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Polysaccharides from the green seaweed *Codium decorticatum*. Structure and cell wall distribution

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ABSTRACT

The cell wall polysaccharides from *Codium decorticatum* and their assembly were studied and these results were compared with those obtained previously for this genus. The water soluble polysaccharides are: (i) Pyruvylated and sulfated 3- and 6-linked β-D–galactans with sulfate mainly on C-4 and also on C-6. Pyruvate ketals are linked to O-3 and O-4 of terminal β-D-galactose or O-4 and O-6 of 3-linked β-D-galactose. (ii) Sulfated 3-linked β-L-arabinans substituted on C-2 or C-2 and C-4 predominantly with sulfate, but also with single stubs of arabinose, and (iii) 4-linked β-D-mannans with a low degree of sulfation on C-2. The whole polysaccharide system comprises 6.9% of sulfated polysaccharides and 32.9% of fibrillar polysaccharides, mostly insoluble mannans. By *in situ* localization it was possible to detect two similar fibrillar layers separated by a zone rich in charged polymers. Besides, arabinogalactan proteins co-localized with the fibrillar components.

1. Introduction

The genus *Codium* is comprised by more than 100 species, widely distributed in marine environments throughout the world, being found mainly in temperate and subtropical zones (Lam & Zechman, 2006; Oliveira-Carvalho, Oliveira, Pereira, & Verbruggen, 2012; Guiry & Guiry, 2014). The sulfated polysaccharides from a few species of this genus have been studied in detail (Bilan, Vinogradova, Shashkov, & Usov 2007; Ciancia et al., 2007; Estevez, Fernández, Kasulin, Dupree, & Ciancia 2009; Farias et al., 2008; Fernández, Estevez, Cerezo, & Ciancia 2012; Fernández et al., 2013; Love & Percival, 1964a; Ohta, Lee, Hayashi, & Hayashi 2009).

Codium species biosynthesize sulfated galactans constituted by 3-linked β-D-galactopyranose residues partially sulfated on C-4 and/or C-6, with ramifications of the backbone on C-6 and important amounts of pyruvate. For galactans from *Codium yezoense*, it has been shown that this substituent was forming mainly five-membered cyclic acetals (*S* isomer) with O-3 and O-4 of non-reducing terminal β-D-galactose residues, but also a minor part of pyruvate formed six-membered cyclic acetals with O-4 and O-6 (*R* isomer) (Bilan, Vinogradova, Shashkov, & Usov 2006; Bilan et al., 2007). The relative amount of these structural units in the galactan varies with the species, and for different fractions of the same species (Bilan et al., 2007; Estevez et al., 2009; Farias et al., 2008).

Besides, highly sulfated pyranosic arabinans were detected and characterized as part of the sulfated polymers from *Codium dwarkense*, *Codium fragile* and *Codium vermilara* (Ciancia et al., 2007; Estevez et al., 2009; Siddhanta, Shanmugam, Mody, Goswami, & Ramabat 1999), and recently those from the latter seaweed were isolated in a pure form and their structure was determined (Fernández et al., 2013).

Interesting biological activities have been described for some of these compounds. Antiherpetic activity and activity on the immune

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system were determined for sulfated galactans from *C. fragile*, which do not have significant anticoagulant effect, while highly sulfated arabinans from *C. vermilara* showed an important anticoagulant activity by mechanisms different to those described for heparin (Fernández, Arata, & Ciancia 2014), this basic research could led to application in the pharmaceutical industry in the future.

In addition, hydroxyproline-rich glycoproteins (HRGPs) have been found in minor quantities (Estevez et al., 2009; Fernández, Ciancia, Miravalles, & Estevez 2010). A small fraction from *C. fragile* showed neutral arabinan sequences composed by 3-linked and 5-linked α-L-arabinofuranose residues; it has been suggested that this structure could be part of the carbohydrate moiety of HRGPs (Estevez et al., 2009).

Seaweeds from the genus *Codium* have β-(1 → 4)-mannans instead of cellulose as major fibrillar component (Ciancia et al., 2007; Estevez et al., 2009; Huitzing & Rietema, 1975; Kaihou, Hayashi, Otsuru, & Maeda, 1993; Love & Percival, 1964b; Mackie & Sellen, 1969). Besides, a sulfated mannan was isolated from the hot water extract of *C. vermilara* (Fernández et al., 2012) and it was shown that its backbone is constituted by 4-linked β-D-mannose units, as the major fibrillar polysaccharides, but 23% of these units are sulfated on C-2. These sulfate groups should be the reason for higher solubility of this polymer.

The utricle cell wall in *C. vermilara* showed a sandwich structure of two fibrillar-like layers of similar width delimiting a middle amorphous-like zone. By immuno- and chemical-imaging, the *in situ* distribution of β-(1 → 4)-D-mannans and HRGP-like epitopes was shown to consist in two distinct cell wall layers, whereas sulfated polysaccharides are distributed in the middle area of the wall (Fernández et al., 2010). Some important differences were found in the distribution of polymers in the cell wall of the utricle of *C. fragile* (Estevez et al., 2009).

Codium decorticatum (Woodward) M.A. Howe, is a widely distributed species around tropical and subtropical regions of the world. In the Atlantic coast of South America it is distributed from 3°S to 42°S (Guiry & Guiry, 2014; Liuzzi, Gappa, & Piriz, 2011). This species grows on hard substrate in subtidal marine habitats.

