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Agarans from the red seaweed *Polysiphonia nigrescens* (Rhodomelaceae, Ceramiales)

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Abstract—*Polysiphonia nigrescens* was sequentially extracted with water at room temperature, 70 and 90 °C. The extracts were analyzed and the major one, isolated at 70 °C, was fractionated by ion-exchange chromatography, eluting with water and solutions of increasing sodium chloride concentration; five main fractions were separated. Structural analysis, carried out by methylation analysis and NMR spectroscopy, showed that four of these were partially cyclized agarans that were highly substituted on C-6 mainly with sulfate, although methyl ether and single stubs of β-D-xylose were found in minor proportions. A fifth fraction comprising 6-sulfated agarose was also isolated. The use of 2D NMR techniques allowed us to assign the ^{1}H and ^{13}C NMR resonances of the G6S→L6S diad for the first time.

Keywords: Algal polysaccharides; Sulfated galactans; Agarans; Structural analysis; *Polysiphonia nigrescens*; Rhodomelaceae

1. Introduction

The family Rhodomelaceae comprises a wide range of algae among which the genus *Polysiphonia* is included. The chemical structures of polysaccharides of a few species of this genus have been determined: *Polysiphonia lanosa*,[1] *Polysiphonia morrowii*,[2,3] *Polysiphonia strictissima*,[4] *Polysiphonia abscissoides*[4] and *Polysiphonia atterima*.[5] With the exception of the studies reported for the polysaccharides of *P. lanosa* and *P. morrowii*, the rest were mainly carried out by ^{13}C NMR spectroscopy of the native and alkali-treated polysaccharides. These polysaccharides are sulfated galactans of the agaran type consisting of linear chains of alternating 3-linked β-D-galactopyranosyl and 4-linked α-L-galactopyranosyl units; some of the latter also occur in the 3,6-anhydro form. This regular backbone is usually masked by different O-linked groups, particularly methyl ether, sulfate ester and β-D-xylopyranosyl residues.

This paper describes the characterization of the polysaccharides of *Polysiphonia nigrescens* obtained by sequential extraction of the seaweed with water at different temperatures, the fractionation of the main extract and structural analysis of the major fractions.

2. Results and discussion

2.1. Analysis of the native polysaccharides

The seaweed was sequentially extracted with water at room temperature, 70 and 90 °C. Table 1 depicts the yield and composition of the different extracts obtained at each temperature. The highest total yield (ca. 9%) was obtained after extraction at 70 °C. The monosaccharide composition of all the extracts showed mainly the presence of galactose and 3,6-anhydrogalactose. In addition, minor quantities of 6-*O*-methylgalactose (mol %, 2–6%)

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Table 1. Yields and analysis of the products obtained by extraction with water at room temperature, 70 and 90 °C

Product	Yield[a] (%)	Molecular weight (kDa)	Sulfate (% $NaSO_3$)	Gal:AnGal:sulfate (molar ratio)	Protein %	Monosaccharide composition (mol %)						
						D-Gal	L-Gal	L-AnGal	6-Me L-Gal	3-Me Gal	Xyl	Glc
RT-1	3.6	7.0	25.0	1.0:0.3:0.7	5.5	48	26	19	2	—	3	2
RT-2	1.3	6.0	24.8	1.0:0.3:0.8	10.4	47	23	23	1	—	4	2
RT-3	0.8	6.0	22.0	1.0:0.3:0.7	18.0	48	24	21	—	—	5	2
70-1	3.1	10.6	25.4	1.0:0.6:1.0	5.0	40	15	31	1	—	10	1
70-2	5.0	25.1	25.6	1.0:0.5:1.0	8.5	41	17	31	3	—	7	1
70-2T[b]	69.2[c]	7.6	21.0	1.0:1.0:1.0	3.0	46	—	48	tr[d]	1	4	1
70-3	0.8	19.6	25.8	1.0:0.5:1.4	10.0	37	13	32	6	1	8	3
90-1	2.2.	9.0	25.0	1.0:0.6:1.3	16.5	36	13	30	6	2	11	2
90-2	1.1	7.9	25.1	1.0:0.6:1.3	22.8	40	14	29	5	2	8	2
90-3	0.8	6.1	18.3	1.0:0.4:1.2	36.8	41	16	25	4	2	8	4

[a] Yields are given per 100 g of dry seaweed.
[b] Alkaline treatment of 70-2 afforded 70-2T.
[c] Yield from alkaline treatment.
[d] Percentages lower than 1% are given as traces (tr).

and xylose (3–11%) were detected, together with small quantities of 3-*O*-methyl-/4-*O*-methyl-galactose (1–2%) and glucose (1–4%). Extracts 90-1–90-3 contained considerable amounts of protein (16.5–36.8%) and the highest number-average molecular weights were obtained for 70-2 and 70-3.

