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Xylogalactans from *Lithothamnion heterocladum*, a crustose member of the Corallinales (Rhodophyta)

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ABSTRACT

The main polysaccharides from the red seaweed *Lithothamnion heterocladum* share the general characteristics of corallinans (agar-like xylogalactans), even though this seaweed is crustose, whereas those previously studied in detail were articulated seaweeds. After fractionation by cetrimide precipitation, several fractions were separated and characterized by sugar composition, other components, methylation, ethylation, desulfation–methylation, and NMR analyses. The main group of fractions carry the agaran disaccharidic repeating unit [→3)-β-D-Galp-(1 → 4)-α-L-Gal-(1→] substituted mainly on O-6 of the β-D-Gal unit by β-xylosyl side stubs, and less with sulfate or methoxyl groups, and also on O-2 of the α-L-Gal unit with methoxyl or sulfate, or less on O-3 of the same unit with methoxyl groups. These features are common to those of the polysaccharides of the four members of the order Corallinales already studied. However, a sugar not very common to the order appears in substantial proportions: 2,3-di-*O*-methyl-D-galactose, which acts as a side stub. Another peculiar issue is the presence of significant amounts of 4-linked α-L-Gal units glycosylated on O-6 or O-3. These results confirm that within a common pattern of corallinean xylogalactans, each species has minor characteristics of their own.

1. Introduction

In red seaweeds, sulfated galactans are the main constituents of the intercellular matrix and nonfibrillar cell walls. The structure of sulfated galactans can be represented by linear chains of alternating 3-linked β-galactose units and 4-linked α-galactose residues. Whereas the galactose in the former residues always belong to the D-series, the α-galactose residues may belong to the D- or the L-series, thus allowing to categorize these galactans as carrageenans (those with α-galactose units of the D-series) and agarans (those with 4-linked α-L-galactose residues). This straightforward idea can be obscured by the appearance of 4-linked D- and L-galactose moieties interspersed in the same molecules (DL hybrids), and by substitution with sulfate ester groups, pyruvic acid ketals, methyl ethers, sugar side stubs or the presence of a 3,6-anhydro ring replacing the α-galactose unit (Stortz & Cerezo, 2000).

Many of the red seaweed galactans, produced in high yields, have gelling and thickening properties, and thus carry industrial importance. For this reason, the polysaccharides originated in species of different orders have been studied in more or less detail (Stortz & Cerezo, 2000). On the other hand, those belonging to the order Corallinales have gathered less attention, probably due to a ten-to-thirty-fold decrease in polysaccharide yield, given their strong calcified cover (Bilan & Usov, 2001). After a preliminary report (Turvey & Simpson, 1965), a detailed study was made first with the Atlantic species *Corallina officinalis* (Cases, Stortz, & Cerezo, 1992), where a peculiar agaran was found, carrying methoxyl and/or sulfate groups on positions 2 and 3 of the L-galactose residues, and large amounts of β-D-xylosyl (or minor ones of sulfate) groups attached at the position 6 of the D-galactose units, within other minor features (Cases, Stortz, & Cerezo, 1994a). Similar structures, but with less complexity, were found in the Pacific Ocean species *Corallina pilulifera* (Usov, Bilan, & Klochkova, 1995; Usov, Bilan, & Shashkov, 1997), *Joculator maximus* (Takano, Hayashi, Hayashi, Hara, & Hirase, 1996), *Bossiella cretacea* (Usov & Bilan, 1996, 1998), and in the Atlantic Ocean species *Jania rubens* (Navarro & Stortz, 2008). Floridean starch was also a component of these seaweeds (Cases et al., 1992; Navarro & Stortz, 2002; Takano et al., 1996; Turvey & Simpson, 1965; Usov & Bilan, 1996; Usov et al., 1995), as well as alginate (Usov & Bilan, 1996; Usov et al., 1995). It was recently suggested the presence of a scarcely xylose-branched, highly 6-*O*-methoxylated galactan in genicular segments of an articulated coralline, and of a highly branched xylogalactan in their intergenicular segments (Martone, Navarro, Stortz, & Estévez, 2010).

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The thalli of red seaweeds from the order Corallinales can be either crustose or articulated. So far, only the xylogalactans of articulated corallines were studied in detail. Usov et al. (1995) have screened twenty different algal specimens, including two samples corresponding to the crustose species *Clathromorphum nereostratum* and *Lithothamnion phymatodeum*. They have found the presence of xylose, glucose, galactose and uronides within these seaweeds, though in smaller amounts than those found for most of the other 18 samples of articulated corallines (Usov et al., 1995). Herein, we report the isolation and fractionation of the xylogalactans from another crustose coralline, *Lithothamnion heterocladum* Foslie from the Tierra del Fuego Atlantic coast, as well as the structural characterization of the fractions using chemical and spectroscopical analyses.

2. Materials and methods

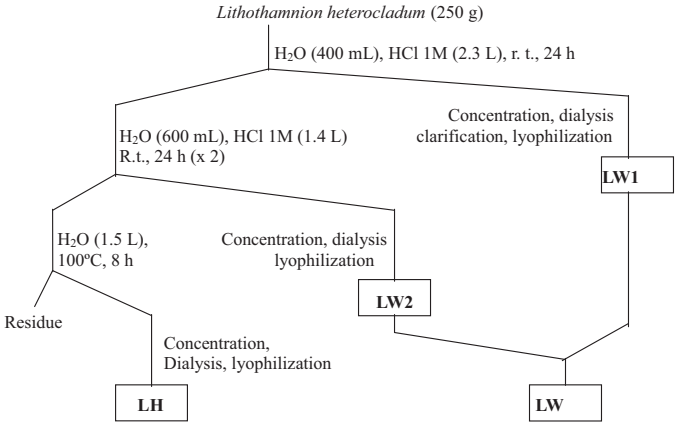
2.1. Materials

Samples of *L. heterocladum* Foslie were collected near Ushuaia (Tierra del Fuego Province, Argentina). The seaweeds were sorted, air dried, cleaned manually and milled to a fine powder before extraction. All chemical reagents were of analytical grade.

