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Structural characterization of cold extracted fraction of soluble sulfated polysaccharide from red seaweed *Gracilaria birdiae*

Jeanny S. Maciel [a], Luciano S. Chaves [b], Bartolomeu W.S. Souza [b], Dárlio I.A. Teixeira [c], Ana L.P. Freitas [b], Judith P.A. Feitosa [a], Regina C.M. de Paula [a,*]

[a] *Departamento de Química Orgânica e Inorgânica, Universidade Federal do Ceará, CP 12.200, CEP 60455-760, Fortaleza, Ceará, Brazil*
[b] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, CP 6020, CEP 60455-760, Fortaleza, Ceará, Brazil*
[c] *Centro de Ciências da Saúde, Universidade de Fortaleza, Fortaleza, Ceará, Brazil*

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Abstract

Water soluble polysaccharide from *Gracilaria birdiae* cultivated along the northeast coast of Brazil was characterized by infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy. The composition of the polysaccharide in wt% was determined as: β-D-gal*p* (50.3%), 3,6-anhydro-α-L-gal*p* (40.5%) and -α-L-gal*p*-6 sulfate (9.2%). The ratio of L/D units (β-D-gal*p* units and 3,6-anhydro-α-L-gal*p* + α-L-gal*p*-6 sulfate) is that of an ideal agarose. The sulfate content calculated by S% accounts for 6.4%. 1D and 2D NMR techniques were employed in order to assign the spin system of polysaccharide without partial degradation. The structure is composed of → 4-3,6-anhydro-α-L-gal*p* (1 → 3)β-D-gal*p* 1 → segments, with the possibility of a α-L-gal*p* unit substituted at the 6-position by sulfate ester.

Keywords: *Gracilaria birdiae*; Polysaccharide; NMR spectroscopy; Agarose; Sulfate ester

1. Introduction

The genus *Gracilaria* of red seaweed is distributed throughout the tropical regions of the world. Algae from this genus are important producers of agar (Marinho-Soriano & Bourret, 2005; Plastino, Guimarães, Matioli, & Oliveira, 1999) and can be found as wild and cultivated species (Critchley, 1993).

Gracilaria birdiae is an economically important marine red alga exploited for the production of agar in Brazil (Plastino, Ursi, & Fujii, 2004). It was first described by Plastino and Oliveira (2002) based on critical comparison with several putative species. Ursi, Pedersen, Plastino, and Snoeijs (2003) studied photosynthesis, respiration and its photoprotective carotenoids. Pigment characterization and growth of a rare strain of *G. birdiae* was reported by Plastino et al. (2004). The species is found along the Brazilian coast from Ceará State to Espirito Santo State (Plastino & Oliveira, 2002). It has been reported that *G. birdiae* cultivated under field conditions in Rio Grande do Norte State (Brazil) over a 6-month period produced biomass ranging from 900 to 3537 g/m^2 (Marinho-Soriano, Moreira, & Carneiro, 2006). To the best of our knowledge, this is the first publication on the chemical characterization of the polysaccharide present in this seaweed.

Polysaccharides from the *Gracilaria* genus are composed mainly of the alternating 3-linked-β-D-galactopyranose unit (G) and the 4-linked-3,6-anhydro-α-L-galactopyranose unit (LA) (Fig. 1). The G unit can be substituted by either a methyl or a sulfate ester groups (Andriamanantoanina, Chambat, & Rinaudo, 2007; Freile-Pelegrín & Murano, 2005; Lahaye & Yaphe, 1988; Melo, Feitosa, Freitas, & de Paula, 2002; Mazumder et al., 2002; Valiente, Fernandez, Perez, Marquina, & Velez, 1992). Sulfate groups can also be found in the α-L-galactopyranose unit (L), the biogenic precursor of the 3,6-anhydro-α-L-galactopyranose unit (LA) (Rees, 1961).

* Corresponding author. Tel.: +55 85 33669973; fax: +55 85 33669978.
E-mail address: rpaula@dqoi.ufc.br (R.C.M. de Paula).