The presence of cations in 70-2 was determined by flame atomic absorption spectrophotometry, which showed an important prevalence of divalent counterions in this extract: Ca^{2+}, 0.160 equiv/100 g; Mg^{2+}, 0.044 equiv/100 g; Na^{+}, 0.021 equiv/100 g and K^{+}, 0.003 equiv/100 g. As expected, the sum of cation equivalents (0.228) is similar to the sulfate equivalents (0.248) measured for this sample, indicating that the former are counterions of the sulfate groups.

In the FTIR spectrum of 70-2 signals at 934 and 822 cm^{-1} were found; the former was attributed to the absorption of 3,6-anhydrogalactose while the latter was indicative of sulfate attached to primary hydroxyl groups. The second derivative spectrum in the region 1250–700 cm^{-1} was similar to those reported previously[6,7] for agar-type polysaccharides showing the two diagnostic bands at 787 and 720 cm^{-1}.

The mol % of D- and L-galactose and the absolute configurations of 6-*O*-methyl-D-galactose and 3,6-anhydro-L-galactose confirmed the presence of agaran structures, no evidence of DL-hybrid galactans[8] was found.

2.2. Fractionation of the major extract

The major extract 70-2 was fractionated on DEAE-Sephadex A-25 (Cl^{-}) by eluting with water and aqueous solutions of increasing sodium chloride concentration, identical chromatographic profiles were obtained in the analytical and preparative fractionations. Table 2 shows the yield and analysis of the isolated fractions. From the data in this table, it can be observed that fractionation in the 0–2.5 M NaCl range was based on the sulfate content, which increased with the molarity of the eluent; fractions with similar sulfate percentages were separated, possibly, according to their molecular weight.

When determined by GLC analysis, the 3,6-anhydrogalactose content showed no significant differences for the same elution range; however, the resorcinol–HCl method[9] suggested decreasing molar percentages with increasing sodium chloride concentration.

It is noteworthy that Fw had a significant sulfate content of 16.6%; anion-exchange chromatography of an extract of *Bostrychia montagnei* (Rhodomelaceae) yielded, after elution with water, a fraction which also contained a considerable amount of sulfate (13.0%).[10]

The composition of F2.7 was similar to that of F2.5 but its number-average molecular weight was slightly lower. The elution of this fraction with a higher sodium chloride concentration could be due to the sulfate distribution, with highly charged zones in the polysaccharide backbone. The separation of F4b was achieved by solubilization in boiling 4.0 M NaCl.

2.3. NMR spectroscopy of the fractions

The main fractions Fw, F1.5, F2.0, F2.5 and F4b were analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy. For Fw, F2.0 and F4b, 2D experiments (^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC) were also carried out.

The ^{13}C NMR spectrum of F4b showed in the anomeric region only two peaks, at 102.8 and 98.9 ppm, which together with a strong resonance at 67.9 ppm and the absence of signals in the 62–61 ppm range indicated a 6′-sulfated agarose backbone[2] (Fig. 1). This result is consistent with the solubilization of this fraction in hot aqueous solution.

The ^{13}C NMR spectra of Fw–F2.5 were more complex showing in the anomeric region the resonances of the G6S→DA diad (nomenclature of Knutsen et al.)[11]

Table 2. Yields and analysis of the fractions obtained by ion-exchange chromatography of 70-2 on DEAE Sephadex A-25

