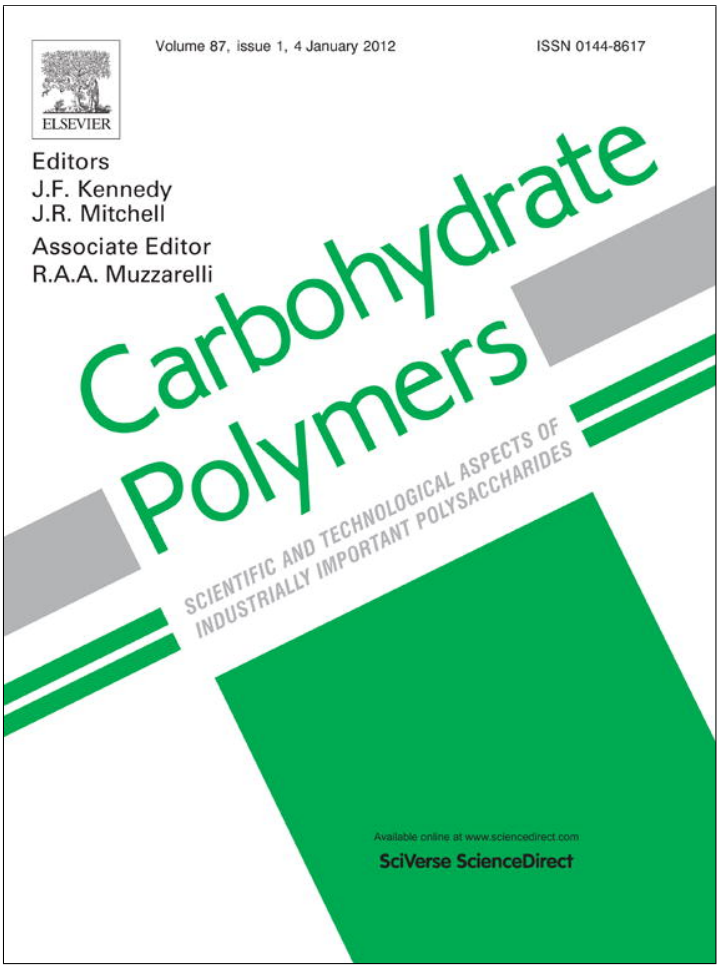

Carbohydrate Polymers 87 (2012) 916–919

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Short communication

Sulfated β-D-mannan from green seaweed *Codium vermilara*

Paula Virginia Fernández [a], José Manuel Estevez [b,1], Alberto Saúl Cerezo [c,1], Marina Ciancia [a,c,*,1]

[a] *Cátedra de Química de Biomoléculas, Departamento de Biología Aplicada y Alimentos, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, 1417 Buenos Aires, Argentina*
[b] *Departamento de Fisiología, Biología Molecular y Celular (IFIByNE-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria – Pabellón 2, 1428 Buenos Aires, Argentina*
[c] *CIHIDECAR-CONICET, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, 1428 Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 18 May 2011
Received in revised form 16 June 2011
Accepted 21 June 2011
Available online 30 June 2011

Keywords:
β-Mannan
Sulfated polysaccharide
Green seaweed
Codium

ABSTRACT

β-(1 → 4)-D-Mannans constitute the major component of the cell wall of seaweeds of the genus *Codium* and replace cellulose as the major fibrillar component. They were found as major constituents of the hot water extracts of green seaweed *Codium vermilara*. By anion exchange chromatography of the first hot water extract, a pure sulfated mannan with a molar ratio carbohydrates:sulfate of 2.7:1 was isolated. The sulfate groups are linked to C-2 of 23% of the mannose units, while most of these units are not substituted. This degree of sulfation would explain the higher solubility of the polymer, compared to that of the non-sulfated fibrillar mannan. Taking into account that the fibrillar polysaccharides form two external layers in the cell wall, while the sulfated polymers are forming an amorphous central layer, it is postulated that these sulfated mannans could act as an interphase region between the neutral and acidic layers.

