Carbohydrate Polymers 136 (2016) 1370–1378

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Carrageenan and agaran structures from the red seaweed *Gymnogongrus tenuis*

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ARTICLE INFO

Article history:
Received 21 August 2015
Received in revised form
30 September 2015
Accepted 3 October 2015
Available online 22 October 2015

Keywords:
Sulfated galactan
Polysaccharide structure
Carrageenan
Agaran
Red seaweed

ABSTRACT

The galactan system biosynthesized by the red seaweed *Gymnogongrus tenuis* (Phyllophoraceae) is constituted by major amounts of κ/ι-carrageenans, with predominance of ι-structures, which were isolated by extraction with hot water in high yield (~45%). A small amount of non-cyclized carrageenans mostly of the ν-type was also obtained. Besides, 12% of these galactans are agaran structures, which were present in major quantities in the room temperature water extracts, but they were also found in the hot water extract. They are constituted by 3-linked β-D-galactose units partially substituted on C-6 with sulfate or single stubs of β-D-xylose and 4-linked residues that comprise α-L-galactose units partially sulfated or methoxylated on C-3 or sulfated on C-3 and C-6 and 3,6-anhydro-α-L-galactose. Related structural patterns were previously found for agarans synthesized by other carrageenophytes. Results presented here show that these agarans are low molecular weight molecules independent of the carrageenan structures, with strong interactions between them.

1. Introduction

Galactans from red seaweeds have linear chains of alternating 3-linked β-galactopyranosyl residues (A-units) and 4-linked α-galactopyranosyl residues (B-units). The A units always belong to the D-series, whereas the B units include residues of the D- or L-series, many times occurring as 3,6-anhydrogalactopyranosyl moieties (Stortz, Cases, & Cerezo, 1997). They are classified in carrageenans and agarans according to whether the 4-linked residues belong to the D- or L-series, respectively. Consequently, seaweeds that produce each of these galactan types as major polysaccharide component were named carrageenophytes and agarophytes, respectively. Although this classification is still pertinent, it has been shown that carrageenophytes from the Phyllophoraceae, as well as those from other families, synthesize small to considerable amounts of agaran or DL-hybrid galactans (Ciancia & Cerezo, 2010; Craigie & Rivero-Carro, 1992; Estevez, Ciancia, & Cerezo, 2001, 2008; Talarico et al., 2004).

Previous work on several seaweeds of the Phyllophraceae, including *Gymnogongrus torulosus* (as *Anhfeltia torulosa*), showed that they produce major amounts of ι-carrageenans, and smaller quantities of other carrageenans, usually κ-carrageenans and precursor units (Cáceres, Carlucci, Damonte, Matsuhiro, & Zúñiga, 2000; Furneaux & Miller, 1985; Kapraun, Dutcher, Bird, & Capecchi, 1993; McCandless, West, & Guiry, 1982; Usov & Shashkov, 1985).

Later, sulfated galactans of the major hot-water extract obtained from cystocarpic and sterile plants of *G. torulosus* were shown to comprise not only carrageenans, but also DL-hybrid galactans or mixtures of carrageenan and agaran structures (Estevez et al., 2001, 2008).

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http://dx.doi.org/10.1016/j.carbpol.2015.10.007
0144-8617/

The presence of agarans and/or agaran DL-hybrid galactans as minor components of the system of many carrageenophytes seems to be a general phenomenon in red seaweeds (Ciancia & Cerezo, 2010; Stortz & Cerezo, 2000); however, it still remains unclear: (i) if they are separate molecules or DL-hybrids, (ii) the importance and significance of their contribution to the whole cell wall polysaccharides, and (iii) the kind of molecular interactions between these galactan types.

Fractionation of the water-soluble sulfated galactans of both types was obtained in some algae, such as *G. torulosus* and *Kappaphycus alvarezii* (Solieraceae), by sequential extraction with water at room temperature and then at higher temperatures (Estevez, Ciancia, & Cerezo, 2000; Estevez et al., 2001; Estevez, Ciancia, & Cerezo, 2004; Estevez et al., 2008). This different behavior of different sulfated galactan structures was not found for other seaweeds, as *Gigartina skottsbergii* and *Sarcothalia papillosa* (Gigartinaceae, the latter as *Iridaea undulosa*), in which most of the galactans were obtained by extractions at room temperature (Matulewicz, Ciancia, Noseda, & Cerezo, 1989; Stortz & Cerezo, 1993). In these last cases, agaran structures were isolated after elaborate fractionation strategies, comprising alkaline treatment and potassium chloride fractionations (Ciancia, Matulewicz, & Cerezo, 1993; Ciancia, Matulewicz, & Cerezo, 1997; Flores, Cerezo, & Stortz, 2002). These products from the Gigartinaceae were obtained in low yields and they usually had rather low molecular weights.

Investigation about the structures of sulfated galactans from *Gymnogongrus tenuis* was carried out with the aim to determine the interest of this seaweed as an industrial carrageenan source, and whether it was possible to generalize previous results on the galactans from *G. torulosus*.

2. Materials and methods

2.1. Algal sample

Non-fertile plants of *G. tenuis* J. Agardh were collected by hand in March, 2006 in Puerto Maya, Estado Aragua, Venezuela. Taxonomic identification was carried out using keys and taxonomic descriptions (Rios, 1972; Taylor, 1960). A voucher material (VEN 383852) was deposited in the National Herbarium of Venezuela. The samples of *G. tenuis* were washed with seawater and analyzed for epiphytic contaminants.

2.2. Extraction of the polysaccharides

The plants, previously freeze-dried, were milled. The milled material was sequentially extracted: three times with methanol for 72 h at room temperature, twice with H_2O at room temperature and then with hot water.