2.2. General methods

Total carbohydrates were determined by the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) standardized as depicted by Cases et al. (1992). Uronic acids were determined using the method of Filisetti-Cozzi and Carpita (1991) using glucuronolactone as standard. The percentages of sulfate were measured by turbidimetry (Dodgson & Price, 1962) after hydrolysis with 1 M HCl, whereas the soluble proteins were determined by the procedure of Lowry, Rosebrough, Farr, and Randall (1951). Average molecular weights were estimated as described by Park and Johnson (1949).

The proportions of monosaccharides constituting the polysaccharides were determined by gas chromatography (GLC) of the hydrolyzates, using different derivatives. The regular hydrolysis procedure was performed by treating the polysaccharides with 2 M trifluoroacetic acid (90 min at 120 °C). The TFA was eliminated by evaporation. Aliquots of the hydrolyzates were converted to their aldononitrile acetates (Turner & Cherniak, 1981) and to the alditol acetates, and analyzed by GLC on an HP 5890A apparatus fitted with a capillary column 30 m × 0.25 mm i.d. 0.20 μm, SP 2330 and equipped with a flame ionization detector operating at 240 °C. The injector temperature was 240 °C and the oven temperature was kept isothermally at 220 °C. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. Aliquots were injected with a split ratio of 80:1. Other aliquots of the hydrolyzates were converted to the acetylated aminodeoxyalditols using (*S*)-1-amino-2-propanol and (*S*)-α-methylbenzylamine (Cases, Cerezo, & Stortz, 1995) and analyzed by GLC as stated therein. In order to avoid destruction of the 3,6-anhydrogalactose, alditol acetates were obtained from the products of a reductive hydrolysis procedure (Stevenson & Furneaux, 1991) slightly modified as shown elsewhere (Navarro & Stortz, 2003). The configuration of the 3,6-anhydrogalactose was determined after mild hydrolysis and derivatization with (*S*)-α-methylbenzylamine, and analyzed as described (Navarro & Stortz, 2003). When necessary, GLC–MS analyses were carried out on a Shimadzu QP 5050 A GC/MS apparatus working at 70 eV using similar conditions to those described above, but using He as gas carrier at a flow rate of 0.7 mL/min and a split ratio of 11:1.



Scheme 1. Extractive sequence for the polysaccharides from *Lithothamnion heterocladum* (residues to the left, supernatants to the right).

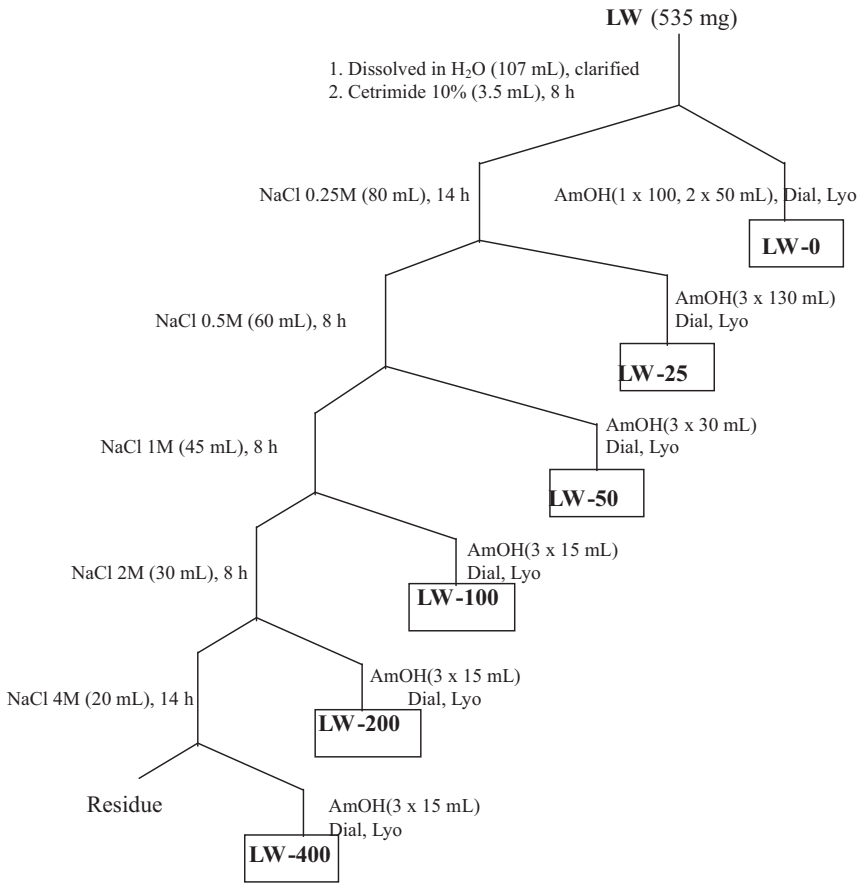
2.3. Extraction and fractionation

The extraction procedures are summarized in Scheme 1. Shortly, the milled seaweed (250 g) was extracted first with water at room temperature to which HCl 1 M was added up to a pH 6 (2.7 L), under mechanical stirring for 24 h. After centrifugation, the residue was reextracted in the same manner with 2 L of the acid mixture. The residues from each step were recovered by centrifugation, and the supernatants were concentrated and dialyzed (m.w. cut-off = 6000–8000). As the composition of both extracts was similar, both were merged to a total extract **LW**. The residue was reextracted with boiling water for 24 h with stirring, and the dissolved polysaccharides were isolated as stated before (**LH**).

