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The system of sulfated galactans from the red seaweed *Gymnogongrus torulosus* (Phyllophoraceae, Rhodophyta): Location and structural analysis

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Abstract

Sulfated polysaccharides were localized in the cuticle, cortex and medulla of the gametophyte thallus, being more concentrated in the intercellular matrix than in the cell walls. During the water extraction sequence, a small percentage of galactan sulfates (5.1% of dry seaweed) with average low M_r (6–11.4 kDa) were extracted at room temperature without disturbing the cellular arrangement, while sulfated galactans of average medium M_r (18–45 kDa) were obtained by further hot-water extractions (52.4% of dry seaweed), with diorganization of the tissue. The residue (40.0% of dry seaweed) still contained carrageenan-type (major) and agaran-type (minor) galactans. Part of these galactans was extracted with 8.4% LiCl solution in DMSO, from which "pure" κ/ι-carrageenans were isolated.

Carrageenans and agarans were extracted in a ratio 1:0.5, showing the highest amount of agaran-structures for a carrageenophyte. The galactans comprise alternating 4-sulfated (major) and non-sulfated (minor) 3-linked β-D-galactopyranose units, and 4-linked α-galactopyranose units with the following substitutions: (i) non-sulfated and 2-sulfated 3,6-anhydro-α-D-galactopyranose residues in the carrageenan-structures, which belong to the κ-family (κ/ι-carrageenans); (ii) 3-sulfated α-L-galactopyranose units and 2-sulfated 3,6-anhydro-α-L-galactopyranose residues in the agaran-structures.

Alkaline treatment and alkaline dialysis of the main extracts gave "pure" κ/ι-carrageenans, showing that carrageenan molecules are extracted together with low M_r agarans or agaran-DL-hybrids.

Keywords: *Gymnogongrus torulosus* (Phyllophoraceae); Red seaweed; Cell wall; Sulfated galactans; Carrageenans; Agarans; L-Galactose

1. Introduction

Cell wall organization of red algae varies with the morphology of the thallus, the tissue under study and the life cycle phases. In multicellular red algae, the cell wall can be divided into two different regions: ordered wall layers surrounding the plasmalemma embedded in a less organized material, which also fills the space between the cells (Kloareg and Quatrano, 1988). In this work, they will be referred to as "cell wall" and "intercellular matrix", respectively (Gordon-Mills, Tas, & McCandless, 1978). The distribution of sulfated galactans in the thallus of red algae is not well determined and only a few species were studied from this point of view (Foltran et al., 1996; Gordon-Mills et al., 1978; LaClaire & Dawes, 1976; Lechat, 1998; Vreeland, Zablackis, & Laetsch, 1992; Zablackis, Vreeland, Doboszewski, & Laetsch, 1991).

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The few studies carried out on cell walls of Florideophyceae showed that most of them are composed of major quantities of (glyco)proteins, minor amounts of skeletal polysaccharides (mainly cellulose) and galactans with alternating 3-linked β-units and 4-linked α-units (Craigie, 1990; Flores, Cerezo, & Stortz, 2000; Flores, Stortz, Rodriguez, & Cerezo, 1997; Lechat, Amat, Mazoyer, Buléon, & Lahaye, 2000). These last components (carrageenans, agarans and DL-galactan hybrids) are much more abundant in the intercellular matrix (30–65% of the dry weight). Other polysaccharides, such as xylomannans, xylans, etc. can also constitute the intercellular matrix, but they are not important in seaweeds of the Phyllophoraceae (Miller, 1997; Stortz & Cerezo, 2000). All these polymers have been associated to the physiological significance of the whole cell in mechanical stress resistance, hydration, ionic and osmotic regulation of the seaweeds in marine environments (Kloareg & Quatrano, 1988).

Previous work (Furneaux & Miller, 1985) on several *Gymnogongrus* species (including *G. torulosus* formerly *Ahnfeltia torulosa*) showed that the crude extracts obtained after boiling-water extraction and alkaline treatment, worked out with spectroscopic techniques, contained major amounts of κ- and/or ι-carrageenan-structures.

Carrageenans are sulfated galactans extracted from seaweeds of the order Gigartinales, and from some genera of Dumontiales, Halymeniales and Rhodymeniales (Chopin, Kerin, & Mazerolle, 1999; Miller, 1997; Stortz & Cerezo, 2000). They are linear polysaccharides comprised of alternating 3-linked β-D-galactose and 4-linked α-D-galactose or 3,6-anhydro-α-D-galactose units, which are usually sulfated at specific positions. They have been classified in different families according to the position of sulfation on the 3-linked β-D-galactose units (Stortz & Cerezo, 2000). On the other hand, the diastereomeric sulfated galactans, in which the α-galactose or 3,6-anhydro-α-galactose units belong to the L-series are called agarans. The main polysaccharide of this group is agarose which is biosynthesized as the major product by seaweeds of the orders Gelidiales and Gracilariales; additional red algae from other orders also produce agarans with different structural variations. Based on these definitions, the seaweeds that produce carrageenan are named "carrageenophytes" and those that biosynthesize agarans, "agarophytes". The regular backbone of these galactans is usually masked by different O-linked groups as methyl ethers, sulfate esters, pyruvic acid ketals and β-D-xylopyranosyl and other sugars residues as side chains (Miller, 1997; Stortz & Cerezo, 2000). The variability in the chemical structure of these galactans, mainly the 3,6-anhydrogalactose content and the position of sulfation is determinant of their physical and biological properties.

In a former work (Estevez, Ciancia, & Cerezo, 2001), the sulfated galactans of the major hot-water extract obtained from *G. torulosus* were shown composed not only by carrageenans, but also by DL-hybrid galactans or mixtures of carrageenan- and agaran-structures. Even if 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 the red seaweeds (Stortz & Cerezo, 2000) still remains unclear: (i) if they are separate molecules or hybrids; (ii) the importance of their contribution to the whole cell wall polysaccharides.