Fraction[a]	NaCl M	Yield[b] (%)	Molecular weight (kDa)	Sulfate (% $NaSO_3$)	Gal:AnGal:sulfate (molar ratio)[c]	Monosaccharide composition[d] (mol %)					
						D-Gal	L-Gal	L-AnGal[e]	6-Me D-Gal	Xyl	Glc
Fw	0.0	38.2	5.8	16.6	1.0:0.4:0.8	38	21	24 (24)	9	5	3
F1.5	1.5	13.7	8.7	18.8	1.0:0.4:0.7	37	19	23 (22)	7	12	2
F2.0	2.0	21.0	20.5	27.4	1.0:0.4:1.1	45	22	27 (20)	4	1	tr[f]
F2.5	2.5	9.5	55.4	27.3	1.0:0.4:1.1	48	20	27 (20)	4	tr	tr
F2.7	2.7	4.1	45.4	24.5	1.0:0.4:0.9	46	22	24 (19)	5	tr	2
F4b	4.0	11.2	8.6	22.7	1.0:0.8:1.1	44	8	42	3	3	—

[a] These fractions showed low content of protein (0.5–2.0%).
[b] Yields are given as percentages of the recovered (73.8%).
[c] Gal:AnGal molar ratio calculated from GLC analysis.
[d] 3-Me Gal was detected in fractions F2.0 (1%), F2.5 (1%) and F2.7 (1%).
[e] In parentheses mol % calculated using the resorcinol–HCl method.
[f] Percentages lower than 1% are considered as traces (tr).

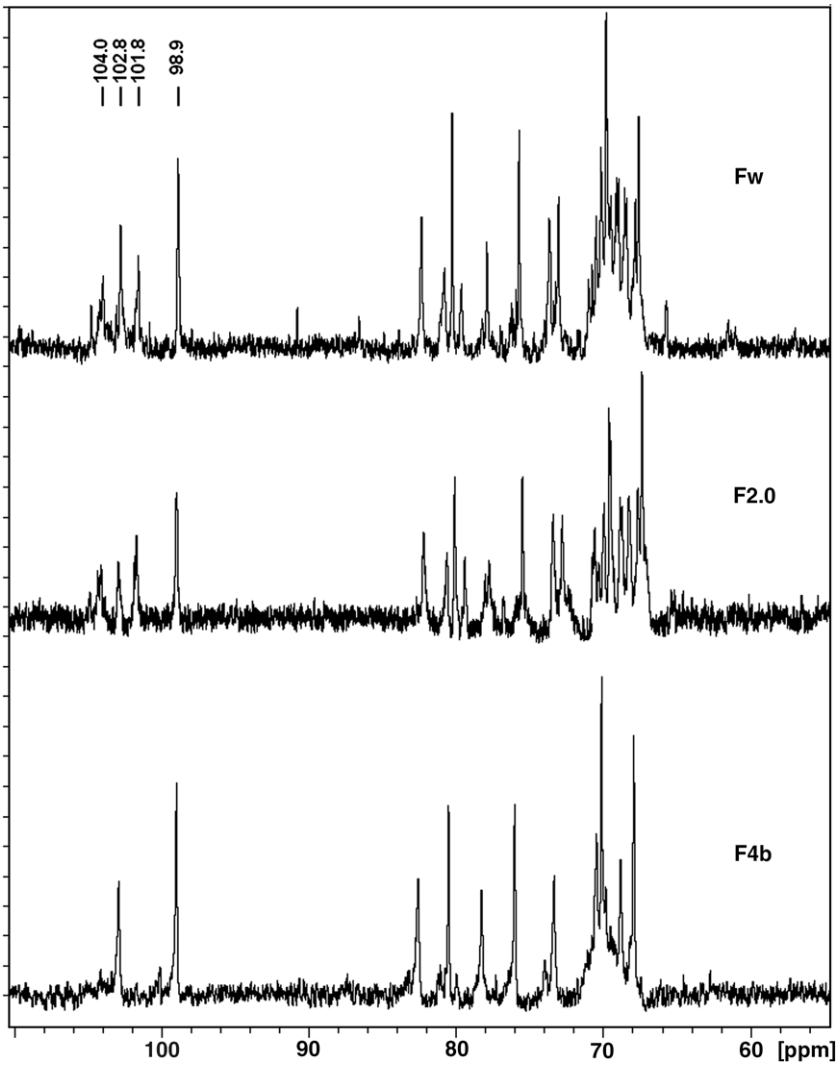

Figure 1. ^{13}C NMR spectra of Fw, F2.0 and F4b. Acquisition parameters are Fw, 90° pulse, acquisition time 1.1 s, relaxation delay 0.1 s; F2.0, 90° pulse, acquisition time 1.1 s, relaxation delay 0.2 s; and F4b, 60° pulse, acquisition time 0.6 s, relaxation delay 0.2 s.

at 102.8 and 98.9 ppm together with signals at 104.0 and 101.8 ppm; in Fw a signal at 90.8 ppm was also detected (Fig. 1).