1. Introduction

Green seaweeds of the Bryopsidales group (including *Bryopsis*, *Codium*, *Debersia*) are interesting because their cell walls are composed of β-(1 → 4) and/or β-(1 → 3)-glycans and they lack cellulose-based polymer as the major structural cell wall polysaccharides (Percival & McDowell, 1981). Particularly, the major fibrillar components of the cell walls of several Bryopsidales are β-(1 → 4)-mannans (Chanzy, Grosrenaud, Vuong, & Mackie, 1984; Ciancia et al., 2007; Estevez, Fernández, Kasulin, Dupree, & Ciancia, 2009; Huizing & Rietema, 1975; Kaihou, Hayashi, Otsuru, & Maeda, 1993; Love & Percival, 1964; Mackie & Preston, 1968). Interestingly, it has been shown that the major fibrillar component of the cell wall can vary in the different life stages (Huizing & Rietema, 1975; Huizing, Rietema, & Sietsma, 1979; Wutz & Zetsche, 1976). Thus, sporophyte macrothalli of *Derbesia* sps. biosynthesize mannans, while gametophyte microthalli of the same genus produce xylans. Similarly, sporophyte microthalli from the genus *Bryopsis* biosynthesize mannans, while gametophyte macrothalli produce xylans.

In addition, in green seaweed *Acetabularia acetabulum* (Dasycladales, Polyphysaceae) it was shown recently that the diplophase biosynthesizes mostly non-crystalline, as well as *para*-crystalline (1 → 4)-β-D-mannans, possibly with low amounts of substitution, while gametangia have a cellulosic wall, with mannans and other polymers representing about a quarter of the mass (Dunn et al., 2007). Also, it has been shown that the unrelated green seaweed *Codiolum pusillum* (Chlorococcales, Endosphaeraceae) synthesizes a β-(1 → 4)-mannan branched at C-6 and carrying sulfate groups on C-2, although this is not the major sulfated polysaccharide biosynthesized by this unicellular seaweed (Carlberg & Percival, 1977).

Sulfated xylomannans have been found in red seaweeds of the Nemaliales, but these polymers are composed of α-D-mannosyl residues (Pérez Recalde, Noseda, Pujol, Carlucci, & Matulewicz, 2009). However, a β-(1 → 4)-mannan with similar substitution pattern to that of *C. pusillum* was isolated from red seaweeds *Chondrophycus papillosus* and *C. flagelliferus* (Ceramiales) (Cardoso, Noseda, Fujii, Zibetti, & Duarte, 2007). This mannan was partially sulfated on C-2 of the mannose units and also substituted on C-6 with single stubs of β-D-xylose, as well as with β-D-mannose 2-sulfate units. All these examples suggest that mannan synthases (ManS), the enzymes in charge of synthesizing mannans, have evolved multiple times along the red and green algae evolution (Popper & Tuohy, 2010).

In this paper we report the isolation and chemical characterization of a partially 2-sulfated β-(1 → 4)-D-mannan from the green seaweed *Codium vermilara*.

* Corresponding author at: Cátedra de Química de Biomoléculas, Departamento de Biología Aplicada y Alimentos, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, 1417 Buenos Aires, Argentina.
Tel.: +54 11 4524 4042/8088; fax: +54 11 4524 8088.
E-mail address: ciancia@agro.uba.ar (M. Ciancia).
[1] Research Member of the National Research Council of Argentina (CONICET).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.06.063

2. Experimental

2.1. Isolation of the mannan

The hot water extract from *C. vermilara* (W1) was obtained as described before (Ciancia et al., 2007). W1 was chromatographed on DEAE-Sephadex A-25. The sample (100 mg) was dissolved in water, centrifuged (36.2% insoluble) and the supernatant was applied to a column (90 cm × 1.5 cm id), previously stabilized in H_2O. The first elution solvent was water and then NaCl solutions of increasing concentration up to 4 M. Fractions of 4 ml were collected. Finally, the chromatographic gel matrix was boiled in 4 M NaCl solution. The presence of carbohydrates in the samples was detected by the phenol sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). After obtaining blank readings, the eluant was replaced by another with higher concentration of NaCl. The products obtained were dialyzed (MWCO 3500) and freeze dried.

2.2. Chemical analysis

The total sugars content was analyzed by the PhOH–H_2SO_4 method (Dubois et al., 1956). Sulfate was determined turbidimetrically (Dodgson & Price, 1962). The protein content was determined by the method of Lowry, Rosenbrough, and Farr (1951). 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. To determine the monosaccharide composition, samples were derivatized to the alditol acetates (Stevenson & Furneaux, 1991).