In this work, determination of the structure of cell wall polysaccharides from *C. decorticatum* collected in the Patagonic coast (Argentina) and their cell wall assembly was carried out. These results were compared with those obtained for other species of this genus.

2. Materials and methods

2.1. Algal sample

Samples of *C. decorticatum* (Woodward) M.A. Howe were collected from a fresh beach-cast at Punta Ambrosetti (42°30′S, 64°30′W, Golfo Nuevo, Chubut province, Argentina) in November 2010. Thalli of *C. decorticatum* were composed by a medulla of densely entangled siphons and a green palisade-like layer of cylindrical utricles, slightly dilated at the apex (Fig. 1). Total length varied among 0.3–0.5 m and mature gametangia were observed. Voucher specimen of *C. decorticatum* was deposited in the herbarium of the Museo Bernardino Rivadavia (BA), Buenos Aires, Argentina (collection number 47427).

2.2. Light microscopy and histochemistry

For light microscopy (LM) semithin sections (10 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 based on

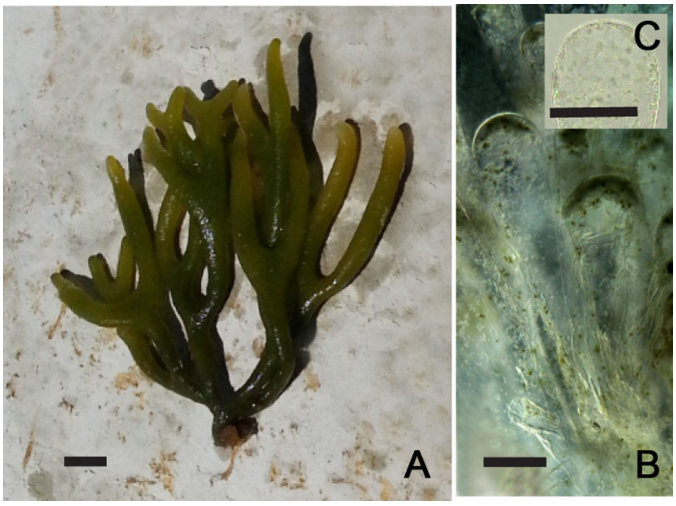

Fig. 1. (A) General aspect of the dichotomous branched thallus of *Codium decorticatum*. Scale bar = 1 cm. (B) The inner structure of the thallus. Scale bar = 1 mm. (C) The utricle view in detail. Scale bar = 200 μm.

Krishnamurthy (1999), were carried out on fixed tissues, included: (1) Toluidine Blue *O*, TB*O* (0.05% w/v) in 0.1 M HCl at pH 1.0 that stains sulfated polysaccharides (red-purple, γ metachromasia); (2) Ruthenium Red, RR (0.0001%) in aqueous solution for acidic polysaccharides (red); (3) Calcofluor White, CW (0.1% w/v) in aqueous solution for (1 → 3), (1 → 4)-β- and (1 → 4)-β-glycans (Wood, Fulcher, & Stone 1983); (5) β-glucosyl Yariv phenyl glycoside (β-GlcYR; 1,3,5-tris-[4-β-D-glucopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) was used for localized total AGPs due to its specificity and α-mannosyl Yariv phenyl glycoside (α-GalYR; 1,3,5-tris-[4-α-D-mannopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) was used as a control (Yariv, Rapport, & Graf 1962). Cross and longitudinal sections of the thallus were incubated overnight at 4 °C in 1% in NaCl (w/v) Yariv solutions.

2.3. Extraction of the polysaccharides

The extraction procedure was described elsewhere (Ciancia et al., 2007). Briefly, the milled plants (100 g) were extracted twice with EtOH (1 L) for 3 h at room temperature. The residue from the alcohol extraction was extracted with H_2O (20 g/L) at room temperature for 18 h giving a product, which was recovered from the supernatant by dialysis and freeze dried (fraction D1). The residue from the first H_2O extraction was extracted one more time in similar conditions to give fraction D2. The residue from the rt H_2O extraction was extracted twice for 3 h with H_2O at 90 °C, giving the soluble fractions C1 and C2, and the residue RC2.

2.4. General methods

The total sugar content was analyzed by PhOH–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) or by the method adapted for insoluble material (Ahmed & Labavitch, 1977). Sulfate was determined turbidimetrically (Dodgson & Price, 1962), except for desulfated F4. For this sample, ion exchange chromatography with conductimetric detection was used: F4 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 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 percentage of pyruvic acid was determined according to Koepsell and Sharpe (1952). The protein content was determined by the method of Lowry, Rosenbrough, and Farr (1951). The absolute configuration of arabinose, mannose, and galactose was estimated by the method of Cases, Cerezo, and Stortz (1995) through their diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino)-alditols. Treatment of C2 with α-amylase was carried out as described by Knutsen and Grasdalen (1987). Dialyses were carried out with tubing with molecular weight cutoff of 3500 Da.

Reductive hydrolysis of the native and methylated samples and acetylation of the sugar mixtures was performed according to Stevenson and Furneaux (1991) (method A). Alternatively, hydrolysis was carried out in 100% TFA for 1 h at 37 °C, 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 (Morrison, 1988); the sugar mixture was derivatized to the corresponding alditol acetates (method B).

GLC of the alditol acetates, as well as those of the partially methylated alditol acetates were carried out on an Agilent 7890 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 160 °C to 210 °C at 1 °C min^{-1}, then from 210 °C to 230 °C at 2° min^{-1} followed by a 30-min hold for partially methylated alditol acetates. 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.