The resonances at 104.0 and 101.8 ppm were in agreement with the chemical shifts previously reported[2] for the C-1 of G and L6S in G→L6S. However, strong

signals at 67.9 ppm in the spectra of all the fractions together with small signals at 61.7 ppm only in the spectra of Fw and F1.5 suggested almost complete sulfation on C-6 and thus the major presence of the G6S→L6S diad. The signal at 90.8 ppm found in the spectrum of Fw was assigned to the hydrated aldehyde of the reducing end-chain 3,6-anhydrogalactose; additional small resonances at 86.4 and 84.0 ppm, due to the C-4 and C-3 of this residue, confirmed this assignment.[12]

Table 3 gives the assignment of the ^{1}H and ^{13}C NMR spectra, which was carried out using ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC experiments for Fw, F2.0 and F4b; Figure 2 shows the HMQC spectrum of F2.0. This assignment is a representative of that carried out for the spectra of the rest of the fractions. The ^{1}H and ^{13}C chemical shifts of the G6S→LA diad agree with the values informed previously.[2,13] The use of 2D NMR techniques also allowed us to assign the ^{1}H and ^{13}C NMR resonances of the G6S→L6S diad for the first time.

The ^{1}H and ^{13}C NMR resonances of G6S in G6S→L6S are almost coincident with those reported[14] for the same residue in a sulfated xylogalactan from *Corallina pilulifera*, where no L6S was detected; thus, it may be concluded that sulfation on the 6-position of the α-unit does not have important influence in the NMR signals of the G6S residue. The ^{13}C NMR resonances of L6S are in agreement with those of the literature[15] for the α-unit in a porphyran (spectrum determined at room temperature); however, according to our spectral analysis, the C-2 of G and the C-3 of L6S should be reversed as suggested by Usov and co-workers.[14]

In the ^{1}H NMR spectra, the ratio of the areas of the signals at 5.30 ppm (H-1 of L6S) and 5.14 ppm (H-1 of LA) is indicative of the proportion of the major diads G6S→LA and G6S→L6S (Table 4);[16,17] the decrease of LA observed from Fw to F2.5 agrees with the trend obtained in the determination of the content by the resorcinol–HCl method[9] (Table 2). It should be noted that in the spectra of Fw and F1.5, additional small signals were found at chemical shifts higher than 5.00 ppm: at 5.02 and 5.38 ppm for Fw and at 5.38 ppm for F1.5. The former correlated in the HMQC spectrum with a ^{13}C NMR resonance at 90.8 ppm and was assigned to the hydrated aldehyde of the reducing end-chain 3,6-anhydrogalactose; the latter, which correlated with a signal at 101.0 ppm, could be due to the H-1 of α-glucopyranosyl residues in floridean starch,[18] as low contents of glucose were detected in both fractions (Table 2).

Fractions Fw and F1.5 contained minor amounts of xylose (mol %, 5 and 12, respectively). The ^{13}C NMR spectra of these fractions showed small signals at 104.7, 74.2, 76.4, 70.3 and 66.0 ppm, which were assigned to C-1–C-5 of β-D-xylopyranosyl branch units linked to the 6-position of β-D-galactose according to the literature.[19] Xylosyl residues at levels detectable by ^{13}C NMR spectroscopy were previously reported for the agaran of *P. atterima*[5] and *P. strictissima*;[4] moreover, for *P. atterima*, single stubs of β-D-xylose were informed to be attached to the 6-position of β-D-galactose.