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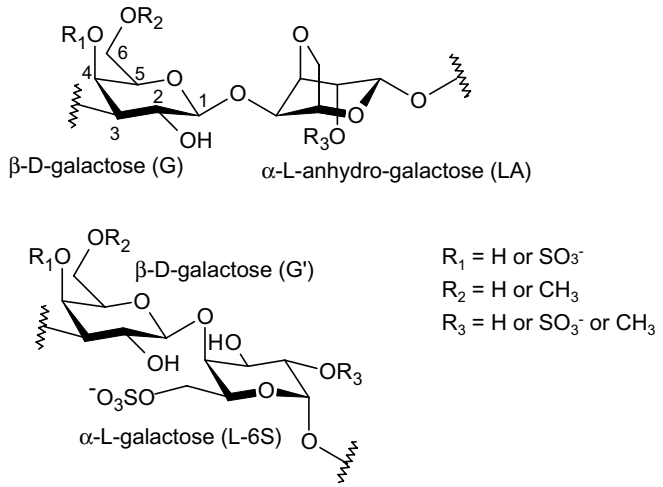

Fig. 1. Structure of the galactan from species of marine red algae.

Despite the low number of scientific studies on *G. birdiae*, it has been cultivated since 2001 along the Ceará coast by a project involving the Federal University of Ceará, University of Fortaleza, and an NGO named the Terramar Institute. The latter is a type of Agribusiness with the participation of local productive cooperations. The alga is cultivated and commercialized by coastal natives, as a way of promoting the social inclusion of people in a situation of poverty. The purpose of this study is the isolation and structural characterization of the water soluble fraction of polysaccharide taken from *G. birdiae* cultivated on the Atlantic coast of Brazil (Fleixeira Beach, Ceará State).

2. Experimental

2.1. Isolation of the soluble polysaccharide

Specimens of the red seaweed *G. birdiae* were collected in September 2006 on the Atlantic coast of Brazil (Fleixeira Beach, Trairi – Ceará). They were cultivated in the sea using seedlings collected during low tide. The seedlings were cleaned and then tied in a structure made of string, which was placed in the sea, where it was anchored and submersed for two months.

After collection, the algae were cleaned of epiphytes, washed with distilled water and stored at −20 °C. The dried tissue (5 g) was dissolved in distilled water (335 ml) and kept under stirring for 15 h at room temperature (25–28 °C). The water soluble fraction was separated from the insoluble fraction by filtration and centrifugation. The supernatant was precipitated with ethanol (1:3 v/v). The precipitate was re-dissolved in distilled water, dialyzed, lyophilized and weighed (0.32 g).

2.2. Composition

Nitrogen, carbon and sulfate content were determined by elemental microanalysis (Perkin-Elmer CHN 2400). Protein content was calculated from %N using the correction factor of 6.25, as proposed by Marks, Baum, and Swain (1985). Moisture was obtained by heating 0.5 g of samples at 105 °C for 24 h.

2.3. Infrared spectroscopy

The Fourier transform IR spectra (FT-IR) were recorded with a Shimadzu IR spectrophotomer (model 8300) between 400 cm^{-1} and 4000 cm^{-1}. The samples were analyzed as KBr pellets.

2.4. Nuclear magnetic resonance (NMR) spectroscopy

^{13}C and ^{1}H NMR spectra of 2.5% w/v solutions in D_2O were recorded at 353 K on a Fourier transform Bruker Avance DRX 500 spectrometer with an inverse multinuclear gradient probe-head equipped with z-shielded gradient coils, and with Silicon Graphics. Sodium 2,2-dimethylsilapentane-5-sulfonate(DSS) was used as the internal standard (0.00 ppm for ^{1}H). A distortionless enhancement by polarization transfer (DEPT 135) spectrum was recorded in order to determine the hydrogenation of each carbon; the acquisition and delay times were 1.0 s. 2D ^{1}H, ^{1}H COSY, TOCSY, ^{1}H, ^{13}C HMQC and 1D selective TOCSY spectra were carried out using the pulse programs supplied with the apparatus.

3. Results and discussion

The soluble polysaccharide of Brazilian *G. birdiae*, obtained by cold extraction is a non gelling polysaccharide and accounts for 6.5% of the seaweed dry weight. This yield is much lower than the value for *Gracilaria cornea* from Brazil obtained also by cold extraction (21.4%; Melo et al., 2002). The low yield might be due to the low extraction temperature. Table 1 shows analytical data for *G. birdiae* polysaccharide. The nitrogen content of sulfated polysaccharide (1.22%) was higher than that reported for *G. cornea* (0.41–0.47%) (Melo et al., 2002), but lower than that obtained for *Gracilaria dura* (2.91%, Marinho-Soriano & Bourret, 2005). Considering the N% the protein content was 7.6%.