Briefly, the dried seaweed after methanol extraction (32 g) was treated with water at room temperature (1380 mL), during 16 h, under mechanical stirring; the residue was removed by centrifugation and the supernatant was dialyzed, concentrated and freeze dried to render A1. This procedure was repeated once more giving A2. The residue obtained after exhaustive extraction of the polysaccharides at room temperature was freeze-dried. A portion of it (19 g) was suspended in H_2O (850 mL) and extracted at 90 °C under mechanical stirring for 4–5 h and, after a similar isolation procedure, extract C1 was obtained.

2.3. General methods

The total sugars content was analyzed by phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) without previous hydrolysis of the polysaccharide. Sulfate was determined turbidimetrically (Dodgson & Price, 1962), except in the case of the desulfated sample for which ion chromatography with conductimetric detection was used. The latter sample was 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 (Sunnyvale CA, USA) 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^{-1}). The number average molecular weight (M_n) was estimated by the method of Park and Johnson (1949) based on the determination of end-chain reducing units. The protein content was determined by the method of Lowry, Rosenbrough, Farr, and Randall (1951).

Dialyses were carried with tubing with molecular weight cutoff of 3500 Da.

For GLC, alditol acetates from native and methylated galactans were obtained by reductive hydrolysis and acetylation of the samples according to Stevenson and Furneaux (1991).

GLC of the alditol acetates, as well as those of the partially methylated alditol acetates were carried out on a Hewlett Packard 5890A gas–liquid chromatograph (Avondale PA, USA) equipped with a flame ionization detector and fitted with a fused silica column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.20 μm film of SP-2330 (Supelco, Bellefonte PA, USA). Chromatography was performed: (a) from 200 °C to 230 °C at 1 °C min^{-1}, followed by a 30-min hold for alditol acetates; (b) from 180 °C to 210 °C at 1 °C min^{-1}, then at 2 °C min^{-1} from 210 °C to 230 °C, followed by a 30-min hold for partially methylated alditol acetates arising from methylation analysis. N_2 was used as the carrier gas at a flow rate of 1 mL min^{-1} and the split ratio was 80:1. The injector and detector temperature was 240 °C.

GC–MS of the methylated alditol acetates was performed on a Shimadzu GC-17A gas-liquid chromatograph equipped with the SP-2330 interfaced to a GCMS-QP 5050A mass spectrometer (Kyoto, Japan) working at 70 eV. Chromatography was performed using the programme temperature (b). The He total flow rate was 7 mL min^{-1}, the injector temperature was 240 °C. Mass spectra were recorded over a mass range of 30–500 amu.

The D:L-galactose ratio was estimated by the method of Cases, Cerezo, and Stortz (1995) through their diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols. The 3,6-anhydro-D:L-galactose and 2-*O*-methyl-3,6-anhydro-D:L-galactose ratios of the native and permethylated polysaccharides were estimated by the method of Navarro and Stortz (2003). The D:L-2,6-di-*O*-methylgalactose ratio was determined on the permethylated polysaccharide by conversion of the monosaccharides, obtained by hydrolysis of the sample, to the diastereomeric acetylated 1-deoxy-1-(1-phenylethylamino) alditols (Errea, Kolender, & Matulewicz, 2001).

2.4. Fractionation of C1

C1 (261 mg) was dissolved in water (100 mL) and the solution was centrifuged to give a residue which was freeze-dried (F1). Solid, finely divided KCl was added to the supernatant in small portions with constant and violent mechanical stirring so that the concentration was increased stepwise. After each addition, stirring was continued for 3–5 h to ensure equilibration of the system. The upper limit of KCl concentration was 4.3 M; eight fractions were isolated (F2–F9). The precipitates as well as the residual solution obtained were dialyzed, concentrated and freeze-dried (total yield, 97.4%). After freeze-drying, solubility of the fractions in water diminished, particularly in the case of F4. Although preparation of a 200 μg mL^{-1} solution, suitable for colorimetric determination, was possible, it was impossible to obtain a solution of the sample adequate to carry out NMR spectra (≥10–15 mg mL^{-1}).

Table 1
Yields and analyses of the extracts obtained from *Gymnogongrus tenuis* by extraction with water at room temperature and at 90°.

Extract	Yield[a] (%)	Carbohydrate (%, anhydro)	Sulfate (% $NaSO_3$)	Protein (%)	Monosaccharide composition[b] (mol %)				Gal:AnGal:sulfate (molar ratio)	Molecular weight (kDa)
					D-/L-Gal	D-/L-AnGal	Xyl	Glc		
A1	0.6	53.2	21.5	14.0	44.6/16.0	3.6/15.2	10.0	10.5	1.00:0.31:0.92	9
A2	0.3	52.5	22.2	5.8	46.0/18.4	7.6/11.5	10.3	6.2	1.00:0.30:1.11	17
C1	47.7	48.3	34.4	3.1	55.7/4.3	33.8/0.8	4.1	1.3	1.00:0.58:1.87	104

[a] For 100 g of the residue from the methanolic extraction.
[b] Small amounts of rhamnose, fucose, arabinose, and 3/4-*O*-methylgalactose were found in A1 (3.1, 2.2, 1.3, 5.2, and 1.7%, respectively) and A2 (1.9, tr., 1.3, 1.4, and 2.2, respectively).

2.5. Methylation analysis

The polysaccharide (10 mg) was converted into the corresponding triethylammonium salt (Stevenson & Furneaux, 1991) and methylated according to Ciucanu and Kerek (1984) using finely powdered NaOH as base. The methylated derivatives were recovered by dialysis and freeze-drying; these samples were hydrolyzed and derivatized to the alditol acetates as described before.