The product **LW** was fractionated with the aid of cetrimide precipitation (Scheme 2): 535 mg of **LW** were dissolved in water (107 mL) and cleared up by centrifugation. A 10% (w/v) aqueous solution of hexadecyltrimethylammonium bromide (cetrimide) was added slowly to the solution until no further precipitation occurred (after 3.5 mL). The mixture was stirred for 8 h, and then the precipitate was centrifuged off. The supernatant (**L-0**) was isolated after washing with 1-pentanol (1× 100 mL, 2× 50 mL), dialysis and lyophilization, whereas the precipitate was suspended in 0.25 M NaCl (80 mL), and stirred overnight. The pellet was centrifuged off, and the supernatant was washed with 1-pentanol (3× 130 mL), dialyzed and lyophilized (**L-25**). The remaining precipitate was submitted to similar consecutive procedures with NaCl concentrations increased to 0.5, 1, 2 and 4 M, yielding fractions **L-50**, **L-100**, **L-200**, and **L-400**, respectively. The amounts of NaCl solutions and washouts with 1-pentanol were decreased, as shown in Scheme 2.

2.4. Desulfation, methylation and ethylation

The triethylammonium salts of the fractions (5 mg) were methylated as described by Ciucanu and Kerek (1984), using NaOH and CH_3I. The ethylation was carried out by a similar procedure, but using ethyl iodide as the alkylating agent (Cases, Stortz, & Cerezo, 1994b). The alkylated products (isolated by dialysis and lyophilization) were hydrolyzed (2 M TFA, 90 min, 120 °C), and the partially alkylated monosaccharides derivatized to the aldononitrile acetates and/or to the alditol acetates were analyzed by GLC with the conditions described elsewhere (Ponce, Pujol, Damonte, Flores, & Stortz, 2003), and characterized by GLC–MS. The determination of the configurations of 2,6-di- and 2,3,4,6-tetra-*O*-methylgalactose were carried out by reductive amination, as described elsewhere (Errea, Kolender, & Matulewicz, 2001; Navarro & Stortz, 2008).



Scheme 2. Fractionation of the polysaccharide **LW** from *Lithothamnion heterocladum* (residues to the left, supernatants to the right). Abbreviations: AmOH, extraction with 1-pentanol; Dial, dialysis (m.w. cut-off = 6000–8000); Lyo, lyophilization.

Desulfation was carried out by either the NaCl–methanol–Dowex resin procedure (Cases et al., 1994a) or solvolytic desulfation, carried out by the microwave-assisted procedure of Navarro, Flores, and Stortz (2007). The best procedure required a two-step reaction of 2× 60 s. The sample was subjected to an *in situ* methylation procedure. The desulfated–methylated product, after hydrolysis, was derivatized to the alditol acetates as described above.

2.5. Nuclear magnetic resonance

The spectra were obtained on a Bruker Avance II 500 spectrometer at 500.13 (^{1}H) and 125.77 (^{13}C) MHz provided with a 5-mm probe, at room temperature, using ca. 20 mg polysaccharide in 0.4 mL of D_2O. Acetone was added as internal standard (referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm in ^{13}C, 2.22 ppm in ^{1}H). Multiplicity determinations and 2D spectra were obtained using standard Bruker software.

2.6. Confocal microscopy

Dry algal thalli were re-hydrated in a HOAc/NaOAc buffer (50 mM, pH 4.5). Calcium carbonate was removed maintaining the pH at 4.5–5 with glacial acetic acid until ceasing of CO_2 bubbling. Decalcified sections (ca. 1 mm^2) were thoroughly washed in filtered seawater and fixed overnight in 1% glutaraldehyde at room temperature. Dehydration proceeded in acetone series followed by inclusion in Spurr resin. Semithin sections (0.5–2 μm) were collected with a wire loop and fixed to microscope slides on hot plate at 60 °C.

Fluorescent alginate probe was synthesized according to Vreeland and Laetsch (1989) by coupling the carboxyl moiety in alginic acid to 5-aminofluorescein in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at pH 4.7. Alginate fluorescent probe gave a single absorption peak at 492 nm.

For hybridization alginate-AF (0.5 μg mL^{-1}) was dissolved in 50 mM Tris buffer (pH 7.6) containing 1 mM EDTA. Semithin sections were covered with 25 μL of this solution plus either EDTA (1 mM) (blank) or 0.25 mM EDTA and $CaCl_2$ (40 mM). Incubation proceeded in the darkness for at least 2 h. Sections were thoroughly washed with 0.25 mM EDTA before observation. Images were obtained in a Leica DMIRE2 Confocal TCS SP2 SE microscope with a 488 nm band excitation filter and a 525–530 nm emission filter.

3. Results and discussion

3.1. Isolation, fractionation and analysis

A sample of the milled seaweed *L. heterocladum* was submitted to the extraction sequence as shown in Scheme 1. The extraction with diluted HCl (pH 6) in order to remove the calcium cover was deemed as necessary for the safe extraction of corallinean polysaccharides (Cases et al., 1992; Navarro & Stortz, 2002). The products from two consecutive extractions were obtained with 0.24 and 0.21% yield, respectively, with respect to the initial dry weight of seaweed. As their compositions in terms of total carbohydrates, sulfate, and constituent monosaccharides were almost identical, both products were merged into one, named **LW**. The analytical characteristics of this product are shown in Table 1. A further hot

Table 1
Yields and analyses of the products extracted from *Lithothamnion heterocladum* and of their fractionation products.