Based on Melo et al. (2002), an approximate DS sulfate was calculated by %S and %C according to Eq. (1). The proposed equation is based on the agarobiose structure (Fig. 1), considering that DS sulfate is defined by the num-

Table 1
Analytical data for *G. birdiae* polysaccharide

Analytical data	Content
Moisture (%)	11.4
N (%)[a]	1.22
S (%)[a]	2.00
C (%)[a]	40.6
Protein (%)[a]	7.6
DS sulfate[b]	0.22

[a] In dried weight.
[b] From Eq. (1).

ber of OSO_3^-, or sulfur atoms, per disaccharide repeat unit, which possess 12 carbon atoms.

$$DS = (\%S/\text{atomic mass of S})/(\%C/\text{atomic mass of C} \times 12) = 4.5(\%S/\%C) \quad (1)$$

The DS sulfate for *G. birdiae* was 0.22. The same value was observed for soluble polysaccharide from the Brazilian *G. cornea* (Melo et al., 2002). Table 2 shows the sulfate content, expressed as $NaSO_3$ (% dry weight), in agar from several *Gracilaria* species. The *G. birdiae* polysaccharide from Brazil has a sulfate content (6.4%) in the range observed for polysaccharides from other *Gracilaria* species (2.3–8.9%).

The FT-IR spectrum of *G. birdiae* from Brazil is depicted in Fig. 2. Bands characteristic of agarocolloids were obtained for *G. birdiae* (1375, 1258, 1076, 933, 890, 775 cm^{-1}). The bands at 1258 and 933 cm^{-1} can be attributed to the S=O vibration of the sulfate groups and the C—O—C of 3,6-anhydro-α-L-galp, respectively. The bands at 1150 and 770 cm^{-1} cannot be assigned, as in the case of those reported by Mollet, Rahaoui, and Lemoine (1998). The region around 800–850 cm^{-1} is used to infer the position of the sulfate group in agarocolloids. The bands at 845, 830 and 820 cm^{-1} are assigned to the 4-sulfate, 2-sulfate and 6-sulfate of D-galactose units, respectively (Chopin & Whalen, 1993; Lahaye & Yaphe, 1988; Mollet et al., 1998; Prado-Fernandez, Rodriguez-Vazquez, Tojo, & Andrade, 2003; Rochas, Lahaye, & Yaphe, 1986). The FT-IR spectrum of *G. birdiae* shows a low intensity band, at 850 cm^{-1}, attributed to the sulfate substitution at the C-4 of galactose. The presence of an almost imperceptible shoulder close to 820 cm^{-1} may suggest a small degree of substitution on C-6. The absence of bands at 805 cm^{-1} indicates that 2-sulfate galactose, and the sulfate on the C-2 of 3,6-anhydro-α-L-galactose were not present.

1D and 2D NMR analyses were employed to investigate the Brazilian *G. birdiae* polysaccharide structure. The ^1H and ^{13}C NMR spectra are shown in Fig. 3. The ^1H NMR spectrum is somewhat complex (Fig. 3a). The signals from the α anomeric proton at δ 5.13 and 5.28 were assigned to 3,6 α-L-anhydrogalactose (LA) and α-L-galactose-6-sulfate (L-6S), respectively. The H-1 of β-D-galactose (G′) was linked to α-L-galactose 6-sulfate and that of β-D-galactose (G) was linked to 3,6 α-L-anhydrogalactose, at δ 4.43 and 4.54, respectively.

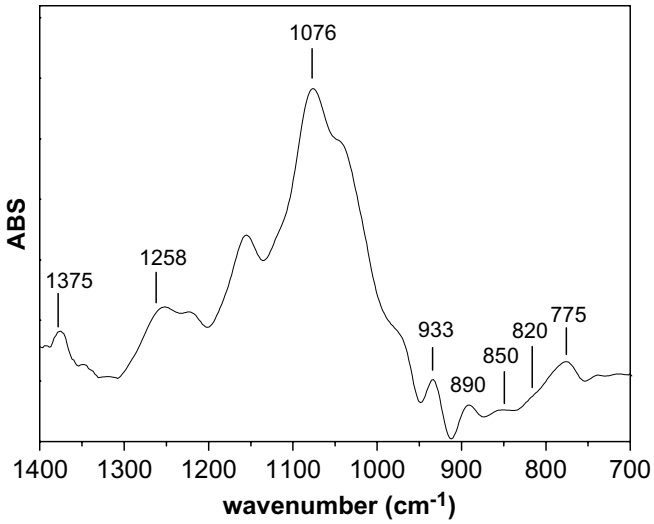

Fig. 2. FT-IR spectra in KBr pellets of *G. birdiae* polysaccharide.