Table 3. Assignments of NMR spectra of fractions Fw, F2.0 and F4b

Residue	Chemical shifts (ppm) in ^{1}H NMR spectrum		Chemical shifts (ppm) in ^{13}C NMR spectrum	
G6S→LA (present in Fw, F2.0 and F4b)				
G6S	H-1	4.60	C-1	102.8
	H-2	3.67	C-2	70.4
	H-3	3.81	C-3	82.5
	H-4	4.16	C-4	68.7
	H-5	3.96	C-5	73.3
	H-6a,b	4.18	C-6	67.8
LA	H-1	5.14	C-1	98.9
	H-2	4.12	C-2	69.9
	H-3	4.58	C-3	80.4
	H-4	4.65	C-4	78.2
	H-5	4.55	C-5	75.9
	H-6a	4.04	C-6	69.9
	H-6b	4.12		
G6S→L6S (present in Fw and F2.0)				
G6S	H-1	4.46	C-1	104.0
	H-2,3	3.74	C-2	71.3
			C-3	81.0
	H-4	4.18	C-4	67.8
	H-5	3.97	C-5	73.9
	H-6a,b	4.18	C-6	68.1
L6S	H-1	5.30	C-1	101.8
	H-2	4.19	C-2	69.3
	H-3	4.12	C-3	69.9
	H-4	4.26	C-4	79.8
	H-5	3.67	C-5	70.4
	H-6a,b	4.18	C-6	68.1

2.4. Linkage analysis of the fractions

Methylation analysis of Fw, F1.5, F2.0 and F2.5 confirmed the presence of highly sulfated agaran structures consisting mainly of G6S→L6S and G6S→LA (Table 5). The absence of 2,3,6-tri-*O*-methylgalactose in permethylated Fw and F1.5 indicated that the resonance at 61.7 ppm found in the ^{13}C NMR spectra of the corresponding native fractions should arise from non-substituted 3-linked galactopyranosyl residues. Moreover, methylation analysis of F1.5 gave a higher 2,4,6-tri-*O*-methylgalactose content than that expected from the 6-*O*-methylgalactose molar percentage of the native fraction, thus confirming the presence of G units (Table 2). Non-substituted 4-linked galactose residues were previously found in the agarans of *P. strictissima*[4] and *P. atterima*.[5] A considerable amount 2,3,4-tri-*O*-methylxylose was detected in permethylated Fw and this result is in agreement with the signals observed in the ^{13}C NMR spectrum of this fraction. The percentage of this monosaccharide (3%) was underestimated in permethylated F1.5, considering the percentage of xylose (12%) in the

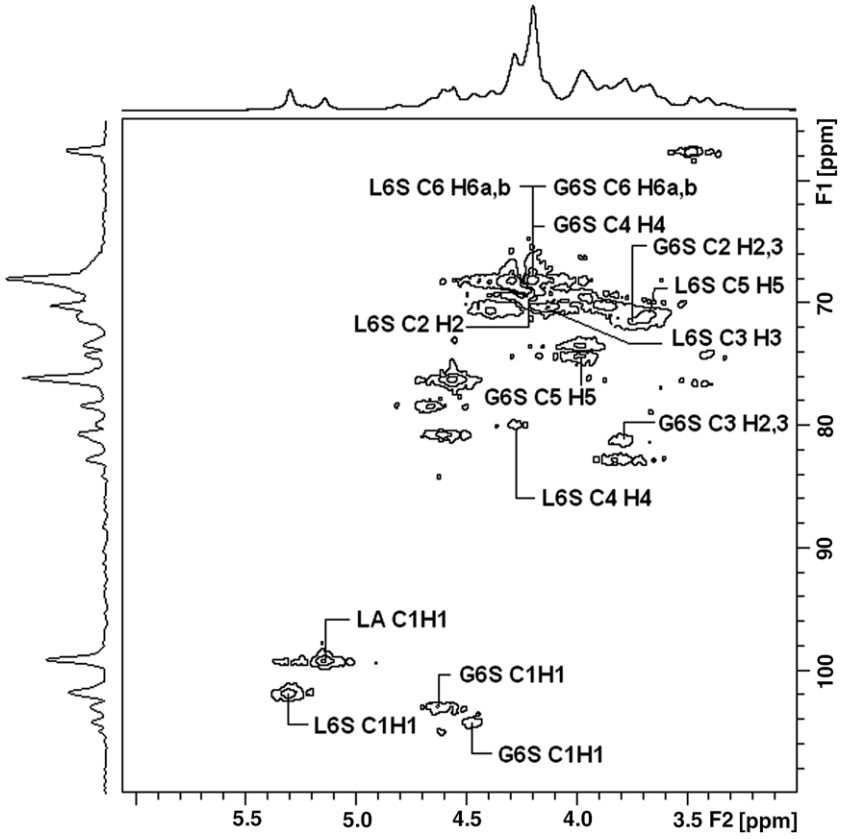

Figure 2. ^{1}H–^{13}C HMQC spectrum of F2.0. Cross-correlations between protons and carbons of the G6S→L6S diad are indicated.