2.6. Desulfation of A1

The reaction was carried out by the microwave-assisted method described by Navarro, Flores, and Stortz (2007). The polysaccharide (40 mg) was converted to the pyridinium salt and dissolved in DMSO (10 mL) containing 2% pyridine. The mixture was heated for 10 s intervals and cooled to 50 °C each time (6×). It was dialyzed and freeze-dried (yield ~60%, considering the sulfate loss) and was methylated as described in Section 2.5. Sulfate content (as $NaSO_3$): 3.5%.

2.7. Alkaline treatment of A2 and C1

The sample (50 mg) was dissolved in H_2O (25 mL), and $NaBH_4$ (3 mg) was added. After 24 h at room temperature, 3 M NaOH was added (12.5 mL) with a further quantity of $NaBH_4$ (1.5 mg). The solution was heated at 80 °C for 3 h, and then cooled to room temperature, dialyzed without previous neutralization to eliminate low-molecular weight material, concentrated and freeze dried to give A2tr (28.5 mg). This sample (24.5 mg) was dissolved in H_2O (10 mL) and KCl was added up to a 2.8 M concentration. The solution was centrifuged and the precipitate and supernatant were dialyzed, concentrated and freeze dried, giving A2tr-pp (7 mg) and A2tr-sol (15 mg), respectively.

Analytical alkaline treatment was carried out with C1 (5 mg) in conditions similar to those described above.

2.8. NMR spectroscopy

Samples (10–20 mg), previously exchanged with deuterium by repeated solubilization in D_2O and freeze-drying, were dissolved in D_2O (0.5 mL) and 5 mm tubes were used. Spectra were recorded at room temperature on a Bruker Avance II 500 spectrometer (Karlsruhe, Germany), with the exception of F4. For ^1H NMR experiments the parameters were: a spectral width of 6.25 kHz, a 76° pulse angle, an acquisition time of 3 s, a relaxation delay of 3 s, for 32 scans. For 125 MHz proton decoupled ^{13}C NMR experiments the parameters were: a spectral width of 29.4 kHz, a 51.4° pulse angle, an acquisition time of 0.56 s, a relaxation delay of 0.6 s, for 25,000 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.

NMR experiments for F4 were carried out using a 8.4% LiCl solution in DMSO, which was prepared by heating 15 mg of the sample in 1 mL solution for 4 h at 100 °C.

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

3. Results and discussion

The residue from the methanol extraction of the milled seaweed was sequentially extracted with water at room temperature, giving extracts A1 and A2, with a total yield of 0.9%. The final residue from the room temperature water extraction was further extracted with hot water, rendering extract C1 (47.7%) (Table 1). Chemical analysis showed that galactan sulfates from all the fractions were composed not only by D-galactose but also by L-galactose units, showing the presence of carrageenan-like and agaran-like structures (Table 1). However, composition of both room temperature (A1 and A2) and hot-water extracted galactans (C1) differed in the presence of major quantities of agaran structures (~62 and 60%, respectively) in the former fractions, while C1 contained ~90% of carrageenan diads. In the first cases, diads of G→L and G→LA[3] types were found (32% and 30% in A1, and 37% and 23% in A2, respectively), but in C1 most diads comprised 4-linked units of the carrageenan type, being only 22% of these non-cyclized α-D-galactose units. These differences were confirmed later from structural analysis. Small amounts of rhamnose, fucose, arabinose, and 3/4-*O*-methylgalactose appeared in A1 and A2, arising possibly from minor amounts of contaminating polysaccharides (Table 1). Besides, these galactans differed also in their molecular weights, rather small in the first two cases (Table 1). Taking into account these important differences, results obtained for both groups of galactans will be treated separately.

3.1. Hot water extract C1

Alkaline treatment of C1 gave a product (C1T) with a galactose:3,6-anhydrogalactose molar ratio of 1.00:0.95, showing that most of the 4-linked α-galactose units were 6-sulfated. Methylation analysis of C1 (Table 2) showed major amounts of ι-carrageenans and less quantities of κ-structure. Only small amounts of precursor units were detected by the presence of 3-*O*-methylgalactose between the partially methylated monosaccharide derivatives, in agreement with the high cyclization rates of these units in alkali reported for carragenans of the kappa-family (Ciancia, Noseda, Matulewicz, & Cerezo, 1993). However, in the ^1H NMR spectrum of this extract, anomeric signals of 4-linked units corresponding to κ-, ι-, and ν-structures (δ 5.11, 5.30, and 5.53, respectively) were present in a ratio 0.6:2.8:1.0. These results indicate that C1 is constituted by major amounts of ι-carrageenans and minor quantities of κ- and ν-diads (Fig. 1A). Small signals at δ 5.42, 5.16, and 5.08 were also present (see later for assignment). In agreement with that, minor percentages of other partially methylated derivatives indicated the presence of 3-linked non-substituted and

[3] Nomenclature of Knutsen, Myslabodski, Larsen, and Usov (1994).

Table 2
Composition (mol %) of monosaccharides produced by permethylation and hydrolysis of the galactans from *Gymnogongrus tenuis*.