The anomeric region of ^{13}C NMR (δ 90–110) shows (Fig. 3b) four main signals, which were assigned based on the literature data (citebib7; Usov, Yarotsky, & Shashkov, 1997; Usov et al., 1980; Valiente et al., 1992) as C-1 of β-D-galactose linked to α-L-galactose 6-sulfate at δ 103.7; C-1 of β-D-galactose linked to 3,6 α-L-anhydrogalactose at δ 102.6; C-1 of α-L-galactopyranose 6-sulfate unit at δ 101.3; and C-1 of 3,6-anhydro-α-L-galactopyranose at δ 98.5. A DEPT 135° experiment was used to investigate the presence of oxymethylene groups, considering that the pulse sequence signals of the carbons bearing two protons have opposite amplitude to the CH and CH_3 carbons. The DEPT 135° spectrum of *G. birdiae* (Fig. 4) shows four CH_2 signals at δ 69.6, 67.7, 61.9 and 61.6 attributed to LA, L-6S, G′ and G residues, respectively. No evidence of a O—CH_3 signal (~δ 59) was observed in this spectrum, indicating that a significant amount of *O*-methyl sugar residue was not present in this polysaccharide.

The ratio between the signal areas of L and D units can be calculated using Eq. (2), as follows:

$$R_{L/D} = A_{102.6} + A_{103.7}/A_{101.3} + A_{98.5} \quad (2)$$

Table 2
Comparison of native agars from different *Gracilaria* species

Polysaccharide source	Gal/anhydrogal molar ratio[a]	L/D molar ratio	$NaSO_3$ (% w/w)	References
G. tikvahiae	1.41	0.83[b]	4.3	Craigie et al. (1984)
G. sjoestedtii	1.15	1.15	2.3	Craigie et al. (1984)
G. textorii	0.94	0.94	13.9	Craigie et al. (1984)
G. domingensis	1.69	nd	7.6	Valiente et al. (1992)
G. mammillaris	1.27	nd	8.9	Valiente et al. (1992)
G. cornea	2.65	1.83	4.8	Melo et al. (2002)
G. birdiae	1.47	1.01	6.4	This study

[a] Gal, galactose; anhydrogal, anhydro galactose.
[b] Including 4-*O*-methyl-L-galactose.