2.3. Methylation analysis

The polysaccharide (10 mg) was converted into the corresponding triethylammonium salt (Stevenson & Furneaux, 1991) and methylated according to Ciucanu and Kerek (1984). The sample was dissolved in dimethylsulfoxide; finely powdered NaOH was used as base. The methylated samples were submitted to reductive hydrolysis and acetylation to give the alditol acetates in the same way as the parent polysaccharides (Stevenson & Furneaux, 1991).

2.4. Gas chromatography

GC of the alditol acetates were carried out on a Hewlett Packard 5890A gas-liquid chromatograph (Avondale PA, USA) equipped with a flame ionization detector and fitted with a fused silica column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.20 μm film of SP-2330 (Supelco, Bellefonte PA, USA). Chromatography was performed: from 200 °C to 230 °C at 1 °C min $^{-1}$, followed by a 30-min hold for alditol acetates. For the partially methylated alditol acetates, the initial temperature was 160 °C, which was increased at 1 °C min^{-1} to 210 °C and then at 2 °C min^{-1} to 230 °C. 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.

2.5. GC–MS

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

2.6. NMR spectroscopy

500 MHz ^{1}H NMR, proton decoupled 125 MHz ^{13}C NMR spectra, and two-dimensional NMR experiments (HMQC and COSY) were recorded on a Bruker AM500 at room temperature, with external reference of TMS. The sample (20 mg) was exchanged in 99.9% D_2O (0.5 ml) four times. Chemical shifts were referenced to internal acetone (δ_H 2.175, δ_{CH_3} 31.1). Parameters for ^{13}C NMR spectra were as follows: pulse angle 51.4°, acquisition time 0.56 s, relaxation delay 0.6 s, spectral width 29.4 kHz, and scans 25,000. For ^{1}H NMR spectra: pulse angle 76°, acquisition time 3 s, relaxation delay 3 s, spectral width 6250 Hz and scans 32. 2D spectra were obtained using standard Bruker software.

3. Results and discussion

Cell walls of *Codium* seaweeds contain important quantities of β-(1 → 4)-mannan (Ciancia et al., 2007; Kaihou et al., 1993; Love & Percival, 1964), but no sulfated mannan-counterpart was described before for any species of *Codium*. The main hot-water extract from *C. vermilara* (W1) that represents 4% (w/w) of the seaweed dry weight, contained 31% of mannose (Ciancia et al., 2007). This extract was fractionated by anion exchange chromatography on DEAE Sephadex A-25. The sample was eluted with water and then with aqueous solutions of increasing NaCl concentrations obtaining 6 fractions (Table 1). The overall recovery was 58.9% of the applied sample, although the last fraction was obtained after boiling the chromatographic matrix with a 4 M NaCl solution. This result indicates that a certain amount of polysaccharide from W1 is retained irreversibly inside the column; a similar behavior was observed previously for other seaweed sulfated polysaccharides (Stortz, Cases, & Cerezo, 1997). Fractions MF1, MF2, and MF3 showed major quantities of mannose, MF2, which eluted with 0.5 M NaCl and was obtained in 7% yield, was chosen for further analysis. MF2 contained carbohydrates and sulfate in molar ratio 2.7:1 and only 2% of proteins. It has a number average molecular weight in the order of 10 kDa, estimated by the reducing end groups (Park & Johnson, 1949). This product is a mannan, containing 94% of mannose; only small quantities of galactose (2%), glucose (3%), and rhamnose (1%) were detected.

Methylation analysis of MF2 (Table 2) showed important amounts of 4-linked non-substituted mannose units, but also 4-linked mannose units substituted on C-2 in molar ratio 2.7:1, confirming substitution with sulfate ester groups. The fact that only a minor amount of terminal non-reducing mannose units are present and that no other terminal non-reducing monosaccharide was detected, confirms the linear structure of the mannan. Very small quantities of 4-linked mannose units substituted on C-6 were also found. Some of this substitution could correspond to single stubs of β-D-mannose. 2,3-di-*O*-Methylmannose units were detected previously by methylation analysis of the parent extract (W1) in significant quantities. The difference in percentages of these units present in both samples was attributed to the fact that MF2 represents only 1/3 of the total mannan in the parent extract, W1, and fractionation by IEC carried out here could have given mannans differing slightly in the sulfate ester substitution pattern (Ciancia et al., 2007).