	LW	LH	LW-0	LW-25	LW-50	LW-100	LW-200	LW-400
Yield (%)[a]	0.45	1.3	6.2	44.5	8.0	0.8	0.7	0.6
Carbohydrates (%)	65	82	51	61	23	nd[b]	nd	nd
Protein (%)	17	7	14	5	6	nd	nd	nd
Sulfate (expressed as % $NaSO_3$)	11	1	tr.	7	8	nd	nd	nd
Mol. weight (kDa)	10.9	12.1	11.3	16.0	33.0	nd	nd	nd
Component sugars (mol/100 mol)[c]								
D-Xyl	21	5	14	25	23	23	33	34
2-*O*-Me-L-Gal	9	1	5	9	6	3	3	1
3-*O*-Me-D-Gal	tr.[d]	–	tr.	tr.	–	–	–	–
3-*O*-Me-L-Gal	1	–	1	1	–	–	–	–
6-*O*-Me-D-Gal	4	2	2	4	11	9	2	–
2,3-Di-*O*-Me-D-Gal	3		2	4	1			
D-Gal	23	4	13	26	28	45	43	45
L-Gal	21		11	24	23			
3,6-An-L-Gal	–	–	–	1	–	–	–	–
L-Fuc	3	–	1	2	7	7	8	2
D-Man	2	1	7	1	tr.	5	5	4
D-Glc	13	88	44	3	1	8	6	14
Ratios								
D/L	1.0	nd	1.0	1.0	1.4	nd	nd	nd
Gals/Xyl[e]	2.9	nd	2.4	2.8	3.0	2.5	1.5	1.4

[a] For **LW** and **LH**, yields from dry seaweed; for the remaining products, yield of fractionation of **LW**.
[b] nd = not determined.
[c] Calculated as an average of determinations made as alditol acetates, aldononitrile acetates and acetylated 1-deoxyaminoalditols, except for minor fractions.
[d] tr. = traces (<1%).
[e] Gals = galactose + methylated galactoses.

water extraction yielded an important proportion of a new product (**LH**), with composition also shown in Table 1. The yields of both products are similar to those found in previous studies (Navarro & Stortz, 2002). Both products were rich in carbohydrates, and also contained protein and sulfate ester. The proportion of uronic acid was very low (ca. 2.0% in **LW**, traces in **LH**). While **LW** was rich in the usual components of corallinean xylogalactans (xylose, D- and L-galactose, sulfate ester, methylated galactoses), with lesser amounts of starch, **LH** showed minor amounts of xylogalactan constituents, and larger proportions of glucose-containing polymers, as expected to occur in a product extracted with boiling water (Navarro & Stortz, 2002). Units of galactose methylated in three out of the four available positions were found in **LW**: those methoxylated in 2 belong to the L-series, whereas those methoxylated in 6 belong to the D-series, as occurred with other similar polysaccharides (Navarro & Stortz, 2008). Both enantiomers of 3-*O*-methylgalactose were encountered in very small proportions. The xylogalactan from *J. rubens* was the only one where both enantiomers were found up to date (Navarro & Stortz, 2008). The polysaccharide was devoid of 3,6-anhydrogalactose, but it contained about 3% of 2,3-di-*O*-methyl-D-galactose, a monosaccharide already encountered in *J. rubens* in lesser proportion (Navarro & Stortz, 2008). The D/L-galactose ratio (1.0, Table 1) is compatible with an agaran structure, whereas the Gal/Xyl ratio (ca. 3) agrees with those found previously in other corallinans (Stortz & Cerezo, 2000).

The **LW** product was fractionated after cetrimide precipitation and redissolution of the precipitate with increasing concentrations of sodium chloride. About 6% of the product remained soluble in the cetrimide solution. This fraction (**LW-0**) is almost devoid of anionic groups (Table 1), as expected, and appears to be a mixture of a glucan (probably floridean starch), a mannose-containing polysaccharide, and a xylogalactan with a monosaccharide composition similar to that redissolved in 0.25 M NaCl solution (**LW-25**), but very mildly sulfated. **LW-25** is, by far, the most important fraction in **LW** (almost one-half). Another significant fraction was isolated after redissolution with 0.5 M NaCl (**LW-50**), whereas three negligible fractions (less than 1% yield for each) were redissolved at higher NaCl concentrations (Table 1). The amounts of these isolated fractions precluded a detailed analysis, but the general characteristics of xylogalactans appear to hold, though increasing xylose content appears when increasing concentrations of NaCl are needed for redissolution. Also, the presence of other 'contaminating' monosaccharides (Table 1) is observed within these fractions. For the main fractions, the D/L-galactose ratio always keeps close to unity (Table 1), suggesting no major deviation from a classical agaran structure. This ratio is higher in **LW-50**, probably because of the presence of a larger proportion of D-galactose side stubs (see Section 3.6). Only in one fraction small proportions of 3,6-anhydrogalactose were encountered, shown to be of the L-series. No cyclizable 6-sulfate units were found in any of the major **LW**-fractions.

In the ^{13}C NMR spectrum of product **LH** (not shown) six signals easily identifiable as becoming from 4-linked α-D-glucose units (Gorin, 1981) appear, with only traces of other signals (as that representing branching on C-6, around 70 ppm). Therefore, this data is compatible with a low-branched starch as the main constituent of **LH**.