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

2.5. Fractionation of the room temperature water extract (D1) with potassium chloride

The raw polysaccharides (0.5 g) were dissolved in water (250 ml, 0.25%). Solid, finely divided KCl was added to the supernatant in small portions with constant and violent mechanical agitation so that the concentration was increased by 0.1 M each time. After each addition, stirring was continued for 3–5 h to ensure equilibration of the system. The upper limit of KCl concentration was 2.0 M. The precipitates obtained by precipitation at 0.2 M and 0.75 M (2.0 and 2.9% of D1, respectively) as well as the residual solution were dialyzed, concentrated, and freeze-dried.

2.6. Ion exchange chromatography (IEC)

The samples (500–20 mg) were chromatographed on DEAE-Sephadex A-25. The mixture was dissolved in water and applied to a column (20–10 × 1.5–1.0 cm i.d.), previously stabilized in H_2O. The first elution solvent was water and then NaCl solutions of increasing concentration up to 4 M. Fractions of 7–2 ml were collected. Finally, the phase was boiled in 4 M NaCl solution. The presence of carbohydrates in the samples was detected by the phenol sulfuric acid method (Dubois et al., 1956); after obtaining blank readings, the eluant was replaced by another with higher concentration of NaCl. The products obtained were dialyzed and freeze dried.

2.7. 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 procedure was repeated to ensure permethylation. The methylated derivatives were recovered by dialysis and freeze–drying. Methylated samples were hydrolyzed with 2 M TFA for 2 h at 120 °C and the partially methylated sugars were converted into the corresponding alditol acetates as described before.

2.8. Desulfation of F4

The reaction was carried out by the microwave-assisted method described by Navarro, Flores, and Stortz (2007). The sample (15 mg) was converted to the pyridinium salt and dissolved in 10 ml of DMSO containing 2% of pyridine. The mixture was heated for 10 s intervals and cooled to 50 °C (×6). It was dyalized 3 days against tap water and then 24 h against distilled water (MWCO 3500) and lyofilized. An aliquot was methylated as described above without previous isolation of the product. By this treatment, 73% of the sulfate present in F4 was lost.

2.9. NMR spectroscopy

Samples (10–20 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 room temperature on a Bruker Avance II 500 spectrometer (Karlsruhe, Germany). 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.

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

Extracts D1 and D2 were obtained by sequential water extraction at room temperature, while extracts C1 and C2 were obtained with hot water, with a total yield of 8.0% of the ethanolic residue. The residue from the exhaustive water extraction (38.4% of the ethanolic residue) contained mannans as the major components, but also proteins and a small amount of sulfate (Table 1). Determination of the absolute configurations of the component sugars showed that galactose and mannose were present only in the D-configuration, while arabinose belonged only to the L-series.

D1 was fractionated with potassium chloride (Fernández et al., 2013), being most of the sample ($D1_{solKCl}$ 84% of the recovered material) soluble at a 2.0 M concentration of this salt. $D1_{solKCl}$ was further fractionated by anion exchange chromatography. Seven fractions were obtained, from which only F1, F3, and F4 gave important yields (Table 2). F1 and F4 were constituted by arabinose as major monosaccharide component, while in F3 galactose predominated. After this process, most of the protein was lost.

Table 2 shows a higher degree of sulfation for F4 than for F1, in agreement with their chromatographic behavior. However, methylation analyses of both fractions (Table 3) gave very similar results for the arabinan moiety being the arabinose units mostly

Table 1
Yields and analyses of the products obtained from *Codium decorticatum* by extraction with water at room temperature (D1 and D2) and at 90 °C (C1 and C2) and of R, the residue of exhaustive water extraction.

Extract	Yield[a] (%)	TC[b] (%)	Sulfate as SO_3Na (%)	Protein (%)	Monosacharides (mol%)						
					Gal	Ara	Man	Glc	Xyl	Rham	Fuc
D1	3.4	48.6	22.5	20.7	36.5	48.8	2.6	5.1	3.0	2.4	1.6
D2	1.0	49.7	18.2	34.5	32.1	23.2	27.2	9.9	2.8	3.0	1.8
C1	2.3	43.0	12.2	34.5	22.9	15.2	18.9	34.9	3.8	2.7	1.7
C2	1.3	27.3	10.1	26.5	13.9	6.6	48.1	25.8	3.4	1.0	1.2
R	38.4	60.5	3.5	18.0	1.2	1.1	95.9	1.9	–	–	–

[a] Expressed as percentage of alcohol insoluble residue.
[b] TC = total carbohydrate content.

Table 2
Yields and analyses of the major products obtained by fractionation of $D1_{solKCl}$ from *Codium decorticatum* by anion exchange chromatography on Sephadex A-25.

Fraction	SolventM, NaCl	Yield[a] (%)	TC[b] (%)	Sulfate as SO_3Na (%)	Pyruvic acid (%)	Protein (%)	Monosaccharides (mol%)						
							Gal	Ara	Man	Glc	Xyl	Rham	Fuc
F1	–	38.5	34.8	10.3	1.3	1.9	25.8	62.4	0.8	3.6	1.7	3.2	2.4
F3	1.0	17.3	41.6	19.0	8.9	7.0	87.6	2.4	1.1	tr[c]	2.9	3.7	1.9
F4	1.5	34.6	53.2	28.2	4.3	0.1	25.3	73.7	–	tr	tr	tr	tr

[a] Expressed as percentage of the total recovered (62%).
[b] TC = total carbohydrate content.
[c] Percentages lower than 1% are given as traces (tr).

disubstituted. In addition, significant amounts of 2-substituted units and terminal reducing units were detected. The different chromatographic behavior of the fractions could be due to aggregation with galactans of different degree of sulfation, or to structural differences not detected by methylation analysis.