NMR spectra of MF2 (Fig. 1) were assigned based on previously published data (Cardoso et al., 2007), confirming the structure of a partially 2-sulfated mannan (Table 3). The excellent resolution of the spectra allowed us to detect some small signals corresponding to reducing and non-reducing terminal units, as those at 94.2/4.82 and 94.3/5.09 ppm, corresponding to C-1/H-1 β- and α-mannose terminal reducing units, at 69.6/3.92, assigned to C-3/H-3 of the

Table 1
Fractionation of the hot water extract of *C. vermilara* by IEC.

Fraction	NaCl (M)	Yield[b] (%)	Carbohydrates (%)	Sulfate as $NaSO_3$ (%)	Proteins (%)	Monosaccharide composition (mol%)						
						Gal	Ara	Man	Glc	Xyl	Rham	Fuc
W1[a]	–	4	58	22	16	26	20	31	21	2	1	–
MF1	0.1	9	29	31	3	3	tr	84	7	2	3	tr
MF2	**0.5**	**7**	**67**	**12**	**2**	**2**	**tr**	**94**	**3**	**tr**	**1**	**tr**
MF3	1.0	6	32	23	28	15	2	56	15	5	5	4
MF4	2.0	26	50	38	34	67	–	33	–	–	–	–
MF5	4.0	45	44	48	4	32	47	16	1	2	tr	tr
MF6	4.0, boiling	7	54	18	2	17	3	7	66	2	3	1

Bold signifies that Fraction MF2 was studied in detail.
[a] Included for comparison.
[b] Yield of W1 is given for 100 g of milled seaweed, for the fractionation, it was calculated from the recovered material (58.9% of the applied sample).

Table 2
Methylation analysis of MF2.

Partially methylated monosaccharide[a]	%	Structural units
2,3,4,6-Man	2.6	Man*p*(1→
2,3,6-Man	63.9	→4)Man*p*(1→
2,4,6-Man	1.3	→3)Man*p*(1→
3,6-Man	23.2	→4)Man*p* 2S(1→
2,3-Man	1.5	→4)Man*p* 6S(1→
3-Man	1.4	→4)Man*p* 2,3S(1→
Man	1.0	–

[a] Small percentages of 2,3,6-Glc (1.5%), 2,6-Gal (1.6%), and Glc (1.8%) were also found.

latter units, and those at 73.4/3.59, 67.3/3.49, and 61.7/3.86–3.67, assigned to C-3/H-3, C-4/H-4, and C-6/H-6,6′of the non-reducing terminal mannose units, respectively (Davis, Hoffmann, Russell, & Debet, 1995).

Analysis of the mannan structure show that the high solubility of these mannans in hot water should be due, mainly, to the substitution with sulfate ester groups in the linear chain, and also perhaps in part to the medium molecular weight of the polymer (~10 kDa). There are two reports about the existence of sulfated β-(1→4)-D-mannans biosynthesized by seaweeds. In one case, a sulfated β-(1→4)-D-mannan branched at C-6 and sulfated on C-2 was obtained from another green seaweed, *C. pusillum*, not taxonomically close to *Codium* (Carlberg & Percival, 1977). In the other case, they were isolated from red seaweeds of the genus *Chondrophycus* (Ceremiales); in this case, the main sulfated polysaccharides were agarans, while mannans constituted ~20% of the polysaccharides extracted with hot-water (Cardoso et al., 2007). All these mannans are sulfated on C-2 and have single stubs of β-D-xylose or β-D-mannose. The sulfated mannan isolated from *C. vermilara* could be considered formally part of a family of β-(1→4)-D-mannans sulfated on C-2 with optional single stubs of β-D-mannopyranose and/or β-D-xylopyranose, family which looks to be more extended in red and green seaweeds than previously know. The specificity of the 2-sulfation pattern in these mannans suggests the presence of a specific sulfotransferase that only adds sulfate groups on C-2.