The aim of the present paper was to determine the location, chemical structure and interactions of the whole family of galactans (carrageenans, agarans and DL-hybrids galactans) biosynthesized by the gametophytes of *G. torulosus*.

2. Materials and methods

2.1. Algal sample

Cystocarpic and sterile plants of *G. torulosus* were collected in March 1998, in Cabo Corrientes (38°03′S, 57°31′W), Mar del Plata, Buenos Aires province, Argentina. Taxonomic identification was based on molecular sequence analysis of the genes: *rcb*L (GenBank AF388561), ITS (AF388644) and SSU rDNA (AF3885604) (Fredericq & Lopez Bautista, 2003). A voucher material has been deposited in the Herbarium of the Museo Bernardino Rivadavia (BA), Buenos Aires, Argentina (Collection No. 35707). Thalli of *G. torulosus* were washed with filtered seawater and analyzed for epiphytic and epizoic contaminants in a Nikon AFX-II macroscope (Nikon, Japan). For checking endophytes, cross-sections were obtained manually with a single-edge razor blade and observed on a Zeiss Axioplan microscope (Oberkochen, Germany).

2.2. Light and Transmission Electron Microscopy

For Transmission Electron Microscopy (TEM), sterile and cystocarpic thalli were fixed at room temperature in 2% glutaraldehyde in sodium cacodylate buffer (0.05 M), postfixed in 2% OsO_4 in the same buffer, dehydrated through a graded Me_2CO series and embedded with Spurr's low viscosity resin by the flat embedding method. All sections were cut with a diamond knife (Diatome Ltd., Bienne, Switzerland) in a Reichert-Jung Ultracut ultramicrotome (C. Reichert Optische Werke, Wien, Austria). Thin sections were mounted on Formvar coated grids, stained with uranyl acetate (40 min) and lead citrate (40 s) and then observed with a Jeol 100 CX-II electron microscope (Jeol Ltd., Akishima, Tokyo, Japan). Ruthenium red (0.0001%) in 2% glutaraldehyde in sodium cacodylate buffer (0.05 M) was used for localization of the acidic polysaccharides in TEM (Krishnamurthy, 1999).

For Light Microscopy (LM), semithin sections (13 μm) were mounted on glass slides and then observed with a Carl Zeiss Axiolab microscope (Carl Zeiss, Jena, Germany). The staining procedures used in LM histochemical characterization, carried out on fixed tissues described before, included: (1) toluidine blue O (0.05% w/v) in 0.1 M HCl at pH 1.0 (O'Brien and McCully, 1981) that stains sulfated

polysaccharides (red-purple, γ metachromasia); (2) ruthenium red (0.0001%) in aqueous solution (Luft, 1971) for acidic polysaccharides (red) and lugol (iodine in 0.5% IK) for α-glycans (brown) (Krishnamurthy, 1999); (3) Basic fuchsine (4% w/v) in NaOH 0.1 M (pH 12.5)-methylene blue (2% w/v) for neutral (pink) and acidic polysaccharides (red-purple), respectively (Robinson et al., 1987); (4) Calcofluor white (0.1% w/v) in aqueous solution (Krishnamurthy, 1999) for β-(1→3) and β-(1→4) fibrillar polysaccharides.

2.3. Extraction of the polysaccharides

The sterile and cystocarpic plants previously dried in the open under strong winds were milled with a mesh of 20. The milled material was sequentially and exhaustively extracted with H_2O (20 g/L) at room temperature and hot-water as previously reported (Estevez et al., 2001). Briefly, the residue of the first room temperature extraction was removed by centrifugation and the supernatant poured into three volumes of *iso*-PrOH, where the polysaccharide precipitated as long fibers. The liquors were decanted and the product dried by solvent exchange (EtOH and Et_2O) and finally *in vacuo*, obtaining extract A1. The residue was extracted twice in the same way (extracts A2–A3). The final residue obtained at room temperature was freeze-dried (RA3). Then, it was re-suspended in H_2O (20 g/1.0 L) and extracted at 90 °C with mechanical stirring for 4–5 h. The extract was treated as described above, obtaining extract C1. The residue was extracted three times in the same way, giving extracts C2–C4 and residue RC4. The residue RC4 (1 g) from the water extraction was extracted with a 8.4% LiCl solution in DMSO (200 mL) at 106 °C for 5 h. The mixture was cooled to room temperature and centrifuged obtaining residue RF. The supernatant was dialyzed (M_r cut off 6.0–8.0 kDa) and freeze-dried, giving the extract F.

2.4. Chemical analysis

The total sugars content was analyzed by PhOH–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) F was also analyzed by the method adapted for insoluble material (Ahmed & Labavitch, 1977). Sulfate was determined turbidimetrically (Dodgson & Price, 1962). The amino sugars in the residues were quantified by Smith and Gilkerson (1979). The number average molecular weight (M_r) was estimated by the method of Park and Johnson (1949) based on the determination of end-chains reducing units. The protein content was determined by the method of Lowry, Rosenbrough, and Farr (1951). Cation analyses were carried out by atomic absorption spectroscopy of the samples in water solutions on a Shimadzu 6800 (Kyoto, Japan) equipped with an autosampler. Commercial standards salts with the desired cations (Merk) were used to calibrate.