Methylation analysis of desulfated F4 (Table 3) clearly showed that: (1) arabinose is in the pyranosic form, as a high percentage of 2,4-di-*O*-methylarabinose was found, while no 2,3,5-tri-*O*-methy-, 2,5-di-*O*-methyl-, 3,5-di-*O*-methyl-, or 5-*O*-methylarabinose were detected by gas chromatography-mass spectrometry of the partially methylated monosaccharides from F4 or its desulfated derivative; (2) at least half of the disubstituted arabinose units are disulfated; and (3) part of the substitution on C-2 could correspond to single stubs of arabinose or galactan side chains; the possibility of covalent linkages between arabinan and galactan structures could not be ruled out. A slight degradation took place, as suggested by the important increase in terminal non reducing units (Table 3).

In the anomeric region of the ^{13}C NMR spectrum of F4 (Fig. 2A), the signals corresponding to arabinose units are not well defined, giving a broad signal at δ 94–96, with a maximum at ~95 ppm. These values confirm that pyranosic arabinose units are in the β-configuration. Besides, ^{1}H NMR spectrum showed three signals at 5.37, which was previously assigned to the disulfated β-L-arabinopyranose units (Fernández et al., 2013), and two other signals at 5.24 and 5.16 (ratio 1:0.6:0.3). Based on methylation and desulfation–methylation analyses, the first one could correspond to β-L-arabinopyranose units substituted on C-2, possibly glycosylated, while the latter one could be attributed to terminal non reducing ends, which could arise from low molecular weight chains or side chains. Besides, signals in the ^{13}C NMR spectrum confirmed the presence of disulfated and 2-sulfated β-L-arabinopynose units (δ 94.8–95.3, 74.3, 70.0, 75.8, and 61.8, corresponding to C1–C5, respectively). In the HMQC spectrum, resolution was not enough to interpret the signals of the arabinan. However, signals of the galactan structure are clear (see below).

On the other hand, NMR spectra of F1 (Fig. 2B and C) are better resolved than those of F4, due, at least in part, to the low molecular weight of the sample, evident by the presence of peaks corresponding to reducing sugars in the anomeric region (δ 97.3/4.51 and 93.0/5.19, from the HMQC spectrum, δ 4.51, $J_{1,2}$ 7.9 Hz, δ 5.19, $J_{1,2}$ 3.9 Hz, from the ^{1}H NMR spectrum). In addition, the presence of small signals at δ 67.3/3.81,3.63, which corresponds to C5/H5,5′ of α-L-arabinopyranose shows the low molecular weight of this arabinan (Wishart et al., 2013).

A detailed analysis of the HMQC spectrum of F1 showed that the disubstituted arabinose units are sulfated on C-2, while the substituent on C-4 would not be sulfate, but possibly a side chain. This assumption is based on the absence of the signal at δ 75.4/4.87, assigned previously to C-4/H-4 of these units. Peaks corresponding to the remaining C1/H1, C2/H2, C5/H5 are clear in the spectrum at δ 95.6/5.37, 74.4/4.55, 61.8/4.05, respectively, confirming substitution on C-2 with sulfate and substitution on C-4, there are signals at δ 74.4/4.73 and 73.5/4.76, one of which could correspond to C4/H4 of this unit. On the other hand, signals of C4/H4 and C5/H5,5′ of 3-linked arabinopyranose units non-substituted on C-4 were also detected at δ 67.0/4.22 and 63.4/4.15, 3.71, respectively. Signals attributed to the galactan moiety are also present in the NMR spectra of F1 (see below).

Table 3
Composition (mol%) of monosaccharides produced by permethylation and hydrolysis of the major fractions obtained from $D1_{solKCl}$ by anion exchange chromatography.

Monosaccharide[b]	Fraction[a]			
	F1	F3	F4	Desulfated F4[c]
2,3,4-Ara	9.3	–	7.6	15.6
2,4-Ara	2.7	–	Tr[d]	19.8
2-Ara	3.9	–	tr	tr.
4-Ara	19.4	–	19.6	23.5
Ara	38.0	1.0	38.8	3.9
2,4,6-Gal	3.2	6.8	1.9	1.3
2,6-Gal	10.2	27.8	10.3	10.3
2,3-Gal	4.0	10.7	2.2	4.5
2,4-Gal	0.8	6.7	0.6	9.6
2-Gal	8.4	45.1	17.8	4.5
Gal (or 4-Gal)	tr	1.7	1.3	5.4

[a] Small amounts to traces of glucose were detected in all fraction.
[b] Methylated at the positions indicated.
[c] 1.6% of 2,3,4,6-Gal was detected in this sample.
[d] Percentages lower than 0.5% are given as traces (tr).