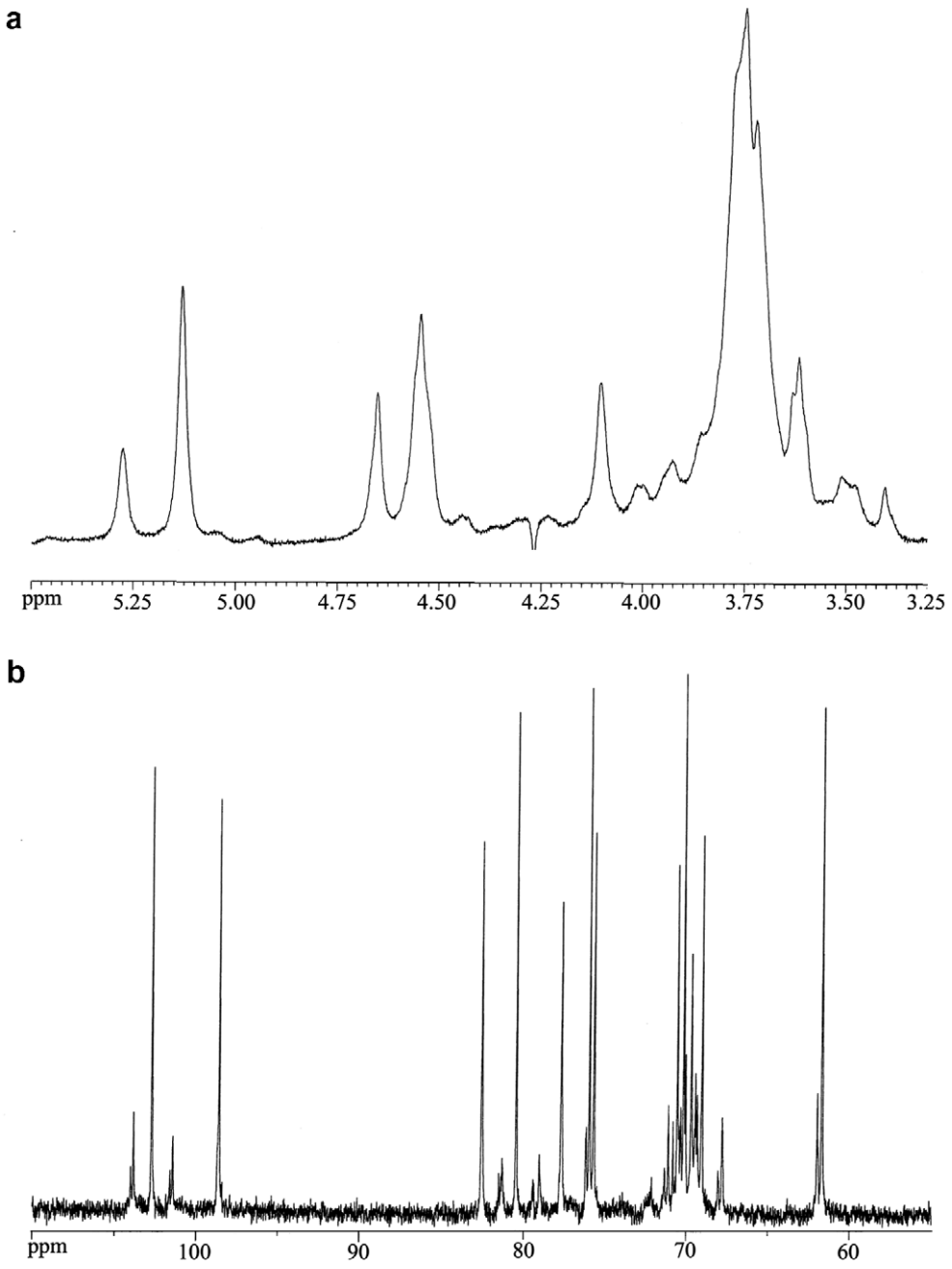

Fig. 3. NMR spectra of *G. birdiae* polysaccharide in D_2O. (a) ^{1}H NMR spectrum; (b) ^{13}C NMR spectrum.

The ratio of L units (3,6 α-L-anhydrogalactose (LA) and α-L-galactose 6-sulfate (L-6S)) to β-D-galactose for *G. birdiae* is 1.01. This value is very close to the ideal agar polysaccharide ratio, as observed for *Gracilaria verrucosa* polysaccharide by Craigie, Wen, and van der Meer (1984) (Table 2). The polysaccharide composition can also be estimated from C-1 signal integrals. Therefore, the *G. birdiae* polysaccharide is composed of 9.2% of the a-L-galactopyranose-6 sulfate unit (L-6S), the biogenic precursor of 3,6-anhydro-a-L-galactopyranose unit; 40.5% of the 3,6-anhydro-a-L-galactopyranose unit (LA); and 50.3% of the b-D-galactose. A low value for the LA units was observed for *G. birdiae* polysaccharide (gal/anhydrogal molar ratio = 1.47) in comparison with other *Gracilaria* polysaccharides (Table 2).

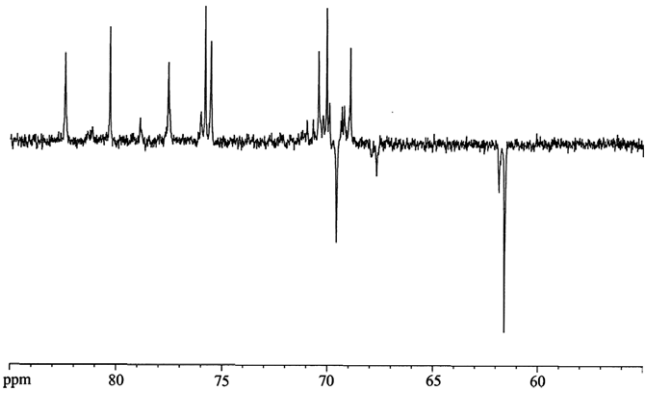

Fig. 4. DEPT spectrum of *G. birdiae* polysaccharide.