For GC, alditol acetates from galactans were obtained by reductive hydrolysis and acetylation of the samples (type A, Stevenson & Furneaux, 1991). The partially insoluble extract F (3 mg) was dissolved in 100% TFA (37 °C, 1 h), followed by dilution of the acid to 80%, heating at 100 °C for 1 h, and further dilution to 2 M to achieve the regular hydrolysis conditions for insoluble polysaccharides (type B, Morrison, 1988); hydrolyzate was derivatized to the corresponding alditol acetates. To quantify the monosaccharide composition of the galactans including 3,6-anhydrogalactose in the insoluble product (F), a complete reductive hydrolysis *in situ* (type C), based on the method described by Usov and Klochkova (1992) was carried out with some modifications. Briefly, the sample (0.5–5.0 mg) was hydrolyzed in 2 M TFA with addition of borane 4-methylmorpholine complex (30–40 mg) during 8 h at 100 °C. Then, a second hydrolysis step was carried out at 120 °C during 2 h (Morrison, 1988). The mixture was evaporated *in vacuo* and EtOH (2 mL) was added to the residue, the suspension was centrifuged and the supernatant was evaporated to dryness, this treatment was repeated twice more. The mixture of monosaccharides was acetylated as described by Stevenson and Furneaux (1991). The ratio D:L-galactose and the configuration of monomethylated galactoses (when the percentages present were high enough) were estimated by the method of Cases, Cerezo, and Stortz (1995) through its diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols; D:L-3,6-anhydrogalactose ratio was estimated by the method of Errea, Ciancia, Matulewicz, and Cerezo (1998) involving an oxidative hydrolysis to obtain the aldonic acids, further converted to the acetylated diastereomeric sec-butylesters. The ratio of 2-*O*-methyl-D:L-3,6-anhydrogalactose in the permethylated polysaccharide was estimated by the method of Navarro and Stortz (2003). 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).

2.5. Alkaline treatment

The sample (106 mg) was dissolved in H_2O (52 mL), and NaBH4 (5 mg) was added. After 24 h at room temperature, 3 M NaOH was added (26 mL) with a further quantity of NaBH4 (5 mg). The solution was heated at 80 °C for 20 min, and then cooled to room temperature, dialyzed (M_r cut off 12.0–14.0 kDa) without previous neutralization, concentrated and freeze-dried to give C1T (90 mg) and A1T (47 mg), respectively.

2.6. GC

GC of the alditol acetates, as well as those of the partially methylated alditol and aldononitrile 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 id × 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) with an initial 2-min hold at 180 °C, then at 2 °C min^{-1} to 210 °C, then at 1 °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. Conversion of GC areas to molar basis was calculated for the partially methylated alditol acetates according to the effective carbon response theory (Sweet, Shapiro, & Albersheim, 1975); for 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol a value of 0.64 was used (Stevenson & Furneaux, 1991).

2.7. GC–MS

GC–MS of the methylated alditol acetates was performed on a Shimadzu GC-17A gas-liquid chromatograph equipped the SP-2330 (see above) interfaced to a GCMS-QP 5050A mass spectrometer (Kyoto, Japan) working at 70 eV. Chromatography was performed on the SP-2330 capillary column. 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.

2.8. Methylation analysis

The polysaccharide (3–6 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 samples were derivatized to the alditol acetates as described for the polysaccharides (Stevenson & Furneaux, 1991). A portion of methylated sample was hydrolyzed with TFA 2 M for 2 h at 120 °C and the partially methylated sugars were converted into the corresponding aldononitrile acetates (Stortz, Matulewicz, & Cerezo, 1982).

2.9. FT-IR

Fourier-transform infrared spectra were recorded from 4000 cm^{-1} to 250 cm^{-1} with a 510P Nicolet FT-IR spectrophotometer (Madison WI, USA), using films prepared by drying aqueous solutions of the polysaccharides of the extracts and KBr discs for the residues. 32–64 scans were taken with a resolution of 2–4 cm^{-1}.

2.10. ^{13}C NMR spectroscopy

Samples (20–30 mg) were dissolved in 1:1 H_2O:D_2O solutions (0.5 ml), agitated 24 h at room temperature and centrifuged. Proton decoupled 125 MHz ^{13}C NMR spectra were recorded on a Bruker AM500 (Germany) at room temperature, with external reference of TMS. The parameters were as follows: pulse angle 51.4°, acquisition time 0.56 s, relaxation delay 0.6 s, spectral width 29.4 kHz and scans 19,000–34,000. Chemical shifts were referenced to internal acetone (δ 31.1 ppm).

2.11. ^{1}H NMR spectroscopy

Samples (20–30 mg) were exchanged in 99.9%D D_2O (0.5 mL) four times, agitated 24 h at room temperature and centrifuged. 500 MHz ^{1}H NMR spectra were recorded on a Bruker AM500 (Germany) at room temperature, with external reference of TMS. The parameters were as follows: pulse angle 76°, acquisition time 3 s, relaxation delay 3 s, spectral width 6250 Hz and scans 32. Chemical shifts were referenced to internal Me_2CO in D_2O (δ 2.175 ppm).

3. Results

The basic structure of the multiaxial thallus of *G. torulosus* is shown in Fig. 1a. It consists of several layers of medullar cells in the inner part of the thallus with a pseudoparenchymatous compacted arrangement, and photosynthetic cortical cells covered by a single-stratified cuticle. A detailed study of the structure of the thallus was reported by Estevez & Cáceres, 2003.

The presence of abundant anionic polysaccharides in the whole thallus is indicated by the positive reaction with methylene blue and ruthenium red (not shown). The red-purple metachromasia obtained by acidic (pH 1) toluidine blue O staining revealed the presence of sulfated polysaccharides in the cell walls and intercellular spaces of all the cells. Staining of these polysaccharides seems to be homogeneous all over the thallus, indicating a similar degree of sulfation in the cortical and medullar zones (Fig. 1b). Reactions for acidic polysaccharides were much higher in the amorphous matrix and less intense in the cell wall (Fig. 1b, c, f and g). Some layers of the cell wall give positive reaction to toluidine blue O and methylene blue (Fig. 1f and g), but no staining is detected with ruthenium red at TEM levels (Fig. 1d, e and h, i).