3.2. Methylation analysis of *LW-25*

The three main fractions (**LW-0**, **LW-25**, and **LW-50**) were submitted to methylation analysis (Table 2). Some degree of undermethylation was observed, as some proportion of monomethylated monosaccharides (ca. 6%) appears in the hydrolyzate. However, the main features given by the methylation pattern are clearly observed: for the most important fraction **LW-25**, 2,3,4-tri-*O*-methylxylose, 2,3,6-tri-*O*-methylgalactose and 2,4-di-*O*-methylgalactose are the main sugars (in similar amounts), as occurred in previous studies of corallinans (Cases et al., 1994a; Navarro & Stortz, 2008). If those sugars would have been alone, a regularly repetitive structure having alternating 3-linked, 6-*O*-xylosylated β-D-galactose units and 4-linked unsubstituted (or 2-*O*-methoxylated) α-L-galactose units should have been postulated. However, other sugars appear in lower but substantial amounts: 2,3,4,6-tetra-, 2,4,6-tri-, 2,6-di-, 2,3-di-, and 3,6-di-*O*-methylgalactose are the most important. All these methylated sugars were also found in previous studies (Cases et al., 1994a;

Table 2
Methylation analysis of three fractions obtained by cetrimide precipitation and NaCl redissolution of the **LW** polysaccharide of *L. heterocladum* (mol/100 mol Gals).[a]

Position of *O*-Me[b]	Structural unit	**LW-0**	**LW-25**	**LW-50**	**LW-25** desulf.
2,3,4-Xyl	Xyl-t	51	25	33	21
2,3-Xyl	→4)-Xyl	7	5	7	4
2,4-Xyl	→3)-Xyl	3	2	3	3
2-Xyl	→3,4)-Xyl	–	2	1	–
2,3,4,6-Gal	Gal-t	11	7	18	7
2,4,6-Gal	→3)-Gal	4	6	6	12
2,3,6-Gal	→4)-Gal	21	29	15	36
2,3,4-Gal	→6)-Gal	4	1	4	1
2,6-Gal	→4,3)-Gal	5	7	6	9
3,6-Gal	→4,2)-Gal	5	4	3	1
2,3-Gal	→4,6)-Gal	14	12	19	7
2,4-Gal	→3,6)-Gal	30	27	18	25
2-Gal	→4,3,6)-Gal	2	3	3	1
3-Gal	→4,2,6)-Gal	4	3	7	1
2,3,4,6-Glc	Glc-t	13	–	–	–
3,4,6-Glc	→2)-Glc	5	–	–	–
2,3,6-Glc	→4)-Glc	43	–	–	–
2,6-Glc	→4,3)-Glc	3	–	–	–
4,6-Glc	→2,3)-Glc	–	1	3	–
2,3-Glc	→4,6)-Glc	15	–	–	–
2,3,4,6-Man	Man-t	4	1	–	2
2,3,6-Man	→4)-Man	6	–	–	–

[a] Proportions lower than 1% are not indicated. Small proportions of non-methylated Gal, Glc, Man and Xyl were found in some fractions. Calculated as an average of the alditol and aldononitrile acetate results.
[b] 2,3,4-Xyl = 2,3,4-tri-*O*-methylxylose, etc.

Navarro & Stortz, 2008; Usov & Bilan, 1998; Usov et al., 1997). However, their relative proportions are very different: the main fractions from *C. officinalis*, *C. pilulifera* and *B. cretacea* have larger proportions of 2,6- and 3,6-di-*O*-methylgalactose, and very low amounts of 2,3-di- and 2,3,4,6-tetra-*O*-methylgalactose (Cases et al., 1994a; Usov & Bilan, 1998; Usov et al., 1997). Similar results were found in *J. maximus*, though the minor sugars were not found (Takano et al., 1996). The main fractions of *J. rubens* are similar with respect to the larger content of 3,6-di-, and of the lower content of 2,3-di- and 2,3,4,6-tetra-*O*-methylgalactose, though they also lacked 2,6-di-*O*-methylgalactose (Navarro & Stortz, 2008). The current study is largely atypical as 2,3-di-*O*-methylgalactose is the most important secondary product, and substantial amounts of the others also appear, including 2,3,4,6-tetra-*O*-methylgalactose. Each of those sugars has a distinctive structural meaning: 3,6-di-*O*-Me-Gal represents a 2-sulfated α-L-Gal unit; 2,3-di-*O*-Me-Gal represents a 6-substituted α-L-Gal unit; 2,6-di-*O*-Me-Gal might represent either a 3-substituted α-L-Gal unit, as occurs in *J. rubens* minor fractions (Navarro & Stortz, 2008) or a 4-sulfated β-D-Gal unit, as occurs in *B. cretacea* (Usov & Bilan, 1998); 2,3,4,6-tetra-*O*-methyl-Gal is indicative of branching. In previous studies, branching with galactose or its mono-*O*-methyl ethers was important for minor fractions but never for the main fractions (Cases et al., 1994a; Navarro & Stortz, 2008). In the present work it has been determined by reaction with (*S*)-methylbenzylamine that the 2,6-di-*O*-methylgalactose belongs to the L-series (suggesting a 3-substituted α-L-Gal unit), whereas the 2,3,4,6-tetra-*O*-methylgalactose belongs fully to the D-series. For the corallinan of *J. rubens* (Navarro & Stortz, 2008) terminal galactose units belonging to either series have occurred. The appearance of glucose just as its 4,6-di-*O*-methyl derivative, and of mannose as its tetramethylated derivative do not allow any structural conclusion, as their proportion was very low. On the other hand, 2,3,4-tri-*O*-methylxylose was not the only xylose derivative found. Other dimethylated and monomethylated derivatives were found (Table 2) in lower amounts. This might be due to undermethylation; however, the presence of these sugars in the desulfated sample (which suffers less undermethylation) is suggesting that, possibly, some double or multiple stubs of xylose might be occurring, linked either on O-3 or on O-4. In any case, their proportion should be low. The presence of a contaminating neutral xylan should be discarded in a fraction with anionic features. These less methylated xylose derivatives have already been observed (Navarro & Stortz, 2008).