Monosaccharide	Structural unit[a]	C1	A1[b]	A1D	A2[b]
2,3,4-Me_3 Xyl[c]	Terminal	4.1	9.9	10.0	10.2
2,3,4,6-Me_4 Gal	Terminal	–	1.4	–	2.0
2,4,6-Me_3 Gal	G	1.6	15.2	38.9	15.9
2,3,6-Me_3 Gal	D/L	2.6	2.8	11.4	4.7
2-Me AnGal	DA/LA	6.9	16.3	13.2	14.1
AnGal	DA2S/LA2S	33.3	7.1	2.7	6.5
2,3-Me_2 Gal	D6S/L6S or D6X/L6X	–	2.1	–	3.4
2,6-Me_2 L-Gal[d]	L3S	2.9	9.7	10.4	10.0
2,6-Me_2 D-Gal[d]	G4S	41.6	7.1		7.6
2,4-Me_2 Gal	G6S or G6X	2.1	20.5	7.2	16.4
2-Me Gal	G4,6S or D3,6S/L3,6S	2.3	4.0	–	4.0
3-Me Gal	D2,6S/L2,6S	2.6	1.9	–	2.0
Gal	nd[e]	–	2.0	6.2	3.2

[a] Nomenclature of Knutsen, Myslabodski, Larsen, and Usov (1994).
[b] Small amounts (>2%) of 2-Me Rha, 3,4,6-Me_3 Gal, and Man were detected in A1, while low percentages (>2%) of 2,3,6-Glc and Glc were found in A1 and A2.
[c] The 2,3,4-Me_3 Xyl content was corrected according to Table 1 for losses during derivatization of the methylated sample (Stevenson & Furneaux, 1991).
[d] 2,6-Me_2 Gal and 4,6-Me_2 Gal are not separated in the conditions used in this work, however, no evidence of the presence of 4,6-Gal was found by GC–MS.
[e] nd = not determined.

6-substituted galactose units and 4-linked non-substituted and 3-substituted galactose units. ^{13}C NMR spectrum of this sample was quite noisy, however, it was possible to detect all the signals corresponding to the major carrageenan structures (see below and Table 5).

C1 was fractionated by stepwise precipitation with potassium chloride (Table 3). When this sample was dissolved in water, a small amount of C1 remained insoluble (F1). The major fraction, F4, was obtained by precipitation at 0.6 M KCl. Even though the classical fractionation with potassium chloride is usually carried out up to a 1.5/2 M concentration (Pernas, Smidsrød, Larsen, & Haug, 1967), this is an arbitrary and historic limit used to separate κ- and λ-carrageenans before the discovery that cystocarpic plants from the Gigartinaceae and Phyllophoraceae synthesized carrageenans from the κ-family, while tetrasporophytes produced λ-carrageenans (McCandless, Craigie, & Walter, 1973; McCandless et al., 1982; Pickmere, Parsons, & Bailey, 1973). In this case, addition of this salt until saturation of the aqueous solution was carried out trying to avoid complexation of the different galactan molecules. In this way, precipitation at 2.8 M and 4.3 M (F7 and F8) was obtained, while 15.1% of C1 remained soluble even at 4.3 M KCl (F9). All the fractions contained galactose and 3,6-anhydrogalactose in molar ratios from 1:0.63–0.78 with the exception of F9 in which this ratio was only 1:0.43. The galactose:3,6-anhydrogalactose ratio could not be correlated with the concentration of potassium chloride in which precipitation took place, indicating that other factors like the amount of sulfate, and the diversification of 4-linked units, which inhibit the symmetry of the backbone favoring random coil conformations, could play a role in the solubilization of these galactans in potassium chloride. The amount of sulfate in the different fractions was also variable. However, the high solubility of F9 at high concentrations of potassium chloride could be correlated with the lower amount of 3,6-anhydrogalactose (Table 3). All the fractions in which enantiomeric analysis was performed showed small amounts of L-galactose and 3,6-anhydro-L-galactose. Considering results from Table 3, some interesting facts can be observed: ~82% of the backbone of polysaccharides in the major fraction, F4, corresponds to carrageenans, 68% to cyclized units and 14% to precursor units. On the other hand, although 50% of the polysaccharides in F9 correspond to cyclized and 20% to non-cyclized carrageenan units, in this fraction there is also a high percentage (22%) of non-cyclized agarans.

Methylation analysis of the fractions, except F1, F3, and F8, which were not further analyzed due to their low yield, showed that the scheme of structural units is qualitatively similar to that of the parent extract, indicating that 3-linked β-D-galactose

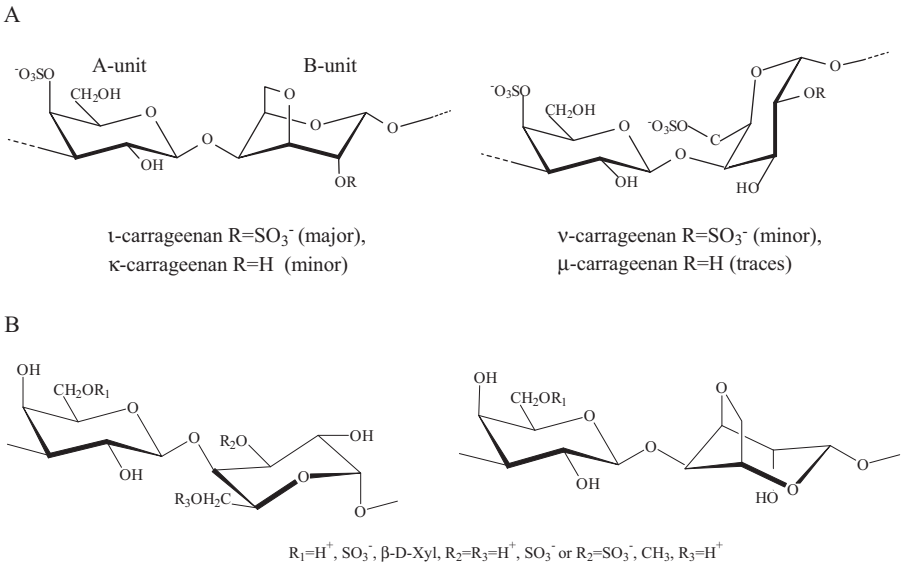

Fig. 1. Major diads of (A) carrageenans and (B) agarans from *Gymnogongrus tenuis*.