Table 4. Ratio of L6S:LA calculated from the areas of the signals at 5.30 and 5.14 in the ^{1}H NMR spectra

Fraction	Ratio L6S:LA
Fw	1.0:1.1
F1.5	1.0:0.8
F2.0	1.0:0.7
F2.5	1.0:0.7
F4b	1.0:6.3

Table 5. Composition (mol %) of monosaccharides produced by permethylation and hydrolysis of Fw, F1.5, F2.0, F2.5 and F4b[a]

Monosaccharide	Deduced linkage and position of substitution	Fw	F1.5	F2.0	F2.5	F4b
2,3,4-Me_3 Xyl	Single stubs	9	3	1	2	1
2-Me AnGal	LA	21	20	23	23	39
2,4,6-Me_3 Gal	G6M + G	10	15	5	9	6
AnGal	LA2S	3	3	4	4	3
2,3-Me_2 Gal	L6S	25	30	27	27	12
2,4-Me_2 Gal	G6S	32	29	40	35	38

[a] The molar percentages of 2-Me AnGal + AnGal are normalized according to the GLC values shown in Table 2.

native fraction. This discrepancy has been observed before and attributed to the volatility of the acetylated 2,3,4-tri-*O*-methylxylitol that is lost, in part, during the sample workup.[20,21]

Methylation analysis of F4b indicated, in agreement with NMR spectroscopy results, that the main diad was G6S→LA. In addition, in all the methylated fractions small molar percentages of 3,6-anhydrogalactose (3–4%) were detected. Sulfation on the 2-position of 3,6-anhydro-L-galactose has been reported[22,23] for the agarans of *B. montagnei* and for the DL-hybrid galactans of *Cryptonemia crenulata*.

2.5. Alkaline treatment of the extract 70-2

When 70-2 was submitted to an analytical alkaline treatment, it gave a modified polysaccharide with galactose:3.6-anhydrogalactose molar ratio of 1.0:1.0. The preparative alkaline treatment of this extract led to a modified polysaccharide with a galactose:3,6-anhydrogalactose:sulfate molar ratio of 1.0:1.0:1.0 and the ^{13}C NMR spectrum (not shown) was similar to that of F4b and thus consistent with the structure of 6′-sulfated agarose. Thus, the alkaline treatment confirmed the general structure previously proposed by NMR spectroscopy, that is, a partially cyclized 6-sulfated porphyran backbone.

2.6. Characteristics of extracts obtained at room temperature and 90 °C

The major extract obtained at room temperature RT-1 and at 90 °C, 90-1 was also analyzed by ^{13}C NMR

spectroscopy; the first was determined at room temperature but the second was run at 70 °C due to the viscosity of the sample. In both the spectra (not shown) G6S→LA and G6S→L6S were present as the major diads. In RT-1 a small signal was detected at 61.7 ppm and methylation analysis confirmed that some of the 3-linked units were non-substituted. The fact that 90-1 gave more viscous solutions could be due to the presence of major blocks with a 6′-sulfated agarose-like structure, which would also be responsible for the greater association of this polysaccharide extract to the cell wall.

3. Conclusion

P. nigrescens biosynthesizes partially sulfated cyclized agarans highly substituted on C-6, mainly with sulfate, although methyl ether and single stubs of β-D-xylose were also found. Important amounts of precursor units are present in all the extracts. However, fractionation of 70-2 led to the isolation of a fraction composed by almost pure sulfated agarose.

Dried biomass of *P. lanosa* has been studied in terms of Cu(II) biosorption performance.[24] The high level of sulfation of the polysaccharides from *P. nigrescens* suggests that this seaweed could constitute an important biomass for removing heavy metals from aqueous environments.

The substitution pattern of the polysaccharides of the different species of this genus, studied until now, is very similar, in spite of the fact that agarans of the Rhodomelaceae show a great variety of structures,[25] differing in the degree and position of sulfation and methylation of their chain.