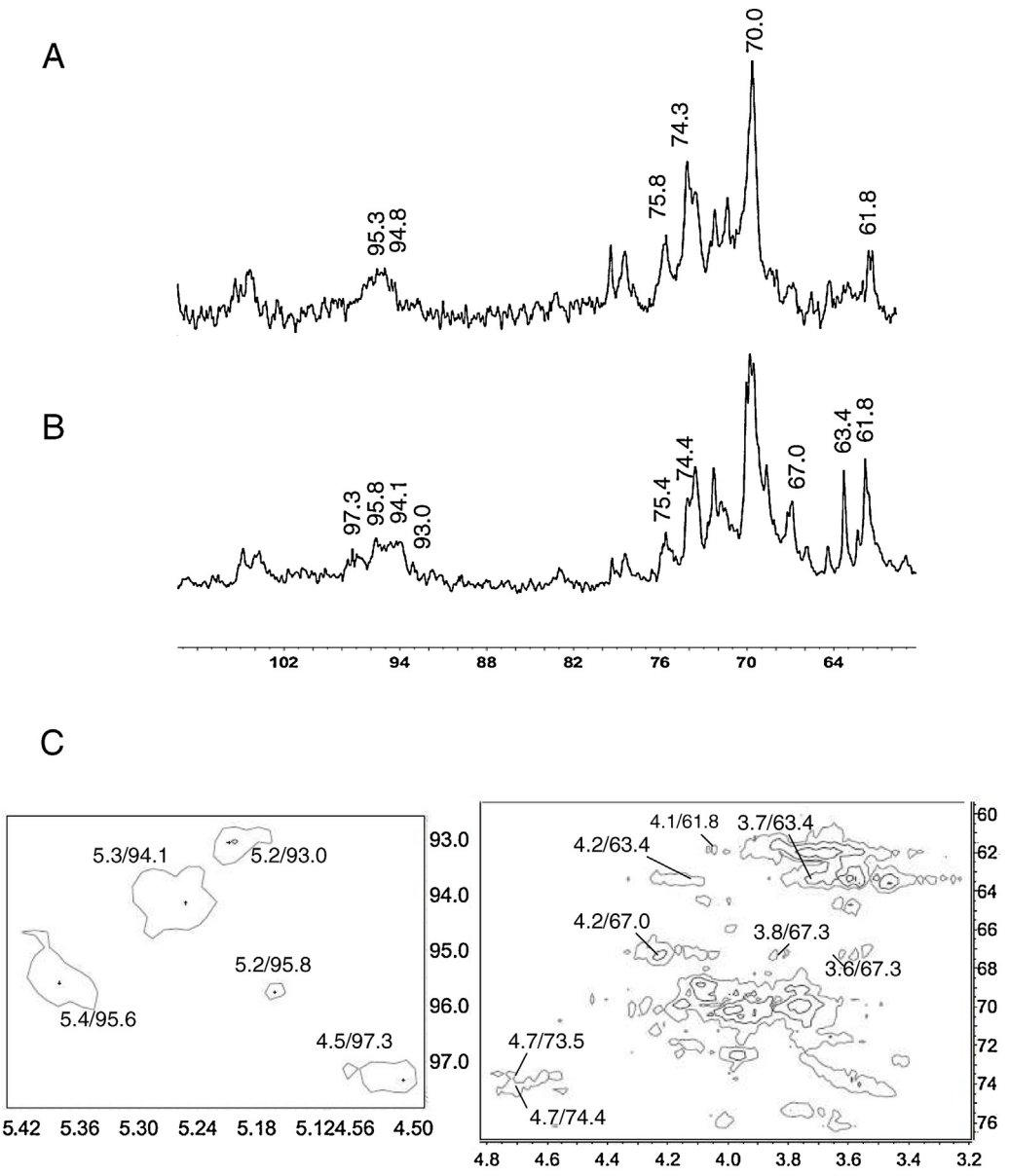

Fig. 2. NMR spectra of arabinan-rich fractions F1 and F4: (A) ^{13}C NMR spectrum of F4. (B) ^{13}C NMR spectrum of F1. (C) HMQC spectrum of F1. Only assignments corresponding to arabinan signals are indicated.

In the anomeric region of this spectrum there is also a signal at δ 94.1/5.25, which was tentatively attributed to 3-linked arabinopyranose units with single stubs or side chains linked to C-2. In this spectrum, it was also possible to detect that the signal at 5.15, similar to that found in the ^{1}H NMR spectrum of F4 and attributed to H1 of terminal non-reducing arabinopyranose residues, correlates with that at δ 95.8.

Only galactose partially methylated derivatives were obtained from F3. The percentage of pyruvic acid found in this fraction (Table 2) indicated that ~45% of the galactose units are pyruvylated. Besides, the presence of this substituent is clear in the NMR spectra forming two different ketal types, assigned based on work reported before (Bilan et al., 2006), namely, (1) a 5-membered ring linked to O-3 and O-4 of galactose units (*S* isomer), which is characterized by the presence of signals at δ 24.3/1.6 assigned to C3/H3 of this substituent and a signal at δ 108.8, clear in the ^{13}C NMR spectrum, but not present in the HMQC spectrum, corresponding to C2, and (2) a 6-membered ring linked to O-4 and O-6 of galactose units (*R* isomer), which is characterized by the presence of signals at δ 26.1/1.4 corresponding to C3/H3 and a signal at δ 101.9, only present in the ^{13}C NMR spectrum, assigned to C2 of this substituent (Fig. 3). Integration of signals of methyl group in the ^{1}H NMR spectrum gave a ratio of 1.0:1.2, indicating that ~20% of the galactose units have a 6-membered ketal, while 25% of these units have this substituent forming a 5-membered ring. Besides, there are no significant peaks in the region between 102 and 90 ppm, indicating that the galactose units are β-anomers (Bock & Pedersen, 1982), moreover, these signals correlate with proton signals between 4.9 and 4.5 ppm, which confirms the assignment. Based on this information and results from methylation analysis, it was deduced that F3 is composed by terminal 3,4-pyruvylated β-D-galactose units (~25%), 3-linked 4,6-pyruvylated β-D-galactose units (~20%), the characteristic signals of the latter units corresponding to C5/H5 and C6/H6,6′ at δ 67.0/3.6 and 65.9/3.9 are very clear in the ^{13}C and HMQC NMR spectra, 3,6-linked β-D-galactose 4-sulfate units (21%), 6-linked 4-sulfated β-D-galactose residues (11%), and 3- and 3,6-linked non-substituted β-D-galactose residues (7% for each unit type). The characteristic signal at 68.2/4.2 was found in the HMQC