However, the gal/anhydrogal molar ratio was similar to the observed by Craigie et al. (1984) for *Gracilaria tikvahiae*.

In order to assign the spin system for the *G. birdiae* polysaccharide, the 2D NMR technique was used. The values obtained are given in Table 3. The HMQC spectrum shows the correlation of the anomeric carbons with their respective protons (Fig. 5) δ 103.7/4.43 for G′ residue; δ 102.6/4.54 for G residue; δ 101.3/5.28 for L-6S residue and δ 98.5/5.13 for LA residue. The four C-6 atoms at opposite amplitudes in the DEPT spectrum exhibited correlations with their protons at δ 69.60/4.10; 67.7/4.30; 61.6/3.76 and 61.9/3.51, respectively, for LA, (L-6S), G and G′. A very low correlation for carbon at δ 59.1/3.40 is observed in the HMQC spectrum, which suggests the presence of trace amounts of methoxyl carbon in the galactose residue.

The 2D COSY was used to determine the proton resonances sequence (Fig. 6). The four H-1 anomeric protons are coupled with the respective H-2 resonances H-1LA/H2LA at δ 5.13/4.09; H-1L-6S/H-2L-6S at δ 5.29/3.85; H-1G′/H-2G′ at δ 4.43/3.72 and H-1G/H-2G at δ 4.54/3.62. The H-2 protons assigned using the COSY spectrum show a correlation on the HMQC spectrum with the C-2 atom (C-2 LA δ 70.4; C-2 (L-6S) δ 69.9; C-2G′ δ 70.8

Table 3
^{1}H and ^{13}C NMR chemical shifts for residues of *G. birdiae* polysaccharide

Residue	^{1}H chemical shift (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6
β-D-galactose (G)	4.54	3.62	3.75	4.12	3.72	3.76
3,6 α-L-anhydrogal (LA)	5.13	4.09	4.53	4.64	4.55	4.10
α-L-galactose-6 sulfate (L-6S)	5.28	3.85	3.94	nd	nd	4.30
β-D-galactose (G′) linked to L-6S units	4.43	3.72	nd	nd	nd	3.51
	^{13}C chemical shift (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
β-D-galactose (G)	102.6	70.5	82.4	68.9	75.5	61.6
3,6 α-L-anhydrogal (LA)	98.5	70.0	80.5	77.7	75.9	69.6
α-L-galactose-6 sulfate (L-6S)	101.3	69.9	69.3	78.9	71.0	67.7
β-D-galactose (G′) linked to L-6S units	103.7	70.8	80.3	69.2	76.0	61.9

nd, not detected.