4. Experimental

4.1. Materials

Native population of *P. nigrescens* was collected in Cabo Corrientes (38° 03′ S, 57° 31′ W, Mar del Plata, Provincia de Buenos Aires, Argentina) in March of 2000, dried in the open under strong winds and carefully hand sorted and identified according to the literature.[26] A voucher specimen (BAc 46.664) was deposited in the herbarium of the Museo de Ciencias Naturales Bernardino Rivadavia (Buenos Aires, Argentina).

4.2. Extractions

The dry seaweed (42 g), previously milled, was extracted with water (2.0 L) with mechanical stirring for 4 h at rt. The residue was removed by centrifugation and the supernatant was dialyzed (see Section 4.5), concentrated and freeze-dried (RT-1). The residue was extracted (×2) in the same way to afford RT-2 and RT-3. Further extractions were carried out with water at 70 °C (×3) (70-1–70-3) and with water at 90 °C (×3) (90-1–90-3).

4.3. Ion-exchange chromatography

An analytical column (1.0 × 15 cm) was filled with DEAE Sephadex A-25 (Cl^-), which had been previously swollen in water for 2 h in a boiling water bath. 70-2 (30 mg) was dissolved in water (3 mL), which was used as the first eluant; then increasing concentrations of NaCl were applied. Fractions of 3 mL were collected and aliquots were assayed by the phenol–H_2SO_4 method,[27] using galactose solution as reference for the carbohydrate content. After obtaining blank readings, the eluent was replaced by another with higher NaCl concentration. The upper concentration was 4 M. Then the stationary phase was removed from the column and boiled in 4 M NaCl (30 mL) for 15 min, and centrifuged. This procedure was repeated ×3, the solutions were pooled and assayed by the phenol–H_2SO_4 method.

For the preparative fractionation, a column (1.5 × 45 cm) was used with 70-2 (600 mg) in water (60 mL); the flow rate was 1 mL/min. Fractions of 10 mL were collected and assayed as mentioned above. The polysaccharide solutions were dialyzed, concentrated and freeze-dried.

4.4. Alkaline treatment

The one-pot alkaline treatment technique described by Navarro and Stortz[28] was carried out. The sample 70-2 (1 mg) was dissolved in water (0.2 mL) and $NaBH_4$ (1 mg) was added. After 1 h, 3 M NaOH (0.1 mL) was added and the resulting solution was heated at 80 °C in a water bath; this procedure was performed simultaneously in four vials. Vials were removed from the bath after 60, 120, 180 and 240 min and the solutions were neutralized with 3 M CF_3CO_2H (0.12 mL). The solvent was evaporated and the residue was derivatized to the acetylated alditols. The samples were analyzed by GLC at 220 °C as reported below and compared with a control sample, which was left at 4 °C after adding 3 M NaOH. Constant 3,6-anhydrogalactose content was observed after 1 h. For the preparative treatment, 70-2 (118 mg) was dissolved in water (50 mL) and $NaBH_4$ (5 mg) added. After 16 h at rt, 3 M NaOH was added (30 mL) with a further quantity of $NaBH_4$ (3 mg). The solution was heated at 80 °C for 1 h. The solution was cooled at room temperature, dialyzed, concentrated and freeze-dried. Yield of 70-2T, 82 mg.

4.5. General methods

Carbohydrate content was analyzed by the phenol–H_2SO_4 method[27] without previous hydrolysis of the

polysaccharide using galactose as the standard. The presence of 3,6-anhydrogalactose was determined independently by the resorcinol–HCl method[9] with fructose as the standard. Sulfate was measured using the turbidimetric method of Dodgson and Price[29] after hydrolysis of the samples with 1 M HCl for 4–5 h at 105–110 °C, or by ion chromatography with conductimetric detection. For the latter, samples were hydrolyzed in 2 M CF_3CO_2H at 121 °C for 2 h, evaporated to dryness under nitrogen and redissolved in high purity water from a Milli-Q system. A DIONEX DX-100 ion chromatography system was used with an AS4A column (4 × 250 mm), an AMMS-II micromembrane suppressor and a conductivity detector (eluent: 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$, flow rate: 2 mL/min). Protein was estimated by the method published in the literature.[30] Number-average molecular weight was determined using the colorimetric method of Park and Johnson,[31] which measures the content of reducing end-chain residues based upon the reduction of ferricyanide ions in alkaline solution followed by the formation of Prussian blue (ferric ferrocyanide).