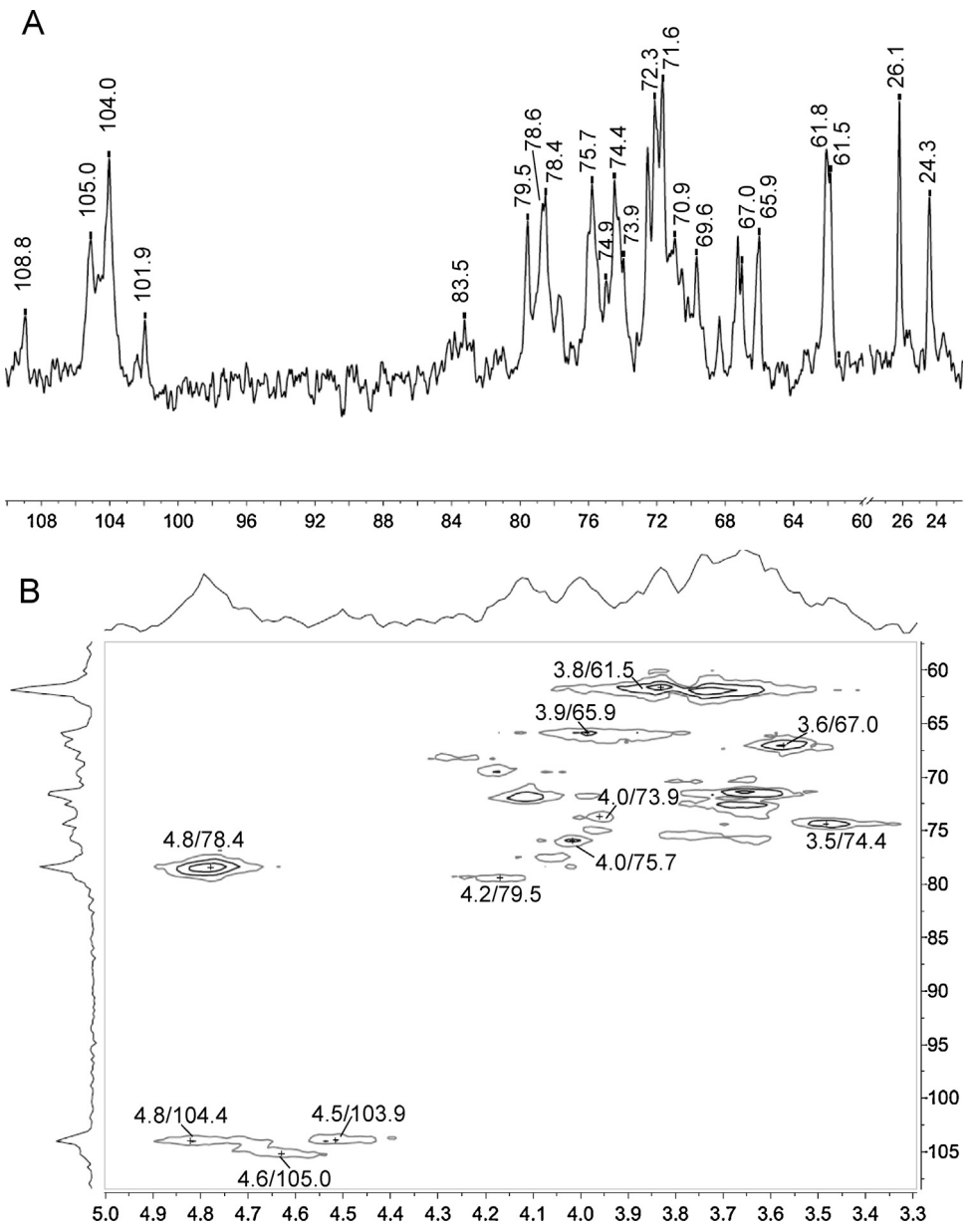

Fig. 3. NMR spectra of galactan-rich fraction F3: (A) ^{13}C NMR spectrum. (C) HMQC spectrum.

spectrum suggesting that a small amount of 3-linked 6-sulfated and/or 4,6-disulfated units could be present. Based on previous data (Bilan et al., 2007; Estevez et al., 2009) and analysis of the NMR spectra, it was possible to assign the signals of the most important units in this galactan (Fig. 3, Table 4).

In F4 the galactan moiety shows a high degree of substitution by pyruvic acid ketals (molar ratio galactose:pyruvic acid 1:0.59) and there is a predominance of pyruvic acid forming a 5-membered ring (ratio 1:0.3, calculated from integration in the ^{1}H NMR spectrum of the peaks at δ 1.6 and 1.4, respectively), closer to data

Table 4
NMR signals assignment of galactans from *C. decorticatum*.