The milled seaweed was extracted exhaustively with water at room temperature giving fractions A1–A3, with a total yield of 5.1% of the seaweed dry weight (Table 1). The final residue from the room temperature extraction (RA3) was exhaustively extracted with hot-water (90 °C) four times, obtaining fractions C1–C4 (52.4% of the dry seaweed) and a residue, RC4 (40.0% of the dry seaweed). RC4 was treated with 8.4% LiCl in DMSO giving extract F and residue RF (6.6% and 26.4% of the dry seaweed, respectively). RA3 shows the ordered structure of the thallus, where the effect of the water extraction is not obvious, but RC4 only shows some individual cortical and medullar cells together with cell walls (results not shown). Yields, analysis and characterization of extracts are shown in

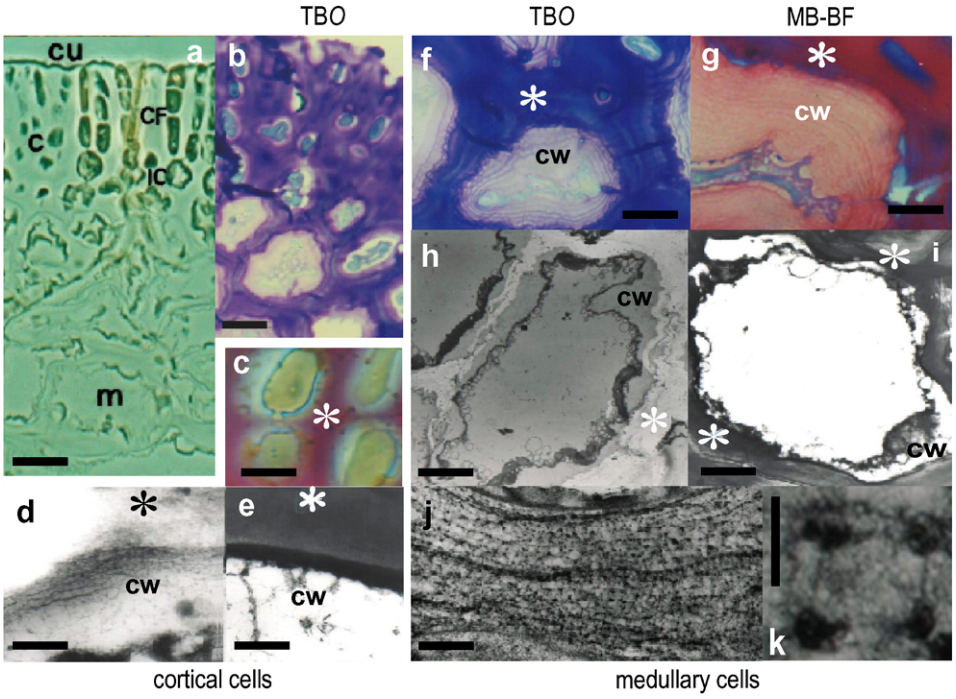

Fig. 1. Semithin cross-section of the thallus of *Gymnogongrus torulosus* showing the localization *in situ* of anionic polysaccharides. (a) Cross-section without staining showing an outer cuticle (cu), cortical filaments (cf) and inner cortical cells (ic) composing the cortex and an extended medullar zone (m). Scale bar = 15 μm. (b) Semithin sections showing the localization *in situ* of anionic polysaccharides. Toluidine blue O staining (pH 1, purple) for sulfated polysaccharides. Scale bar = 12 μm. (c–e) Cortical cells in detail. (d) Ruthenium red (red, asterisk labeling) for anionic polysaccharides and lugol (brown) for α-glycans. Scale bar = 6 μm. (d) and (e) Electron Microscopy (TEM) of cortical cells. (d) Semithin sections without staining. Scale bar = 1.5 μm. (e) Ruthenium red-TEM staining in the intercellular space (asterisk) and cell wall (CW). Scale bar = 1.5 μm. (f–k) Medullar cells in detail. (f) Sulfated polysaccharides located in the intercellular with toluidine blue O. Scale bar = 5 μm. (g) Anionic polysaccharides in intercellular spaces (purple-blue, asterisk labeling) and neutral polysaccharides (pink) in the inner cell wall, stained with methylene blue and basic fuchsin, respectively. Scale bar = 3 μm. (h) Cell wall and intercellular space (asterisk) in TEM. Semithin section without staining. Scale bar = 5 μm. (i) Medullar cell stained with ruthenium red-TEM. Scale bar = 5 μm. (j) Cell wall microfibrills organization by TEM. Scale bar = 0.5 μm. (k) Detail of cell wall structure. Scale bar = 0.1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1. The galactan sulfates from the fractions are composed not only by D-units, but also L-units suggesting the presence of carrageenan- and agaran-structures. In order to test if the agaran-structures are forming hybrid molecules with the carrageenan-ones, the main extract C1 (molar ratio Gal:AnGal ca.1:0.72) was submitted to a short alkaline treatment using conditions suitable to avoid degradation (Ciancia, Noseda, Matulewicz, & Cerezo, 1993a). The treated product was dialyzed in the alkaline medium (M_n cut off 12–14 kDa) to eliminate small fragments, giving C1T (85.1% yield) with a molar ratio of Gal:AnGal ~ 1:1. Neither 3,6-anhydrogalactose nor galactose in the L-configuration were detected, indicating that C1T, in contrast with C1, is a "pure" κ/ι-carrageenan. A similar treatment was carried out on A1 (molar ratio Gal:AnGal ca. 1:0.40) and the treated product, (A1T) recovered after dialysis (44% yield) had a molar ratio of Gal:AnGal ca. 1:0.67 and was also a "pure" carrageenan.

Composition of both room temperature (A1–A3) and hot-water (C1–C4) extracted galactans is similar, not only in monosaccharide composition but also in enantiomeric ratios (Table 1), differing only in M_r and monomethylated galactose units content. After alkaline treatment the monomethylated units disappeared. Composition of F (Table 1) confirms that major quantities of sulfated galactans are still present in RC4. The low M_r of F is noteworthy, but the value obtained by the reducing end method (10.9 kDa) is in the same range of that deduced by methylation analysis (15–16 kDa). Enantiomeric analysis of F (Table 1) indicated the absence of galactose and 3,6-anhydrogalactose in the L-configuration.

Based on the absolute configuration of the (3,6-anhydro)-galactose units in all the fractions obtained, a ratio ca.1:0.5 of carrageenan-:agaran-structures was estimated for the water-soluble sulfated galactans *G. torulosus*.

