_1 (Figure 2), although the signal correspondent to the residual water has been observed (data not shown) (CAMPO et al., 2009). Single structural difference due to sugar type and anomeric configuration could promote great changes on biological action of SPs

(POMIN; MOURÃO, 2008). In a recent report, it was demonstrated that a SPs fraction with anticoagulant effect from *C. cupressoides* var. *lycopodium* (Cc-SP_2, Table 1) contained various polysaccharides of different molecular weights (RODRIGUES et al., 2013a). Based on these our previous findings, low molecular weights SPs (oligosaccharides) could be used to a more detailed structural investigation of these molecules, and arousing thus a new importance to gain insight into the complexity of these polysaccharides (CAMPO et al., 2009).

Conclusion

The coenocytic green seaweed *Caulerpa cupressoides* var. *lycopodium* contains sulfated polysaccharides fractions that possess some structural features similar to others studied *Caulerpa* sulfated polysaccharides. However, the structural analysis by ^{1}H NMR technique from a polysaccharidic fraction indicates new importance to gain insight in the study of Caulerpaceae.

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