Table 3
Fractionation of C1 by precipitation with increasing KCl concentrations.

Fraction	KCl (M)	Yield[a] (%)	Carbohydrate (%, anhydro)	Sulfate (% KSO_3)	Gal:AnGal:sulfate (molar ratio)	Monosaccharide composition (mol %)[b]		
						D-/L-Gal	D-/L-AnGal	Xyl
F1	0	4.4	35.5	17.6	1.00:0.64:1.15	58.8 (51.3/7.5)	33.2 (29.3/3.9)	4.3
F2	0.1	6.6	30.9	21.5	1.00:0.64:1.61	58.5 (51.1/7.4)	33.3	4.6
F3	0.3	3.0	34.9	24.1	1.00:0.76:1.65	57.1	38.5	4.4
F4	0.6	44.5	42.9	24.7	1.00:0.68:1.31	60.2 (55.6/4.6)	36.1 (33.7/2.4)	3.7
F5	1.1	6.2	41.3	41.0	1.00:0.78:2.41	56.1	43.9	tr
F6	1.8	10.4	45.1	28.8	1.00:0.73:1.44	60.4 (58.2/2.2)	39.6 (26.4/12.8)	tr
F7	2.8	6.7	39.9	41.9	1.00:0.76:2.43	59.0	39.7	1.3
F8	4.3	3.3	30.6	24.9	1.00:0.63:1.76	63.2	35.3	1.4
F9	Sol[c]	15.1	28.3	18.9	1.00:0.43:1.41	64.6 (56.3/11.7)	24.5 (25.3/0.5)	6.0

[a] From the total recovered.
[b] Minor quantities of 3/4-Me Gal and Glc (<3%) were detected in F1, F2, and F9.
[c] Soluble in 4.3 M KCl.

4-sulfate and 4-linked 3,6-anhydro-D-galactose 2-sulfate are the major structural units (Table 4). The only other structure detected in considerable amounts is the κ-structure. In addition, all these fractions showed small amounts of 3-linked non-substituted and 6-substituted galactose units and 4-linked non-substituted (none in F7) and 3-substituted galactose units, as found in C1. Enantiomeric analysis showed the presence of L-galactose derivatives in small quantities. For F9, the amount of 2,6-Me_2 L-Gal detected between the partially methylated monosaccharides was more important.

Great efforts were carried out to obtain good spectra of the major fraction, F4, as during the sample workout, insolubilization took place and it was not possible to obtain a suitable solution in water for NMR. This high degree of insolubilization could be due to the previous extraction of the seaweed with methanol. Methanol is a well known dehydrating agent for cell wall preparations for microscopy. However, hot methanol extraction was used by Usov and Shashkov (1985) previous to extraction of carrageenans from *Phyllophora brodiaei* and no indication of a similar behavior was informed.

Moreover, attempts to dissolve F4 in 8.4% LiCl/DMSO solution at room-temperature (Petruš, Gray, & BeMiller, 1995) were unsuccessful. Finally, by heating this mixture at 100 °C for 4 h, a solution was obtained.

As expected, in the ^{13}C NMR spectrum signals corresponding to ι-carrageenans predominated, and those of κ-structure were also present. In addition, peaks of C-1 and C-5 of terminal β-D-xylose linked to C-6 of some galactose units were evident. No reducing end anomeric resonances were detected, confirming that the product was not degraded, in spite of the conditions used to dissolve it. It should be pointed out that signals did not appear exactly at the same displacement values as those found for spectra run using water as solvent (Fig. S1). This effect could be due to the solvent change as well as to interaction of the polysaccharide with Li^+. A similar effect was found comparing published NMR data for a 3-linked α-D-glucan in both solvents (Chen, Zhou, & Zhang, 1998; Synytsya & Novak, 2013).

On the other hand, by dissolution of F9 in water good spectra were obtained. Peaks corresponding to ι-structure were very clear and they were fully assigned (Greer, Rochas, & Yaphe, 1985; Jouanneau, Boulenguer, Mazoyer, & Helbert, 2010; van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002) (Table 5, Fig. S2). In the anomeric region of the HMQC spectrum, the signals corresponding to ν-diad are also very clear at δ 105.1/4.70 and 98.7/5.53, which correspond to A- and B-units of this structure, respectively (Bellion, Brigand, Prome, Welti, & Bociek, 1983; Ciancia, Matulewicz, Finch, & Cerezo, 1993). In addition, only ^{13}C NMR spectroscopic data about ν-carrageenan were reported previously (Ciancia, Matulewicz, et al., 1993, Stortz, Bacon, Cherniak, & Cerezo, 1994). As only small amounts of this structure were present in F9, the HMQC spectrum of 1C3 from *G. skottsbergii* (Ciancia, Matulewicz, et al., 1993), which contained important amounts of ν-carrageenan, was carried out. Although full assignment was still not possible, some further data were obtained (Table S1). These results were useful for further assignment of spectra of the polysaccharides analyzed in this work. Besides, the signals at δ 102.6/4.67 and 95.2/5.11 were assigned to C-1/H-1 of the A- and B-unit of the κ-structure, respectively. Signals from C-2–C-6 of both diads were found in the ^{13}C NMR, but the low amounts present precluded complete assignment in the HMQC spectrum (Ciancia, Matulewicz, et al., 1993; Usov, Yarotskii, & Shashkov, 1980; van de Velde et al., 2002). In the ^{1}H NMR spectrum anomeric signals of 4-linked units corresponding to κ-, ι-, μ-, and ν-structures (δ 5.11, 5.30, 5.24, and 5.53, respectively) are

Table 4
Composition (mol %) of monosaccharides produced by permethylation and hydrolysis of the fractions obtained from C1 of *Gymnogongrus tenuis*.