In the starting material and A1 the monovalent cations predominated, while in C1and F, Ca^{2+} and Mg^{2+} represent the major counter-ions. The presence of less than 2.8 meq/100 g of Li^{+} in F and RF indicates that this cation was not interchanged during the LiCl/DMSO extraction procedure.

FT-IR spectra of extracts (not shown) are very similar, showing absorptions at 932–930 cm^{-1} corresponding to the structure C–O–C of the 3,6-anhydro ring. Another band was observed at 847–851 cm^{-1}, due to stretching vibrations in the structure C4–O–S of the axial sulfate group in 3-linked β-D-galactose 4-sulfate units, and at 801–807 cm^{-1} of the axial sulfate group in the 4-linked 3,6-anhydro-α-D-galactose 2-sulfate residues. No absorp-

Table 1
Yields, analyses and monosaccharide composition of the extracts

Samples	Yield [a] %	Carbohydrates %	Sulfate as SO_3Na %	Proteins %	Molecular weight M_n KDa[b]	Type of hydrolysis [c]	Monosaccharide composition (mol %)									
							D-Gal	L-Gal	6-Gal	3-Gal	2-Gal	D-AnGal	D-AnGal	Xyl	Glc	Man
Extracts																
A1	3.7	53.6	24.6	2.3	11.4	A	54.1	9.6	Tr. [d]	1.0	1.2	23.1	3.4	4.1	2.3	1.2
A1T[h]	44.4	–	–	–	–	A	57.0	–	–	–	–	38.0	–	2.6	2.4	Tr.
A2	0.8	50.5	26.0	8.8	6.0	A	45.1	12.0	Tr.	Tr.	2.9	29.4	6.4	1.9	2.3	Tr.
A3	0.6	46.1	20.2	9.4	9.7	A	40.1	6.7	Tr.	1.8	1.2	24.4	8.4	9.9	4.7	2.8
C1[e]	31.1	51.3	25.1	Tr.	44.0	A	54.2	6.1	Tr.	–	–	28.7	9.6	1.4	Tr.	Tr.
C1T[h]	85.1	–	–	–	–	A	47.5	Tr.	–	Tr.	–	42.2	Tr.	3.3	3.2	3.8
C2	10.1	54.6	19.2	4.4	45.0	A	55.5	5.9	1.4	Tr.	Tr.	22.4	11.0	1.4	2.4	Tr.
C3	7.6	54.5	17.0	9.5	23.0	A	55.7	6.8	2.3	Tr.	Tr.	19.9	10.0	1.0	4.3	Tr.
C4	3.6	49.6	14.4	16.8	18.0	A	41.6	8.4	3.7	Tr.	Tr.	18.6	12.0	6.4	9.3	Tr.
F	6.6	46.3[f]	19.0	12.2	10.9[g]	B	←66.2→		–	1.1	–	←17.9→		3.0	26.8	2.9
						C	45.1	Tr.	–	–	–	37.6	Tr.	2.6	14.7	Tr.

[a] Yield of the extracts are given for 100 g of seaweed. In parentheses, they are given based on carbohydrate and sulfate content.
[b] Determined by the method of Park and Johnson (1949).
[c] (A) reductive hydrolysis (Stevenson & Furneaux, 1991), (B) acid hydrolysis for insoluble materials (Morrison, 1988) and (C) reductive hydrolysis *in situ* (Usov & Klochkova, 1992). For details see Section 2.
[d] Percentages lower than 1% are given as trace (Tr.).
[e] Included for comparison (see Estevez et al., 2001).
[f] 17.9% of the total carbohydrate content corresponds to 3,6-anhydrogalactose (Yaphe & Arsenault, 1965).
[g] In these condition of hydrolysis, the 3,6-anhydrogalactose units were degraded and, consequently, not detected.
[h] After alkaline treatment and alkaline dialysis.

tion around 830–820 cm^{-1} due to the C2–O–S in the equatorial sulfate group and stretching of C6–O–S of the primary sulfate group were observed (Prado Fernandez, Rodríguez Vazquez, Tojo, & Andrade, 2003).

Linkage analyses (Table 2) confirmed that in all the extracts the κ/ι-carrageenan-structures predominate. Only small differences were found, i.e. the higher diversity of minor structural units in the low-molecular weight products. Precursor units were poorly detected in consistency with the balance of methylation- and cyclization-reaction rates of these units in carrageenans of the κ-family during methylation (Ciancia, Matulewicz, Finch, & Cerezo, 1993b). In methylated A1-A3 the κ-structure slightly predominates over the ι-one (κ/ι: ~1.1–1.25), in the same derivative of C1–C4 both structures are present in rather similar amounts (κ/ι: ~0.85–1.07) and in F, the ι-substitution is about 67% of the cyclized diads (Table 2). This trend is also seen in the corresponding resonances in the ^{13}C NMR spectra (Fig. 2, Table 3).

Table 2
Methylation analyses of the extracts

Monosaccharides[a,b]	A1	A2	A3	C1[f]	C2	C3	C4	F[c]
2,3,4,6-Gal	1.3	1.2	1.7	Tr.[d]	Tr.	Tr.	Tr.	1.4
2,4,6-Gal	2.6	1.5	3.3	1.6	3.0	2.3	2.4	3.1
2,3,6-Gal	2.3	1.3	2.1	3.0	2.6	2.5	2.8	2.3
2,6-D-Gal	45.7	53.4	43.2	50.7	50.2	48.5	48.6	52.2
2,6-L-Gal	3.1	1.9	4.3	2.0	2.8	2.5	3.4	Tr.
2,4-Gal	2.3	Tr.	2.6	–	1.1	Tr.	1.6	1.0
6-Gal	2.0	2.0	2.3	–	Tr.	1.1	1.5	3.8
3-Gal	3.5	2.2	2.4	–	1.9	1.4	1.7	1.3
2-Me-D-AnGal	19.0	18.9	17.0	18.9	16.6	18.3	11.1	12.3
D-AnGal	15.2	15.3	15.4	19.3	19.5	17.1	13.2	24.6
L-AnGal	2.0	1.3	3.6	4.5	2.3	4.3	10.7	Tr.
κ/ι-Structures ratio[e]	1.25	1.24	1.1	0.98	0.85	1.07	0.85	0.49
2,3,4-Xyl	1.0	1.0	1.9	Tr.	Tr.	1.0	3.0	1.3