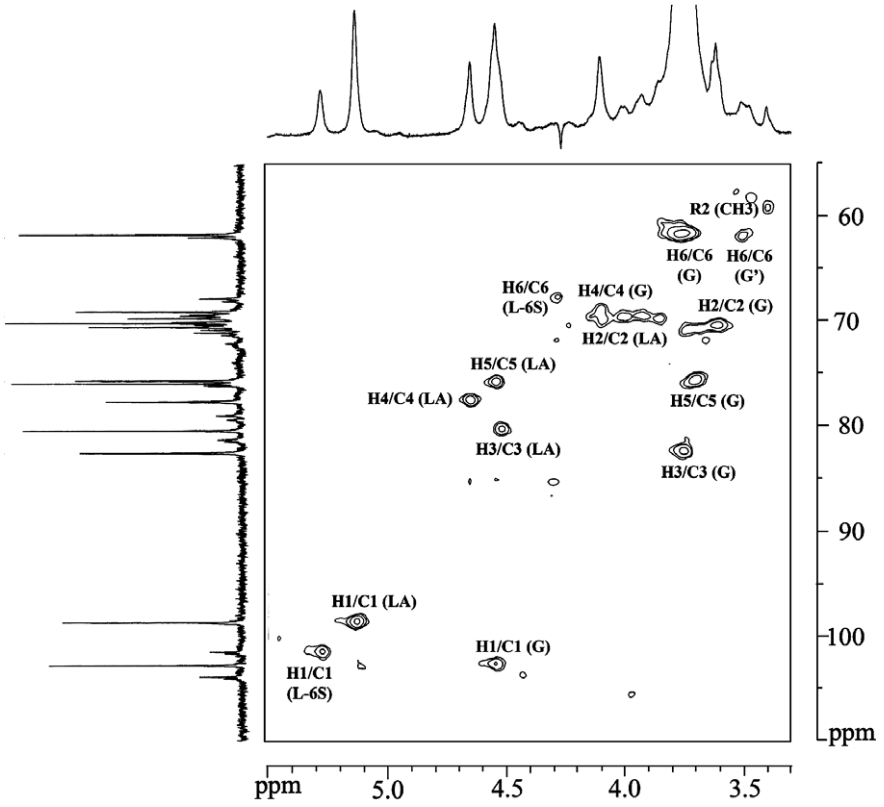

Fig. 5. HMQC spectrum of *G. birdiae* polysaccharide in D_2O.

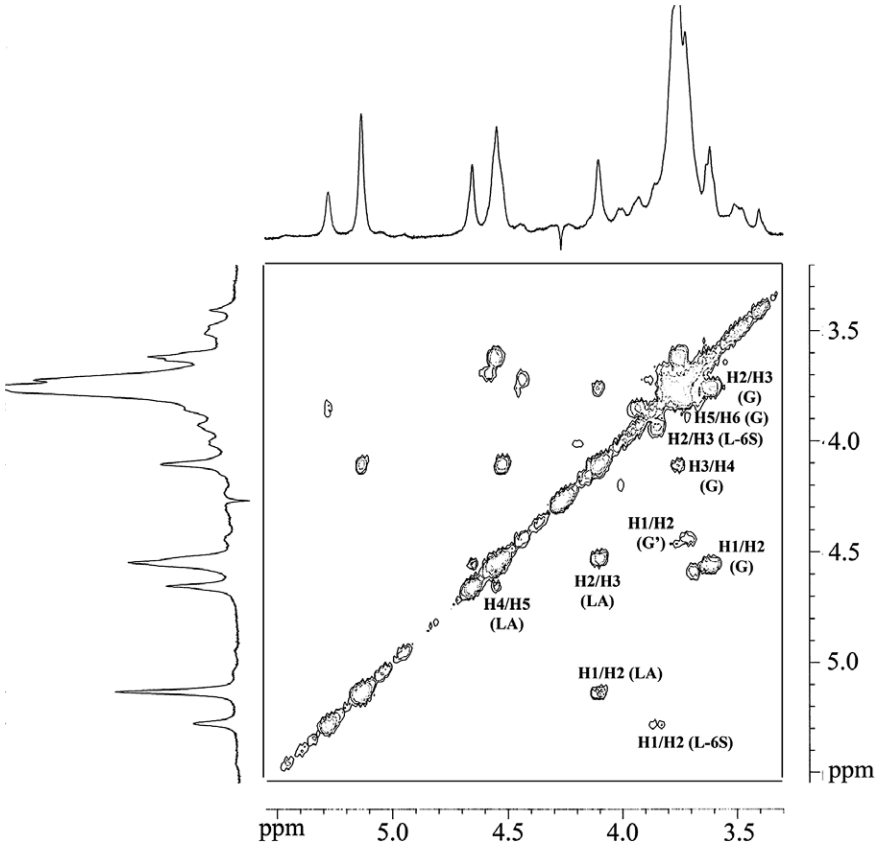

Fig. 6. Cosy spectrum of *G. birdiae* polysaccharide in D_2O.