Monosaccharide	Structural unit[a]	F2	F4	F5	F6	F7	F9
2,3,4-Me_3 Xyl	Terminal	1.3	tr[b]	tr	–	–	1.5
2,4,6-Me_3 Gal	G	2.3	1.9	1.2	2.0	tr	5.6
2,3,6-Me_3 Gal	D/L	2.6	2.5	tr	tr	–	3.0
2-Me AnGal	DA/LA	6.7	6.4	6.6	7.6	7.8	6.5
D-AnGal	DA2S	29.5	28.6	32.0	28.5	32.0	20.8
L-AnGal	LA2S	tr	1.0	tr	4.3		
2,3-Me_2 Gal	D6S/L6S	–	1.1	tr	tr	tr	1.9
2,6- Me_2 D-Gal	G4S	48.2	44.8	49.7	50.9	55.5	39.8
2,6- Me_2 L-Gal	L3S	2.2	2.3	3.3	1.3	2.0	6.4
6-Me Gal	G2,4S or D2,3S/L2,3S	3.5	1.9	1.1	1.0	tr	2.0
2,4-Gal	G6S	1.3	2.5	1.5	1.1	tr	3.0
2-Me Gal	G4,6S or D3,6S/L3,6S	2.4	2.9	1.4	tr	tr	3.1
3-Me Gal	D2,6S/L2,6S	tr	4.1	3.2	3.3	2.7	6.4

[a] S indicates substitution, in most cases, with sulfate (nomenclature according to Knutsen et al., 1994), but also single stubs of β-D-xylose may be present.
[b] Percentages lower than 1% were considered as traces (tr).

Table 5
NMR signal assignments (ppm) of galactans from F9.[a]

Structural unit[b,c]	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H6,6′
ι-diad						
G4S	102.6/4.67	69.5/3.64	77.4/4.04	72.4/4.92	75.3/3.83[1]	61.8/3.82
DA2S	92.5/5.30	75.5/4.71	78.2/4.86	78.8/4.71	77.4/4.69	70.3/4.16, 4.28
ν-diad						
G4S	105.1/4.70	71.5/3.70[2]	80.6	71.4/3.67[2]	75.4/3.77[1]	61.8/3.83
D2,6S	98.7/5.53	77.0/4.85	68.7/4.32[3]	80.3/4.37	68.7/4.20[3]	68.3/4.28, 4.31
κ-diad						
G4S	102.6/4.67	69.6/3.57	79.6	74.3/4.87	75.4	61.8/3.83
DA	95.2/5.11	69.8/4.15	79.6/4.55	78.7/4.55	77.3	70.0/4.10, 4.20

[a] Nomenclature of Knutsen et al. (1994).
[b] For (1), (2), (3), assignments could be interchanged.
[c] A signal at δ 73.5 was assigned to C-4 of the A-unit of a μ-structure, which is present in small quantities, so the other peaks are overlapped with those of other structures. In the anomeric region of the ^{13}C NMR spectrum, small peaks at δ 104.1, 101.4, and 99.6 were attributed to small amounts of agaran structures, while that at δ 100.6/5.54 derives from reserve α-glucans.

present in a ratio 1.0:2.2:0.5:1.0. Also minor peaks at δ 5.42 and 5.20 were detected (see later for assignment). Enantiomeric analysis of F9 (Table 3) and of its methylated derivative (Table 4) indicated that in this fraction, agaran structures of the G → L type were significant and that the 4-linked L-galactose units were mostly substituted on C-3, and, possibly, also in part non-substituted.

3.2. Room temperature water extracts A1 and A2

Analysis of A1 and A2 gave very similar results, suggesting that both samples differed mostly in their molecular weight (Table 1). This was confirmed analyzing the pattern of partially methylated derivatives obtained by methylation analysis (Table 2).

When 15 mg of A1 were dissolved in 1 mL of D_2O, only partial solubility was possible. The ^{13}C NMR spectrum of the soluble moiety of A1 showed major amounts of κ-structure and minor quantities of ι-carrageenans (Fig. S3). Some other minor signals were also present. On the other hand, in the ^{1}H NMR spectrum, anomeric signals of 4-linked units corresponding to κ-, ι-, μ-, and ν-structures (δ 5.11, 5.30, 5.23, and 5.53, respectively) are present in a ratio 5.9:2.9:1.0:1.7. There are other minor signals at δ 5.42 and 5.33 (see later for assignment). These results are not in agreement with those from Table 1, which clearly show that this extract is mainly composed by agarans, so it was concluded that the agaran structures precipitated when the solution for NMR was prepared, and that is why only carrageenans of A1 was detected in the spectra. Similar difficulties were found with A2 (results not shown).