3.3. Ethylation and desulfation–methylation analysis of ***LW-25***

In order to determine the location of naturally methylated sugars, ethylation (Cases et al., 1994b) was carried out on the main fraction, **LW-25**. The ethylation data (not shown) agree with those obtained by methylation, and with the proportions of naturally methylated sugars determined by direct analysis. The results show that the original 2,3-di-*O*-Me-D-Gal gives rise exclusively to the tetra-*O*-alkylated product, indicating that it acts only as a side chain. This was shown previously to occur for the minor fractions of the corallinan of *J. rubens* (Navarro & Stortz, 2008). The 2-*O*-MeGal gives rise just to 2,3,6-tri-*O*-alkylgalactose, and the 6-*O*-MeGal yields exclusively 2,4,6-tri-*O*-alkylgalactose. At last, the small amounts of 3-*O*-MeGal yield traces of 2,3,4,6-tetra- and 2,3,6-tri-*O*-alkylated products, suggesting that the D-enantiomer is a side stub, and the L-enantiomer is part of the main chain, as occurred with the xylogalactans from *J. rubens* (Navarro & Stortz, 2008). An interesting issue is that all of the 2,3,4,6-tetra-*O*-alkylgalactose appearing after ethylation becomes from naturally methylated galactoses, either in 2,3- (mostly), or 3-, and none from plain galactose.

In order to complete the chemical determination of the structure, a desulfation analysis followed by methylation was necessary. Attempts to desulfate by the old methanolic hydrogen chloride (Cases et al., 1994a) method were unfruitful. The use of the microwave-assisted solvolytic desulfation and then by the *in situ* methylation procedure developed in our lab (Navarro et al., 2007) only resulted in considerable desulfation after a two-step procedure. Neither a single 60 s treatment nor a 90 s treatment gave results distinguishable from that of the original **LW-25**. The proportion of alditol acetates after methylation and hydrolysis is shown in Table 2. A coarse analysis of the results indicates that the amount of 2,3,6-tri-*O*-methylgalactose increased at expenses of 2,3-di- and 3,6-di-*O*-methylgalactose (ca. 8%), indicating that the L-galactose unit is partially sulfated at O-2 and/or O-6. An increase of 2,4,6-tri-*O*-methylgalactose at expenses of 2,4-di- and 2-*O*-methylgalactose is also observed, suggesting the presence of some 6-sulfate in the

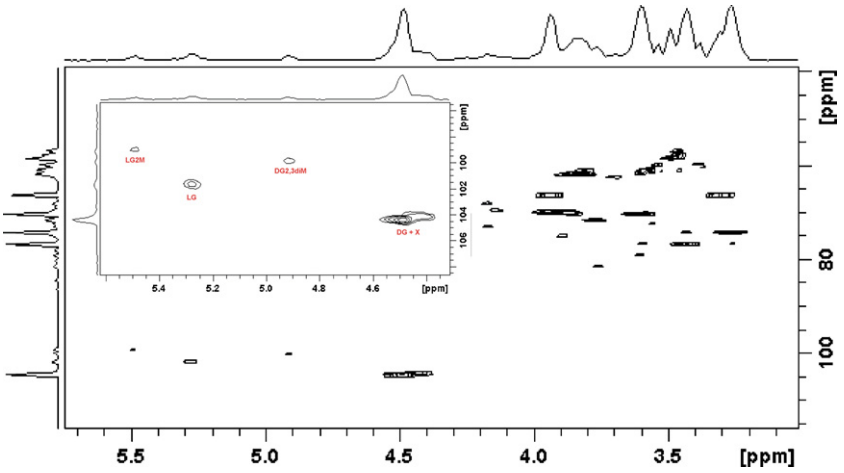


Fig. 1. 500 MHz HSQC spectrum of fraction **LW-25**. The inset represents an enlargement of the anomeric region, with the corresponding assignments.

3-linked D-galactose units. No major change in the proportion of 2,6-di-*O*-methylgalactose is observed, suggesting that the units leading to those methylated sugars are either not sulfated or carry a sulfate group non susceptible to solvolytic desulfation. The constancy in the proportion of tetramethylated galactose might be indicating that no major degradation took place during the desulfation procedure.

3.4. Structural analysis of *LW-25* by NMR

The ^{13}C NMR spectrum of **LW-25** (not shown) is very similar to those already published for other corallinans (Navarro & Stortz, 2008; Takano et al., 1996; Usov & Bilan, 1998; Usov et al., 1997). Five sharp peaks dominate the spectrum: they correspond to the signals assigned to the β-D-xylosyl side chains at 104.3 (C-1), 76.4 (C-3), 73.9 (C-2), 70.1 (C-4) and 65.9 ppm (C-5). This is consistent with a constant upfield shift of 0.6 ppm with respect to the work of Usov et al. (1997). For the remaining signals, this spectrum was too complex. The HSQC NMR spectrum of **LW-25** is shown in Fig. 1. Four anomeric signals were found. In the β-region, the signal at 104.1/4.43 ppm corresponds to all of the β-D-Gal units, either unsubstituted or substituted at O-6 with sulfate, methoxyl or xylosyl side stubs (Navarro & Stortz, 2008). In the α-region, the signals at 101.6/5.28 and 98.9/5.50 ppm correspond to the anomeric carbon/proton of 4-linked α-L-Gal units and 2-*O*-Me-α-L-Gal, respectively (Navarro & Stortz). The signal for 2-sulfated α-L-Gal units (99.5/5.50 ppm) could only be observable after raising the noise level. A signal appearing at 99.8/4.91 ppm (Fig. 2, inset) has never been observed previously in corallinans or other agarans. The higher field where the proton appears agrees with that observed for an α-D-Gal unit (Vinogradov, Kubler-Kielb, & Korenevsky, 2008). Together with the chemical determinations, it can be assumed that this signal corresponds to the 2,3-di-*O*-Me-D-Gal side stubs, indicating that their configuration is α. In the methoxyl region, the signals for 6-*O*-Me-D-Gal and 2-*O*-Me-L-Gal can be easily recognized at 59.4/3.38 and 58.2/3.50 ppm, respectively (Navarro & Stortz, 2008). The signals belonging to 2,3-di-*O*-Me-D-Gal were recognized with the aid of the spectrum obtained for fraction **3.1** of *J. rubens* (Navarro & Stortz, 2008), which also contained this sugar. They should correspond to those appearing at 56.6/3.46 and 57.5/3.46 ppm, with a probable assignment of the former to the methoxyl on O-3.