[a] Mol % of monosaccharide having methyl groups at the positions indicated.
[b] Small percentages of non-methylated glucose, mannose and xylose, 4,6-Gal (tr. −1.6%), 2-Gal (tr. −1.4) and 2-Me-L-AnGal (tr. −2.2) and 2,3,6-Glc (tr. −3.6%) were detected in all samples.
[c] Small percentages of 2,3,4,6-Glc (1.8%), Glc (1.2%) and Man (1.4%) were detected in this sample.
[d] Percentages lower than 1% are given as trace (Tr.).
[e] Based on the amounts of 2-*O*-methyl-3,6-anhydro-D-galactose (κ-structures) and 3,6-anhydro-D-galactose units (ι-structures).
[f] Given for comparison, see Estevez et al. (2001).

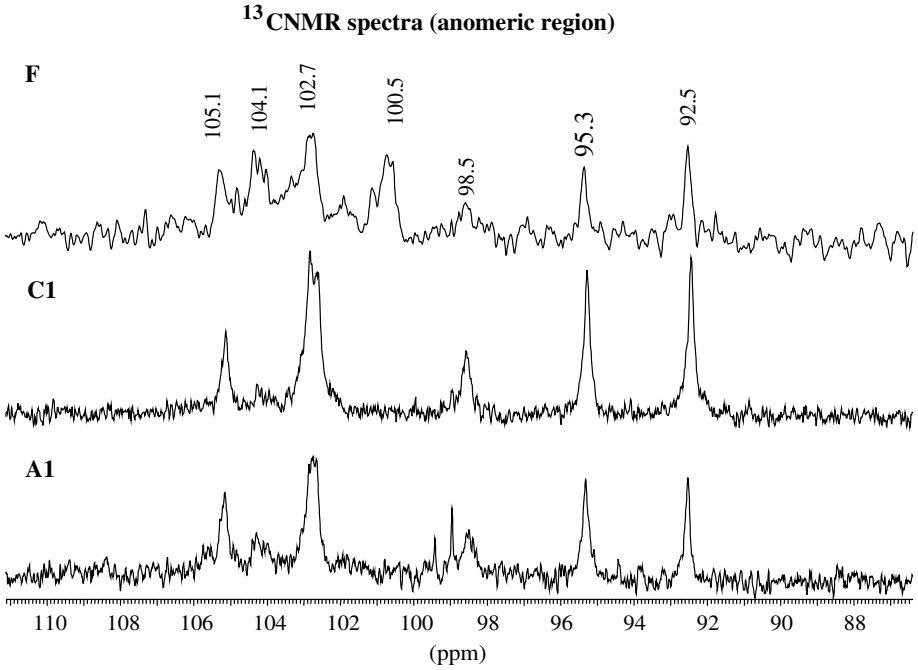

Fig. 2. Anomeric region of the ^{13}C NMR spectra of the main extracts obtained with water at room temperature (A1) and at 90 °C (C1) and of the extract obtained with 8.4% LiCl in DMSO (F). The spectra of the other extracts in both sets obtained with water are similar. Only main resonances are indicated in each spectrum (see Table 4 for the structural assignments).

Table 3
Assignments (in ppm) of the ^{13}CNMR spectra of the extracts

Residue	Reference[a]	C-1	C-2	C-3	C-4	C-5	C6
Carrageenan diads							
G4S		102.7	69.9	77.2	72.4	75.4	61.8
DAG2S	Usov and Shashkov (1985)	92.5	75.4	78.2	78.8	77.3	70.3
G4S		102.7	70.1	78.8	74.3	75.3	61.8
DAG	Usov and Shashkov (1985)	95.3	70.3	79.6	78.8	77.2	70.0
G4S	Ciancia et al. (1993b)	105.1	70.9	80.4	71.4	75.3	61.8
DG2S6S	Stortz, Bacon, Cherniak, and Cerezo (1994)	98.5	76.7	68.6	79.6	68.6	68.2
Agaran diads							
G		103.9–104.1	70.1	81.3	69.9	75.9	61.7
LG	Miller and Blunt (2002)	101.3	69.6	71.2	79.3	72.4	61.6
LG3S	Kolender and Matulewicz (2002)	101.4	67.8	79.1	77.0	71.6	6.41
LG6S	Lahaye et al. (1989)	101.5	69.5	71.6	79.2	70.3	67.9
G4S		104.2	71.9	78.4	78.2	72.4	62.0
LG2S3S	Miller (2001)	99.1	73.6	73.9	77.1	75.8	61.4
tXyl	Furneaux, Miller, and Stevenson (1990)	104.3	73.9	76.8	70.3	66.0	
Floridean starch	Knutsen, Myslabodski, and Grasdalen (1990)	100.5	76.2	74.3	72.7	68.2	61.8

[a] Assignments based in these references.

Table 4
Carrageenan- and agaran-structures present in the extracts of *Gymnogongrus torulosus*

Extracts	Yield[a]	D-units[b] (%)	L-units[b] (%)	Galactan structures		[C]:[A] ratio
				Carrageenan [C] (%)[c]	Agaran [A] (%)[c]	
A1–A3	3.9	82.8	17.2	2.7	1.2	1:0.44
C1–C4	43.3	81.0	19.0	28.7	14.7	1:0.51
F	4.2	100.0	–	4.2	–	1:0.00
Σ	53.8	–	–	35.6	15.9	–

[a] Yields were calculated based on carbohydrate and sulfate content of each extract.
[b] Only Gal and AnGal were considered as backbone units and Xyl, Glc and Man were considered as side chains or contaminants.
[c] Given as dry weight.