Unless otherwise stated, dialyses were carried out with tubing with a molecular weight cutoff of 6000–8000 Da.

Ca^{2+}, Mg^{2+}, Na^+ and K^+ determinations of F70-2 were carried out by flame atomic absorption spectrometry using a Shimadzu AA 6800 atomic absorption spectrometer.

The Fourier-transform infrared spectrum of 70-2 was recorded with a 510P Nicolet FTIR spectrophotometer, using a KBr pellet, at 4000–250 cm^{-1}; 32–64 scans were taken with a resolution of 2–4 cm^{-1}.

Reductive hydrolysis of the native and permethylated samples, and acetylation of the sugar mixtures was performed as described in the literature.[32]

GLC of alditol acetates was carried out on a Hewlett–Packard 5890A gas chromatograph equipped with a flame-ionization detector and fitted with a fused-silica column (0.25 mm i.d × 30 m) WCOT-coated with 0.20 μm film of SP-2330. Chromatography was carried out at (a) 220 °C isothermally for alditol acetates; (b) from 180 °C (2-min hold) to 230 °C at 1 °C/min, followed by a 30-min hold, for partially methylated alditol acetates. Nitrogen was used as a carrier at a flow rate of 1 mL/min. The split ratio was 75:1. The injector and detector temperature was 240 °C.

Conversion of GLC areas to molar basis was calculated for the partially methylated alditol acetates according to the effective carbon response theory.[33] For 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrolgalactitol and 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol values of 0.72 and 0.64, respectively, were used.[32]

GLC-MS of the methylated alditol acetates was carried out on a GCMS-QP 5050A gas chromatograph/mass spectrometer (Shimadzu Corporation). Chromatography was performed on the SP-2330 capillary column using the programme temperature (b). The He total flow rate was 4.4 mL/min, the head column pressure 12 psi, the injector temperature 250 °C and the split ratio 10:1. Mass spectra were recorded over a mass range of 30–600 Da, using an ionization potential of 70 eV.

The percentages of D-and L-galactose and the absolute configuration of 6-*O*-methyl-D-galactose in the native and alkali-treated samples were determined by the method of Cases and co-workers.[34]

The absolute configuration of 3,6-anhydrogalactose was determined by the procedure of Navarro and Stortz.[28]

4.6. NMR spectroscopy

Samples (10–30 mg), previously exchanged with deuterium by repeated evaporations in D_2O, were dissolved in D_2O (0.5 mL) and 5 mm tubes were used. Spectra were recorded at rt on a Bruker Avance II 500 MHz spectrometer. For 500-MHz ^1H NMR experiments the parameters were a spectral width of 6.1 kHz, a 90° pulse (7.0 μs), an acquisition time of 2.7 s and a presaturation time for solvent suppression of 7.0 s; for 16–32 scans. For 125 MHz ^{13}C NMR experiments, the parameters were a spectral width 29.8 kHz, a 60/90° pulse (10/15 μs), an acquisition time of 1.1 s and a relaxation delay of 0.1/0.2 s; for 1024–3200 scans. In all the cases, signals were referenced to internal acetone at 2.21 ppm for ^1H NMR and 31.1 ppm for ^{13}C NMR experiments, respectively.

Pulse sequences for ^1H–^1H COSY and ^1H–^{13}C HMQC techniques were supplied by the spectrometer manufacturer; spectra were recorded at rt and were obtained at a base frequency of 500 MHz for ^1H and 125 MHz for ^{13}C.

4.7. Methylation analysis

Fw (20.7 mg), F1.5 (6.3 mg), F2.0 (10.0 mg), F2.5 (11.6 mg) and F4b (10.0 mg) were converted into the corresponding triethylammonium salts[32] and were methylated by the method of Ciucanu and Kerek (NaOH–iodomethane).[35] For F2.0, the procedure was repeated to ensure the permethylation and similar results were obtained in both the methylation steps. The methylated derivatives were recovered by dialysis (molecular weight cutoff 3500 Da) and freeze-drying. Yields: Fw, 15 mg; F1.5, 8.0 mg; F2.0, 10.3 mg; F2.5, 14.4 mg and F4b, 7.0 mg; in the second methylation step, methylated F2.0 (8.0 mg) gave a yield of 7.6 mg.

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