Structural unit	Chemical shift (ppm)					
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
→3)β-D-Gal*p*(1→	105.0/4.6	71.6/3.7	83.5/3.9	69.6/4.0	75.7/3.6	61.8/3.7
3,4Pyr β-D-Gal*p* (1→[a]	104.0/4.8	74.4/3.5	79.5/4.2	75.7/4.0	73.9/4.0	61.5/3.8
→3)4,6Pyr β-D-Gal*p*(1→[b]	105.0/4.6	69.6/4.2	79.5/4.2	71.6/nd[c]	67.0/3.6	65.9/3.9
→3,6)β-D-Gal*p* 4S (1→	105.0/4.6	72.3/nd	78.6/4.0	78.4/4.8	74.9/nd	70.9/3.6
→3)β-D-Gal*p* 4S (1→[d]	104.0/4.5	71.5/3.7	79.3/4.1	78.4/4.8	75.4/3.9	61.8/3.7
→3)β-D-Gal*p* 4,6S (1→[d]	104.0/4.5	71.5/3.7	79.3/4.1	78.4/4.8	73.6/4.0	68.2/4.3

[a] Signals at δ 108.8 and 24.3/1.6 correspond to C2 and C3/H3 of pyruvic acid ketal linked to O-3 and O-4 of terminal galactose units (*S* isomer).
[b] Signals at δ 101.9 and 26.1/1.4 correspond to C2 and C3/H3 of pyruvic acid ketal linked to O-4 and O-6 of 3-linked galactose units (*R* isomer).
[c] nd: not determined.
[d] Not clear in F3, deduced from spectra of F4.

Table 5
Analysis of samples obtained by treatment of C2 with α-amylase and fraction rich in mannose.

Sample	Yield[a] (%)	TC[b] (%)	Sulfateas SO3Na (%)	Monosaccharides (mol%)						
				Gal	Ara	Man	Glc	Xyl	Rham	Fuc
C2	1.3	27.3	10.1	13.9	6.6	48.1	25.8	3.4	1.0	1.2
TrC2	63.9	32.5	9.5	17.9	15.9	47.1	7.8	3.8	2.3	5.2
Sugars released by dialysis	nd[c]	nd	nd	14.4	5.6	15.8	55.8	5.6	–	2.8
TrC2-2	13.8	nd	7.7	3.9	tr	83.8	7.7	2.2	1.3	1.2

[a] Yield for C2 (included for comparison) is given as percentage of alcohol insoluble residue, for TrC2, as percentage of C2, and for TrC2-2, as percentage of TrC2.
[b] TC = total carbohydrate content.
[c] nd = Not determined.

reported for galactans from other species of *Codium*, as *C. yezoense*, *C. fragile*, and *C. vermilara* (Bilan et al., 2006; Estevez et al., 2009; Fernández et al., 2014). In accordance with these data, the signals of C5/H5 and C6/H6, characteristic of 3-linked 4,6-pyruvylated β-D-galactose units are small. In addition, signals corresponding to 4-sulfation (C4/H4 at δ 78.4/4.80) and 6-sulfation (C6/H6 at δ 68.2/4.25) are important.

These results are in agreement with those from desulfation–methylation studies, which show that: (1) the percentage of 2,6-di-*O*-methylgalactose remains constant and (2) the high percentage of 2-*O*-methylgalactose between the partially methylated monosaccharides obtained by methylation analysis decreases after the desulfation procedure, with an increase in the 2,4-di-*O*-methylgalactose content, indicating the presence of important quantities of 3,6-linked β-D-galactose 4-sulfate units. The high amounts of negatively charged residues in this fraction could be the reason for the behavior of this galactan during the ion exchange chromatographic procedure.

The galactan moiety in F1 gave molar ratio galactose:pyruvic acid 1:0.26, with this substituent predominantly linked to O-3 and O-4 of terminal units (Tables 2 and 4). This suggests that around half of 2,6-di-*O*-methylgalactose derives from these terminal units, and half from 3-linked 4-sulfated units. The signal at δ 78.6/4.80, characteristic of C-4 of these units is very clear in the HMQC spectrum, as well as the other signals corresponding to this unit. Also, 3,6-linked β-D-galactose 4-sulfate and 6-linked β-D-galactose 4-sulfate are present in important quantities. Sulfation on C-6 was not detected.

By treatment of C2 with α-amylase, the percentage of glucose decreased, but there was no concomitant increase in the percentage of mannose (Table 5). Analysis of the monosaccharides released by dialysis showed a high percentage of glucose, as expected, but also some other sugars were lost. The treated product (TrC2) was fractionated by anion exchange chromatography obtaining six fractions, from which TrC2-2, which was eluted from the column with 0.25 M NaCl solution, was the richest in mannose (Table 5). Methylation analysis showed a high percentage of 4-linked non-substituted mannose units, but also 4-linked 2- and 6-substituted units (Table 6). The fact that this fraction has 7.7% of sulfate and no other sugar is present in significant quantities suggests that sulfate groups are linked to mannose units. In agreement with this, the high amount of mannose terminal units was attributed to the low molecular weight of this sample. These results suggest a similar sulfation pattern to that reported for the water soluble mannan from *C. vermilara*. Although TRC2-2 is less sulfated than the mannan isolated from *C. vermilara* (Fernández et al., 2012), the high solubility in water would be increased by the low molecular weight of the sample.

Table 6
Methylation analysis of mannan-rich fraction TrC2-2.