3.5. The structure of *LW-25*

The polysaccharides from only five species from the order Corallinales have been studied in detail up to the moment, all of them very similar: they share an agaran backbone, with C-6 of the β-D-Gal unit almost completely substituted, mostly with β-xylosyl side stubs, and less by sulfate, and with C-2 of α-L-Gal residues

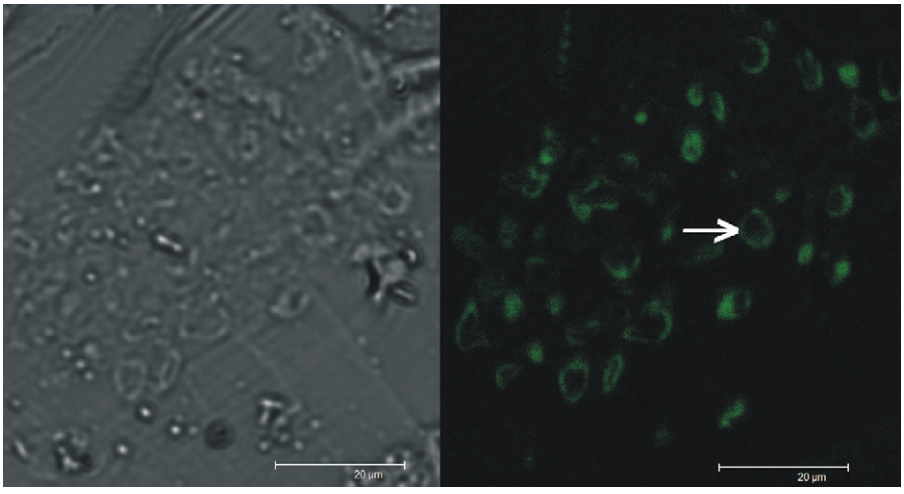


Fig. 2. Cross semithin sections of perithallial cells of *Lithothamnion heterocladum*. Left panel shows micrograph in bright field; right panel, confocal image of alginate-AF labeled cells (exc 488 nm, em 525–530 nm).

partly substituted by methoxyl and sulfate groups. In the most simple corallinan, that of *J. maximus* (Takano et al., 1996) most of the C-6 of the β-D-Gal units are substituted by xylosyl side stubs (50%) or sulfate ester (30%), whereas about half of the α-L-Gal units were substituted, in similar amounts in C-2 by a methoxyl group, in C-2 by a sulfate group and in C-3 by a sulfate group. For the xylogalactan from *C. pilulifera* (Usov et al., 1995, 1997) a similar pattern was found, but with more complexity: the D-Gal unit is mostly substituted with xylose, but some methoxyl substitution appears. For the other unit, a small proportion of 3-*O*-Me-Gal also appears, giving three different methoxylated Gal units. In *B. cretacea* (Usov & Bilan, 1996, 1998) the α-L-Gal units have similar substitution patterns to those of *J. maximus*, but in the β-D-Gal units the C-4 appears partly (ca. 15%) sulfated, a fact usual in carrageenans but not in agarans (Stortz & Cerezo, 2000), at expenses of 6-sulfated units. For *C. officinalis*, many fractions were isolated (Cases et al., 1992, 1994a). A similar pattern, though more complex, is observed. For the main fractions, which represent two-thirds of the extract, the substitution of the β-D-Gal unit is similar to that of *C. pilulifera*, whereas the α-L-Gal unit is much more substituted: larger sulfation of C-3, and larger methoxylation on C-2. Also, additional branching with 4-*O*-Me-Gal appears. In the main fractions of *J. rubens* within a similar general pattern, 3,6-anhydrogalactose is found for the first time in corallinans, C-3 of the α-L-Gal unit is partly substituted by methoxyl, and C-3 and C-6 of the α-L-Gal unit are partly substituted by glycosidic stubs (mainly 3-Me-D-Gal, but possibly others as Xyl, 2,3-di-Me-D-Gal or 3-Me-L-Gal). The main polysaccharide from *L. heterocladum* (**LW-25**) shows similarities with all the articulate corallinean polysaccharides found so far, but its structure appears to be closer to that of *J. rubens*, also a representative of the Atlantic Ocean. However, the current polysaccharide resembles better the minor fractions found in *J. rubens*, and not the major structures, as in the current polysaccharide important proportions of 2,3-di-*O*-methyl-D-galactose appear as side stubs, and significant proportions of the 3- and 6-positions of 4-linked α-L-galactose moieties appear glycosylated, or (in minor proportions) sulfated. The average of data corresponding to NMR, methylation, desulfation and ethylation analyses allow to determine that the main agaran structure has, for the 3-linked β-D-Gal unit a pattern characterized (for every 50 units) by unsubstituted (6 units), 6-glycosylated (34 units), 6-sulfated (8 units), and 6-methylated (6 units) moieties. This pattern for the β-units is almost identical to that found for fraction **2** or **3a** (minor fractions) of *J. rubens* (Navarro & Stortz, 2008). On the other hand, the α-L-Gal unit appears with a larger variability: the pattern for every 50 units can be defined by unsubstituted (15 units), 2-methylated (13 units), 2-sulfated (6 units), 3-glycosylated (8 units), 6-glycosylated (6 units) plus traces of 3-methylated, 6-sulfated and 3,6-anhydrogalactose. This pattern resembles the early-eluting minor fractions of *J. rubens* regarding glycosylated and 2-sulfated units, but the proportion of unsubstituted and 3-methylated units is more alike the major fractions, and the proportion of 2-methylated units is larger than that found for any fraction of *J. rubens*. The larger amount of glycosylation in α-L-Gal units matches the larger amount of 2,3-di-*O*-methylgalactose, which has been shown to act just as a side chain.