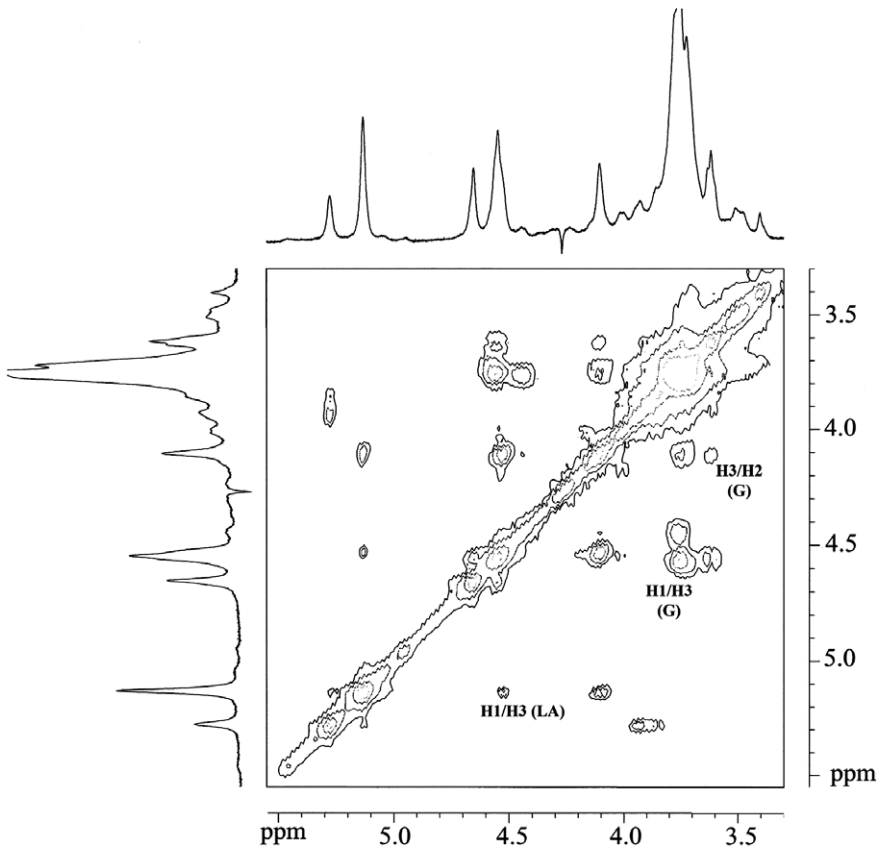

Fig. 7. TOCSY spectrum of *G. birdiae* polysaccharide in D_2O.

and C-2G at δ 70.5). The H-2/H-3 and H-3/C-3 correlations were also identified for the three residues on the COSY and HMQC spectra, respectively: H-2/H-3 of G δ 3.62/3.75 (C-3 δ 82.4); H-2/H-3 of LA at δ 4.09/4.52 (C-3 δ 80.5) and H-2/H-3 of L-6S δ 3.85/3.94 (C-3 δ 69.3). The H-6/H-5 and H-3/H-4 correlations for the G and H-5/H-4 for LA residues were also detected on the COSY spectrum: H-6/H-5 for G δ 3.76/3.72 (C-5 δ 75.5); H-3/H-4 for G δ 3.75/4.12 (C-4 δ 68.9) and H-5/H-4 for LA at δ 4.55/4.64 (C-4 δ 77.7; C-5 δ 75.9).

The TOCSY experiment (Fig. 7) confirms the H-1, H-2 and H-3 assignments for the LA residue (δ 5.13, 4.09 and 4.52, respectively) and for the G residue (δ 4.54, 3.62 and 3.75, respectively). In selective TOCSY experiments (t_{mix} 60 ms) irradiation at ~4.10 δ (4.09–4.12) led to peaks at 5.13 and 4.52 ppm (H-1 and H-3 of LA residue) and also signals at 3.75 and 3.62 ppm (H-3 and H-2 of G residue). This result confirms that a signal at around δ 4.10 is an overlapped signal.

Carbon assignments for G′ and L-6S residues (low intensity signals) were obtained by comparison with the literature data (Lahaye, Yaphe, Viet, & Rochas, 1989; Valiente et al., 1992) as shown in Table 3.

4. Conclusions

Water soluble sulfate polysaccharide from *Gracilaria birdeae* extracted at room temperature (25–28 °C) is composed of β-D-gal*p* (50.3%), 3,6-anhydro-α-L-gal*p* (40.5%) and -α-L-gal*p*-6 sulfate (9.2%). The sulfate content is 6.4%. The ratio of L/D units (β-D-gal*p* units and 3,6-anhydro-α-L-gal*p* + -α-L-gal*p*-6 sulfate) is 1.01, very close to the ideal agarose ratio. The structure is formed of $\rightarrow$4-3,6-anhydro-α-L-gal*p*(1$\rightarrow$3)β-D-gal*p* 1$\rightarrow$ segments, with the possibility of a α-L-gal*p* unit substituted at the 6-position by sulfate ester.

Acknowledgements

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