Mannose units[a]	Structural Unit	Unit (%)
2,3,4,6-Man	tMan	11.4
2,4,6-Man	3-Linked Man	3.0
2,3,6-Man	4-Linked Man	74.7
3,6-Man	4-Linked Man 2-sulfate	5.1
2,3-Man	4-Linked Man 6-sulfate	2.3
Man	nd[b]	3.5

[a] Methylated at the positions indicated. Small amounts of glucose derivatives were also detected.
[b] nd = Not determined.

Fig. 4 shows the polymer distribution pattern in the cell walls of *C. decorticatum*. The utricle cell wall shows an external cuticle and two fibrillar-like layers delimiting a middle amporphous region (Fig. 3A). Calcofluor white (CW) staining suggests that the fibrillar β-(1 → 4)-mannan is localized in two well-defined layers with similar development, whereas the central layer does not react (Fig. 3B). On the other hand, Toluidine Blue (TBO) and Ruthenium Red (RR)

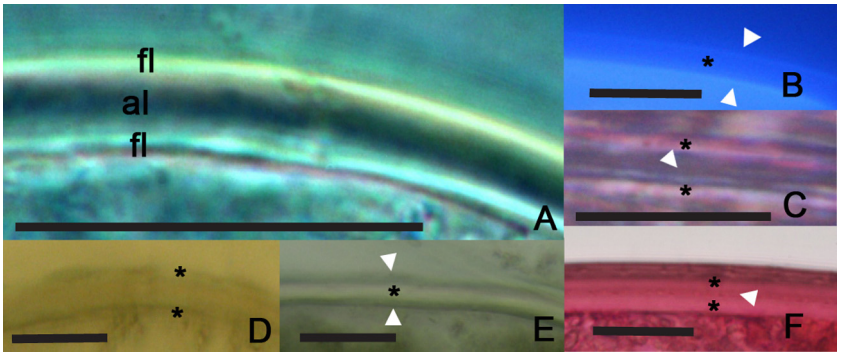

Fig. 4. *In situ* distribution of major polymer components in cell walls of *Codium decorticatum*. Positive staining areas are indicated with arrowheads and the absence or low staining is shown with asterisk (*) Scale bars = 10 μm. (A) Fibrillar (fl) and amorphous (al) layers of the utricle in longitudinal section observed with phase contrast microscopy. (B) Calcofluor White staining showing β-(1 → 4)-mannan localization. (C) Toluidine Blue *O* staining showing sulfated polysaccharides localization. (D–E) AGP localization. Positive β-glucosyl Yariv staining indicates the presence of AGP epitopes (E), while negative α-mannosyl Yariv staining was observed (D). (F) Ruthenium red staining showing negatively charged polysaccharides localization.

staining showed that sulfated polysaccharides are concentrated in the middle layer of the cell wall (Fig. 3C and F). Finally, β-glucosyl Yariv labeling for AGPs was detected in both marginal layers, that is, overlaping with mannans, whereas incubation with the negative control α-Mannosyl Yariv showed no reaction (Fig. 3D and E). These observations suggest a "sandwich" structure for the utricle cell wall of *C. decorticatum*. This pattern of distribution for the main components of cell wall is in agreement with models proposed for other species, especially *C. vermilara* (Estevez et al., 2009; Fernández et al., 2010).

In summary, 6.9% of soluble and 32.9% of fibrillar polysaccharides were isolated from *C. decorticatum*. The distribution arrangement of these polymers is similar to that found in the cell wall of *C. vermilara*, with some differences with that found for *C. fragile*. These differences are possibly related with the presence of the mucron in the latter, a characteristic structure formed in the utricle tip of this species. The presence of HRGPs in cell walls from *C. decorticatum* was suggested by reaction with β-glucosyl-Yariv reagent, and their distribution is similar to that found before for *C. fragile* (Estevez et al., 2009) and *C. vermilara* (Fernández et al., 2010).

Although the system of polysaccharides from *C. decorticatum* has the same general pattern as that from *C. vermilara* (Ciancia et al., 2007; Fernández et al., 2012, 2013) important differences were found in all the polysaccharide types. These differences were only evident after the detailed isolation and purification process described here, which reveals the interspecific differences in the cell wall polysaccharide components.

The major pyruvylated and sulfated galactan from *C. decorticatum*, F3, is a highly ramified polysaccharide, its backbone is constituted by 3-, 6-, and 3,6-linked, and terminal β-D-galactopyranose units, and sulfation occurs mainly on C-4 of some of these units, and also on C-6 in minor amounts. It has pyruvate ketals linked in both of the ways reported previously for other *Codium* species in high and close percentages. In this sense, this galactan is different to those of *C. vermilara*, *Codium isthmocladum*, and *C. yezoense*, where most of this substituent is linked to O-3 and O-4 of terminal β-D-galactose units, while in F3 from *C. decorticatum*, almost half of the pyruvate is forming a 6-membered ring with O-4 and O-6 of 3-linked β-D-galactose units. On the other hand, galactans present in other fractions showed predominance of the former units.

Besides, arabinans from *C. decorticatum* are less sulfated than that of *C. vermilara* (Fernández et al., 2013) and evidence presented here shows a high degree of branching, possibly with single stubs of arabinose, which had not been detected in the arabinans studied previously.

There are important differences in the degree and pattern of sulfation, as well as in the substitution pattern in the different fractions, which show an important dispersion of structures for the polysaccharides biosynthesized by this seaweed.

The sulfation pattern of the mannan studied in this paper is similar to that found for *C. vermilara*, but the molecular weight appears to be extremely low.

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