Accordingly two different strategies were undertaken for structural analysis of these polysaccharides:

In the first place, A1 was submitted to a desulfation–methylation procedure (Table 2), which clearly showed, as expected, the increase of 2,4,6-Me_3 Gal and 2,3,6-Me_3 Gal and the concomitant decrease of 2,4-Me_2 Gal and 2,6-Me_2 Gal, on one hand, and 2,3-Me_2 Gal and 3-Me Gal, on the other, respectively. It is important to note that when desulfation takes place, the 4-linked galactose 6-sulfate units should lose the sulfate groups, so no cyclization can take place in the alkaline medium during the methylation procedure. Besides, although these results gave evidence about the position of the sulfate groups, they were not conclusive in relation to whether the single stubs of xylose are linked to C-6 of some of the 3-linked β-D-galactose units, or to C-3 of the α-galactose residues, as 2,4-Me_2 Gal (7.2%) and 2,6-Me_2 Gal (10.4%) were detected between the partially methylated monosaccharides. Information regards the agaran and carrageenan structures was obtained from the NMR spectra; in the HMQC only four signals were present in the anomeric region at δ 104.0/4.49 and 101.6/5.36, corresponding to diads of the G → L type, and at 102.8/4.66 and 98.9/5.21, which correspond to diads of the G → LA type. Hence, the only important structures in this sample are galactans of the agaran family. All the signals for both unsubstituted diads were present in the spectra (Lahaye, Yaphe, & Rochas, 1985; Prado, Ciancia, & Matulewicz, 2008; Welti, 1977) (Table 6, Fig. S4). A signal at δ 57.7/3.50 was assigned to a methoxyl group on C-3 of a 4-linked α-galactose unit (Miller & Blunt, 2000; Navarro & Stortz, 2008), in agreement with the small amounts of 3-*O*-methylgalactose detected in the monosaccharide composition of the sample. Other samples also showed small amounts to traces of this sugar. Accordingly a peak at δ 81.1/3.43 was tentatively assigned to C-3 of 4-linked 3-*O*-methylgalactose units.

Table 6
NMR signal assignments (ppm) of galactans from A1D and A2tr-sol.[a,b]

Structural unit[c]	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5,5′	C-6/H6,6′
A1D						
G	104.0/4.49	71.3/3.78	81.2/3.83	68.0/4.31	75.8/3.77	61.9/3.79
L	101.6/5.36	69.3/4.19[1]	70.2/4.18	79.4/4.30	72.3/4.11	61.9/3.79
G	102.8/4.66	70.8/3.73	82.6/3.88	69.2/4.23[1]	75.8/3.77	61.9/3.79
LA	98.9/5.21	70.2/4.35	80.5/4.62	77.8/4.71	76.0/4.62	69.8/4.11, 4.18
A2tr-sol						
G6S (6X)[d]	102.8/4.67	70.6/3.67	82.5/3.86	69.2/4.17	73.5/4.01	67.9/4.25 (69.5)[d]
LA	98.9/5.20	70.2/4.35	80.5/4.64	78.5/4.70	75.8/4.63	69.8/4.11, 4.18
tX	104.2/4.55	74.0/3.51	76.5/3.50	70.6/4.02	66.0/4.00,3.38	
G	104.2/4.46	71.5/3.84		67.8/4.19	76.1/3.80	61.9/3.80
L3S	101.3/5.42	67.7/4.09	81.2/4.47	77.05	71.55	61.1/3.91

[a] Nomenclature of Knutsen et al. (1994).
[b] Signals at δ 57.7/3.50 in A1D and at δ 57.2/3.50 to 57.2/3.56 in A2tr-sol were assigned to a methoxyl group on C-3 of a 4-linked α-galactose unit; a resonance at δ 81.1/3.43 in the spectrum of A1D was tentatively assigned to C-3 of this latter residue.
[c] For (1), assignments could be interchanged.
[d] The signal at δ 69.5 corresponds to C-6 of G6X.

The second strategy to study the structures of these agarans was based on the fact that controlled modifications of the galactans, like alkaline treatment, produce known changes in the structure of the polysaccharide, but, at the same time, they modify their solubility behavior (Estevez et al., 2004). Hence, alkaline treatment of A2 was carried out, and the modified product, A2tr, was fractionated by precipitation with 2.8 M KCl. Most of the modified sample (A2tr-sol, 68% of the recovered, molar ratio galactose:3,6-anhydrogalactose:sulfate 1.00:0.37:0.54) remained soluble in these conditions. The NMR spectrum of A2tr-sol was analyzed considering data from A2 (Tables 1 and 2), which indicate that the precipitate (A2tr-pp) should be composed mostly by carrageenans. The ^{13}C NMR spectrum of the A2tr-sol showed signals corresponding only to agaran structures, as expected. Besides, a signal at δ 66.1 corresponds to C-5 of xylose units. The anomeric signal for this unit should be that at δ 104.2/4.55 in the HMQC spectrum (Fig. S5) (Navarro & Stortz, 2008). Previously published data showed that galactans substituted with single stubs of β-D-xylose at C-3 of α-L-galactose should appear at higher fields, at $\delta \sim$ 101–102 (Usov & Elashvili, 1991). However, in this range only a signal at δ 101.3/5.42 was found in the HMQC spectrum of A2tr-sol, which cannot be assigned to these units, but to 4-linked α-L-galactose units. This indicates that single stubs of β-D-xylose are linked to C6 of the 3-linked β-D-galactose units (Table 6, Fig. S4). Considering results from methylation analysis of A2 and analyses of A2 and A2tr-sol, the major structures of the agaran in A2tr-sol are: G(6X)(6S) → L(3S)(3,6S)(3Me), LA (Fig. 1B). Table 6 indicates assignment of the corresponding spectra, based on chemical analysis and previously reported information (Kolender & Matulewicz, 2002; Navarro & Stortz, 2008; Prado et al., 2008; Usov, Bilan, & Shashkov, 1997).

In addition, two small signals at δ 57.2/3.56 and 57.2/3.50 were assigned to a methoxyl group on C-3 of a 4-linked α-galactose unit, in agreement with the small amounts of 3-*O*-methylgalactose detected in the monosaccharide composition of the sample (differences in chemical shifts between them were attributed to slightly different environment).