3.6. Methylation analysis of minor fractions

As pointed out in Section 3.2, fractions **LW-0** and **LW-50** were also submitted to methylation analysis (Table 2). For **LW-0**, besides the xylogalactan, Glc- and Man-containing polysaccharides are present. The glucose-containing polysaccharide is mainly floridean starch, as shown by the presence of 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3-di-*O*-Me-Glc as the main derivatives. Their amounts suggest a branching of about 25% of the chain units, indicating that the starch is split according to its branching degree: that with higher branching is extracted at room temperature, whereas the low-branched starch is extracted with hot water (**LH**), as suggested by its ^{13}C NMR spectrum (see Section 3.1). Mannose appears mainly as 2,3,6-tri-*O*-Me-Man, suggesting the presence of a 4-linked mannan. However, the presence of 2,3,4,6-tetra-*O*-Me-Man without any branching point indicates that mannose can also be part of a heteropolysaccharide, probably combined with xylose. Xylomannans are common polysaccharides in red seaweeds (Cardoso, Noseda, Fujii, Zibetti, & Duarte, 2007; Kolender, Pujol, Damonte, Matulewicz, & Cerezo, 1997), though never reported on the Corallinales. Some Glc derivatives methylated at 3,4,6- and 2,6- also appear; they might become from undermethylation of starch or from other polysaccharides.

The xylogalactans present in **LW-0** and **LW-50** share common features with those of **LW-25**. However, the quantitative features differentiate them. For the 3-linked β-D-Gal units the trend is clear: the proportion of 6-substituted (by glycosyl groups or sulfate) units decreases with an increase in the NaCl concentration, whereas the unsubstituted and 4-substituted units increase. On the other hand, for the 4-linked α-L-Gal units, the unsubstituted (or 2-methoxyl substituted) units peak for the **LW-25** fraction and decrease for **LW-0** and further for **LW-50**, whereas the substitution on C-6 and on C-2 + C-6 increases in the same direction. The substitution on C-2 is more evenly distributed for the three fractions. It is noteworthy that these two fractions, for which methylation shows larger proportion of branching on the α-Gal units (indicated mainly by the 2,3-di-*O*-methylgalactose) are richer both in terminal galactose and xylose units, suggesting that the C-6 position of these α-Gal units is glycosylated with galactose or some of its methyl ethers, and/or with xylose.

3.7. Histochemical detection of alginic acid

In situ alginate-FITC probe hybridization (Fig. 2) shows that fluorescence is restricted to cell walls. Note that when cross section traverses the central plane of the cell, an homogeneous weak labeling appears in the whole contour (Fig. 2, arrow), in agreement with the estimated alginate content. To the best of our knowledge this is the first report on *in situ* localization of alginate in the cell wall of a coralline alga. Recently, Stanley (2008) proposed that biomineralization in coralline algae proceeds with little control of the organism over its skeletal mineralogy, being calcite precipitation a consequence of the photosynthetic removal of CO_2 from the internal fluids. Such mechanism for calcite deposition confers protagonism to the organic matrix in the cell walls in the nucleation of calcite crystals, as suggested by Bilan and Usov (2001) and Martone et al. (2010). Nevertheless the occurrence of sulfated galactans and alginates is widespread in non-calcified red and brown algae, respectively. The question remaining is whether the initiation of $CaCO_3$ deposition is determined by a particular assembly of cell wall amorphous matrix polysaccharides in the cell wall. This might turn out to be not a minor item facing the role of coralline algae in oceanic biogeochemistry upon the challenge of ocean acidification and global warming (Kuffner, Andersson, Jokiel, Rodgers, & Mackenzie, 2008; Ries, Cohen, & McCorkle, 2009).

3.8. Can methylated sugars act as biological markers?

It is noteworthy the presence of several distinctive monosaccharides as side chains of corallinans. In *C. officinalis* (Cases et al., 1994a), 4-*O*-methyl-L-galactose acted as side stub, especially in some early-eluting fractions. In *J. rubens* (Navarro & Stortz, 2008), both 3-*O*-methyl- and 2,3-di-*O*-methyl-D-galactose acted as side stubs, the former more abundantly in all fractions, but especially in the less-abundant early-eluting ones. In the current xylogalactan from *L. heterocladum* the 2,3-dimethylated galactose unit appears

in larger amounts, and also solely as site stub. These three sugars have a common feature: they cannot be inserted into the regular agaran chain, as they have methoxylated the position where they should be linked (C-3 on D-Gal units, C-4 on L-Gal units). Thus, assuming a regular agaran structure, they can only appear as side stubs, and so they occur. A possible explanation could be that these methoxyl groups act as some sort of biological (species-specific?) marker, as occurs with minor sugars (2-*O*-Me-Fuc, 2-*O*-Me-Xyl, aceric acid, etc.) in the rhamnogalacturonan (RG-II) of flowering plants (Carpita & Gibeaut, 1993). The possible role of putative methoxyl transferases in the calcification process of corallinean cell walls has already been suggested (Martone et al., 2010). The presence of higher amounts of these methoxylated side stubs, as compared with those at the articulated species previously studied, can be related to the crustose nature of the current seaweed. Markers may be needed, for instance, to allow decalcification to occur during cell division: the newly formed, subapical cells have to move to the surface of the thallus, and such displacement is only possible once the mineral skeleton between the old and the new cells is removed. Pueschel, Judson, and Wegeberg (2005) observed that this demineralization occurs in precise locations in the cell walls, which suggests the presence of markers which direct the enzymatic and secretory machinery.

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