In addition, small percentages of other units were detected in all the extracts. Some of the β-D-galactose units are non-substituted (*G*) or partially substituted on C-6 with single stubs of β-D-xylose (*G6X*). Lower percentages of the residue 2,3,4-tri-*O*-methylxylose (Table 2) than those expected from Table 1 could be due to losses of this residue during derivatization of the methylated sample (Stevenson & Furneaux, 1991). Methylation analysis also showed the presence of minor amounts of non-substituted 4-linked galactose (*G*), L-galactose substituted on C-3 (*LG3S*) and 3,6-anhydro-L-galactose substituted on C-2 (*LA2S*). Although small amounts of 6-*O*-methylgalactose could be attributed either to β-D-galactose 2,4-disulfate (*G2S4S*) or to α-galactose 2,3-disulfate (*G2S3S*), in this case this monomethylated galactose corresponds to the latter residue, according to the ^{13}C NMR spectra.

Diads of the agaran-type were assigned in the ^{13}C NMR spectra (Table 3 and Fig. 2) with the aid of the methylation analysis (Table 2). The peaks at 103.9 ppm and in the range of 101.3–101.9 ppm correspond to diads with the backbone *G–LG*, bearing different substituents (Miller & Blunt, 2002): (i) the disaccharidic repeating unit *G–LG3S* was confirmed in the spectra of A3, C4 and F (Fig. 2) based on signals at 101.3 ppm and 67.8 ppm, corresponding to C-1 and C-2, respectively, of the *LG3S* unit (Kolender & Matulewicz, 2002); (ii) the precursor diad of agarose *G–LG6S* was part of the structure of C3 and C4, with signals at 103.9 ppm and 101.5 ppm corresponding to the anomeric carbons of the units *G* and *LG6S*, respectively (Lahaye, Yaphe, Viet, & Rochas, 1989). Besides, the resonances at 104.2 ppm and 99.0–1 ppm in A1–A3 would correspond to the anomeric signals of the diad *G4S–LG2S3S* (Miller, 2001). In agreement with the linkage analysis, most of the 3,6-anhydro-α-L-galactose units are sulfated on C-2. A linear glucan constituted by 4-linked α-D-glucose units (floridean starch) was detected on the spectra of C4 and F (C-1 100.5 ppm, Tables 3 and 4). The presence of this structure was confirmed by analysis of the ^{1}H NMR spectrum of F, where an important signal at 5.344 ppm was assigned to H-1 of the 4-linked α-D-glucopyranose units. (Ciancia et al., 1993b; Knutsen & Grasdalen, 1987).

4. Discussion

4.1. Sulfated galactans distribution

The sulfated galactans show a homogeneous distribution in the thallus of *G. torulosus*, in a similar way to that

of *Eucheuma nudum* J. Agardh (Solieriaceae, Gigartinales) (LaClaire & Dawes, 1976). On the contrary, in another red seaweed of the latter family, *Kappaphycus alvarezii* (Doty) Doty (Vreeland et al., 1992; Zablackis et al., 1991) and in seaweeds belonging to Gigartinaceae (Flores et al., 1997; Foltran et al., 1996; Gordon-Mills et al., 1978) an uneven distribution of sulfate, which was more concentrated in the cortical regions, was found. On the other hand, in *Gelidium pacificum* Okamura (Gelidiaceae, Gelidiales) (Akatsuka & Iwamoto, 1979), it mainly occurred in the medullar region. Thus, it would appear that the location of sulfated polysaccharides in the thallus could differ between organisms, even phylogenetically close.

At cellular level, in medullar cells, the concentration of acidic polysaccharides in *G. torulosus* was higher in the intercellular matrix than in the cell walls, as it was generally observed in other seaweeds (Akatsuka & Iwamoto, 1979; Gordon-Mills et al., 1978; Kloareg & Quatrano, 1988; LaClaire & Dawes, 1976; Lechat, 1998; McCandless, Okada, Lott, Vollmer, & Gordon-Mills, 1977; Rascio, Mariani, Dalla Vecchia, & Trevisan, 1991). There are few exceptions, like *Gracilaria cf. verrucosa* (Hudson) Papenfuss (Talarico, Guida, Murano, & Piacquadio, 1990) and *I. cordata* (Foltran et al., 1996), in which the charged components were located mainly in the cell walls. In this region, for *G. torulosus*, it was possible to detect layers or zones rich in sulfated galactans negative to ruthenium red from other unstained layers of neutral polysaccharides. These results are in agreement with those reported for *C. crispus* and *K. alvarezii* (Gordon-Mills et al., 1978; Lechat, 1998; McCandless et al., 1977).

4.2. Galactans from G. torulosus

The major polysaccharides of *G. torulosus* are galactans constituted by major amounts of κ/ι-carrageenan-structures as previously suggested (Furneaux & Miller, 1985) and/or κ/ι-carrageenan DL-hybrids with minor but significant quantities of agarans and/or agaran DL-hybrids (Estevez et al., 2001).

Although the presence of agaran-structures in carrageenophytes is already well documented, the ratio carrageenan-: agaran-structures in *G. torulosus* is ca. 1:0.5 showing the highest amounts of agaran-structures for a seaweed considered a "carrageenophyte" (Table 1). Isolation by alkaline treatment and alkaline dialysis of "pure" κ/ι-carrageenans from the main extracts suggests that, at least, some carrageenan- and agaran-structures would be in different molecules.

The carrageenans are mainly κ/ι with small amounts of nu-structures (Estevez et al., 2001 and this study) but. the agarans counterpart do not have a structure similar to that of agarose; moreover, in *G. torulosus* no significant quantities of 3,6-anhydro-α-L-galactose units were detected and all the cyclized α-units present are substituted on C-2. Sulfation on the α-L-galactose residues occurs mainly on C-3 or C-2 and C-3.

Sulfated galactans from red seaweeds can be fractionated according to their water extraction capacity: (i) water extractable galactans and (ii) galactans extracted from the cell wall with highly chaotropic solvents.