It is important to note that the peaks previously unassigned in the ^{1}H NMR spectra of C1, A1, and F9 at δ 5.42, 5.33, and 5.20 correspond to the anomeric signals of small amounts of agaran structures comprising 4-linked α-L-galactose 3-sulfate, α-L-galactose, and 3,6-anhydro-α-L-galactose units, respectively.

3.3. *Carrageenan and agaran structures in* G. tenuis

The galactan system biosynthesized from *G. tenuis* is constituted by major amounts of κ/ι-carrageenans that were extracted with hot water. As expected for haploid plants of red seaweeds from the Phyllophoraceae, ι-structures predominate. A small amount of non-cyclized carrageenans mostly of the ν-type was also present.

Most of the galactans from this seaweed were extracted with hot water, and only trace amounts of polysaccharides were obtained by extraction at room temperature. This behavior was expected, as it is similar to that found for other seaweed of the same genus, *G. torulosus* (Estevez et al., 2008), but different to that observed for galactans from the related family, Gigartinaceae (Flores et al., 2002; Matulewicz et al., 1989). Almost 90% of the galactans obtained from the species studied in the present work are of the carrageenan type, while ∼10% are agaran structures. This result is quite different to that obtained for *G. torulosus*, which showed to have 1/3 of the galactans of the agaran type; however, comparison should be considered tentative because the extraction and fractionation conditions were not the same.

Besides, under these extraction conditions, ∼22% of C1 is constituted by non-cyclized carrageenans mostly of the ν-type, so by the alkaline extraction used industrially, they would be converted to ι-carrageenans. Moreover, in the industrial process, low molecular weight components are lost, and this should include the agaran molecules, showing that this seaweed could be a potential source of ι-carrageenans. In this paper, conditions used for extraction of the polysaccharides intended to be as mild as possible to determine the structure of the original polysaccharides, nevertheless, by alkaline treatment of C1, a galactose:3,6-anhydrogalactose molar ratio close to 1:1 was obtained. This result, together with the structural determination of C1, gives a first evidence of the industrial interest of these carrageenans. However, in this work a persistent problem was the tendency of some of the samples to retrograde. This phenomenon has been observed previously but in this case, the effect was very important. A reason for this behavior could be related with the sample work up during the preliminary methanol extraction.

Currently, the presence of small to considerable amounts of galactans of the agaran type is well documented for many seaweeds considered as carrageenophytes, but details about their structures are scarce. The structural features found generally indicate dispersion in the substitution pattern. However, some structural units are repeatedly found (Table 7). 3-Linked 6-sulfated β-D-galactose units, together with 4-linked 3-substituted and non-substituted α-L-galactose residues, a pattern common to corallinans (Cases, Stortz, & Cerezo, 1994) and other red seaweeds galactans obtained from several species of the Halymeniales (Miller, Falshaw, & Furneaux, 1995; Miller, Falshaw, Furneaux, & Hemmingson, 1997) were detected in most of the studied agarans extracted from carrageenophytes.

In summary, the most particular structural feature in agarans biosynthesized by carrageenophytes is substitution on C-3 of the α-L-galactose units. This substitution pattern was also found in

Table 7
Structural units found in agarans detected in carrageenophyte red seaweeds.

Seaweed	A-unit[a]	B-unit[a,b]	Reference
Kappaphycus alvarezii (Solieraceae)	G, G2S, G4S, G2,4S, G6S	L, L3S, L2,3S, L2M, L3M, LA, LA2S	Estevez et al. (2001, 2004)
Gigartina skottsbergii, *Sarcothalia papillosa*[c] (Gigartinaceae)[d]	G6S, G6X	L3S, L	Ciancia, Matulewicz, et al. (1993), Ciancia, Matulewicz, Finch, et al. (1993), Ciancia, Noseda, et al. (1993), Ciancia et al. (1997), Noseda (1994), Stortz et al. (1997), Flores et al. (2002)
Gymnogongrus torulosus (Phyllophoraceae)	G, G2S, G6X	L3S, L, LA, LA2S	Estevez et al. (2001, 2008)
Gymnogongrus tenuis (Phyllophoraceae)	G, G6X, G6S	L3S, L3M, L3,6S, LA	This work

[a] Nomenclature according to Knutsen et al. (1994).
[b] Most of these products were studied after alkaline treatment, so, part of LA and LA2S units could correspond to L6S and L2,6S, respectively in the untreated samples.
[c] As *Iridaea undulosa*.
[d] In these papers, the presence of agarans was clearly established, however, details of their actual structure were scarce and, possibly their revision, mainly using spectroscopic methods would give some new structural evidences.

agarans from *G. tenuis*, which showed a quite simple structure in comparison to some of those found previously for components of this type (Table 7, Fig. S5).

The methodology used here allowed the isolation and study of the structure of agarans biosynthesized by *G. tenuis*, in part in their native form, and in part after chemical modification, showing that they are low molecular weight molecules, independent of the carrageenan structures. The strong interactions between both polysaccharide types, as well as the low molecular weight of these agarans determine the difficulties found for their detection and structural study and should be the cause for them to go unnoticed for so long.

The biological role of the presence of agarans in cell walls from carrageenophytes is not known. They could contribute to the assembly of the cell wall due to their capacity of complexing with other polysaccharides by crosslinking and thus modifying the accessibility of enzymes involved in cell wall metabolism.

Acknowledgements

This work was supported by grants of the National Research Council of Argentina (112-200801-00234/2009-2011 and 11220110100208/2012-2014), the University of Buenos Aires (20020100100479/2011-2014, 20020130100216BA/2014-2017, and 20020130100576BA/2014-2017) and FONACIT (No. 2012000830) from Venezuela.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2015.10.007.

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