Further fractionation of the water-soluble galactans can be obtained in some algae, such as *G. torulosus* (Phyllophoraceae) and *K. alvarezii* (Solieriaceae), by extracting sequentially with water at room temperature and at higher temperature (Estevez, Ciancia, & Cerezo, 2000, 2004; Estevez et al., 2001). This difference cannot be obtained in other seaweeds, such as *G. skottsbergii* and *S. crispata* (Gigartinaceae), where all the water-soluble carrageenans are obtained by extraction at room temperature (Matulewicz, Ciancia, Noseda, & Cerezo, 1989; Stortz & Cerezo, 1993). Compositional, enantiomeric and structural analysis of galactans from both sets of water extracts in *G. torulosus* showed differences only in: (i) the yields, small for the first set (A_n, fractions ca. 5–6% of the dry seaweed) and high for the second (C_n fractions, ca. 40–65%); (ii) the fact that the first extracted products do not look to play an obvious role in the intercellular matrix or in the cell walls, while the galactans from the hot-water extracts are main constituents of the intercellular matrix; (iii) the different average M_r between both room temperature and hot-water extracts (Table 1). Similar results were also obtained for *K. alvarezii* (Estevez et al., 2000, 2004). It is likely to hypothesize that: (i) the low M_r products would be precursors of the higher ones and they would be synthesized at the membranes of the Golgi apparatus and transferred to the cell wall-matrix to be polymerized, as it was suggested by Vreeland and Kloareg (2000) or (ii) they could be partial degradation products of the high M_r galactans. There are evidences that other red seaweeds also produce these variants of low M_r galactans (Ciancia, Matulewicz, & Cerezo, 1993c; Ciancia et al., 1993b; Matulewicz et al., 1989; Stortz, Cases, & Cerezo, 1997; Stortz & Cerezo, 1993). In cultured maize cells newly synthesized xyloglucan had M_r of 300 kDa, whereas 15 min later, they increased their M_r by sevenfold. In this case, there was a qualitative change of M_r in these polysaccharides from their site of synthesis towards to plasma membrane and ultimate secretion (Fry, 2004). On the other hand, in cultured rose cells, xyloglucans with low M_r (ca. 40 kDa) were sloughed into the medium, whereas the xyloglucans freshly deposited in the cell walls had M_r 4–6 times higher (Fry, 2004).

4.3. Carrageenan/agaran complexes

Alkaline treatment of A1 and C1 produces "pure" κ/ι-carrageenans (Table 1) suggesting that κ/ι-carrageenans biosynthesized by *G. torulosus* were forming aggregates with agarans and agaran DL-hybrids, similarly to those of *K. alvarezii* (Estevez et al., 2004). The changes in fraction C from *K. alvarezii* were explained in terms of complexes of κ-carrageenans and small fragments of agarans and/or agaran DL-hybrids formed, possibly through divalent calcium cations bridging two sulfate groups from different

molecules and being the arrangement stabilized by further complexation of the cation (Estevez et al., 2004; Hunter, Wong, & Kim, 1988; Ruggiero, Fossey, Santos, & Mourao, 1998). Alkaline treatment dissociate these complexes and the agaran fragments were lost during the alkaline dialysis. The fact that F was also a "pure" carrageenan suggests that also strong chaotropic mediums of the LiCl/DMSO type could dissociate these complexes Probably, this scheme of aggregation of small agaran molecules with higher M_r carrageenans is a general phenomenon between the galactan sulfates from carrageenophytes belonging to the Phyllophoraceae and Solieriaceae, and it should be tested for other carrageenophytes of the Gigartinales group. Complex formation between carrageenans and agarans was previously observed in other red seaweeds (Ciancia et al., 1993b, 1993c; Matulewicz et al., 1989; Stortz et al., 1997).

4.4. The mixed system of galactans (or DL-galactan hybrids ?) in red seaweeds

It has been found that "carrageenophytes" of related families as Gigartinaceae, Phyllophoraceae and Solieriaceae produce small amounts of agarans. These agarans were detected on each galactan system obtained from "carrageenophytes" where fractionation and enantiomeric analysis was carried out (Ciancia, Matulewicz, & Cerezo, 1997; Ciancia et al., 1993c; Estevez et al., 2000, 2001, 2004; Stortz et al., 1997). It seems that carrageenophytes from Gigartinales have the biosynthetic ability to produce not only carrageenans as major structures, but also agarans in variable amounts (Chopin et al., 1999; Miller, 1997; Stortz & Cerezo, 2000; Takano, Shiomato, Kamei, Hara, & Hirase, 2003). On the other hand, agarophytes were found to produce not only agarans but also carrageenans as minor components, while other groups of red algae, as Bonnemaisoniales, Halymeniales, Plocamiales, Rhodymeniales, biosynthesize both families of galactans in variable quantities (Stortz & Cerezo, 2000; Takano et al., 2003). Taking into account that agarophyte taxa of the order Bangiales is considered as the ancestral pool from which the evolutionary higher taxa of the Florideophyceae have arisen (Oliveira & Battacharya, 2000), the biosynthetic capacity to produce agarans may represent a primitive condition among the Florideophyceae, as suggested by the fact that seaweeds of the order Ahnfeltiales are agarophytes. These organisms would be interpreted as ancestral to taxa that contain carrageenans as major product in their cell walls (Fredericq, Hommersand, & Freshwater, 1996). Agarans and carrageenans are not always biosynthesized by monophyletic groups of red algae, but they are also present in paraphyletic or polyphyletic groupings (i.e. "agarophyte" red seaweeds comprise the orders Ahnfeltiales, Bangiales, Ceramiales, Gelidiales, Gracilariales). This fact could be explained by: (i) a multiple origin of each type of polysaccharides along the red algal evolution; (ii) differential gene expression by turning-off and turning-on those specific genes related only with carrageenan or only with agarans biosynthesis, assuming that most of the florideophycean seaweeds have the same pool of genes related with these two types of cell wall-polymers. However, the chemical structure of these products seems to be a stable chemotaxonomic character, useful for the characterization at genera or family level (Chopin et al., 1999; Miller, 1997; Stortz & Cerezo, 2000; Takano et al., 2003), but it is highly variable at ordinal or higher groupings.

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