A linear 6-sulfated β-(1→4)-D-glucan with 8.4% of (1→3)-linkages has been isolated from cellulose-based cell walls of the red seaweed *Kappaphycus alvarezii* in low yield (4%; Lechat, Amat, Mazoyer, Buléon, & Lahaye, 2000). Both, the low-sulfated mannan studied here and the sulfated glucan from *K. alvarezii* would be examples of a mechanism by which the cell can build up from a fibrillar polysaccharide structure into new product with a putative different biological functions, by modulating solubility factors like sulfation, degree of polymerization, presence of side chains, etc. It has been shown that the utricle cell wall in *C. vermilara* has a multilayered sandwich structure of two major fibrillar-like

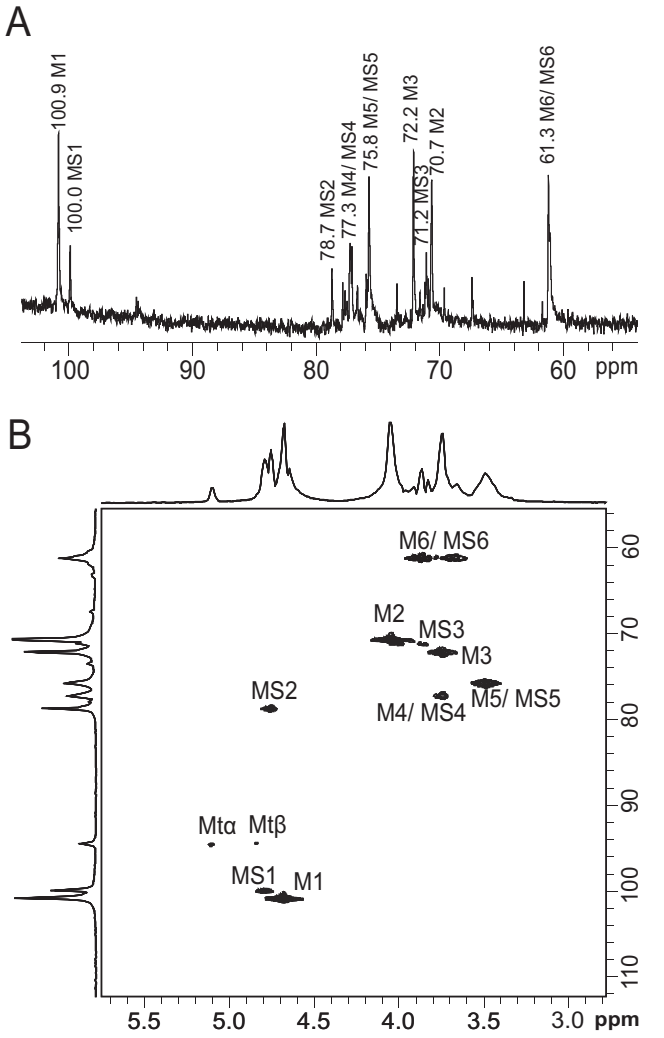

Fig. 1. NMR spectra of mannan MF2. (A) ^{13}C NMR spectrum. (B) HMQC spectrum (M = non-sulfated mannose units, MS = 2-sulfated mannose units, Mt = terminal mannose units).

Table 3
Signal assignments (ppm) of NMR spectra of MF2.

Structural unit	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6,6′
4-linked β-D-Man	100.9/4.69	70.7/4.02	72.1/3.74	77.3/3.76	75.7/3.48	61.2/3.66,3.60
4-linked β-D-Man 2S	99.9/4.78	78.7/4.75	71.1/3.88	77.1/3.76	75.9/3.48	61.1/3.38,3.85

layers of similar width delimiting a middle amorphous-like zone. By chemical imaging, the *in situ* distribution of β-(1 → 4)-mannans was shown to locate in these two distinct cell-wall layers, whereas sulfated polysaccharides, including the sulfated mannans described here, were distributed in the middle area of the wall (Fernández, Ciancia, Miravalles, & Estevez, 2010). These mannans, having a quite low degree of sulfation, could be acting as an interphase region between neutral-fibrillar mannan polysaccharide zones and the highly negatively charged polysaccharides in the central amorphous zone of the cell wall (Fernández et al., 2010). Further localization studies of the sulfated mannans are needed to confirm this idea.

In the last few years, the structures of water-soluble sulfated polysaccharides from different *Codium* species were studied. In all cases, the major sulfated polysaccharide is a pyruvylated β-(1 → 3)-galactan sulfate. Sulfate groups were found mainly at C-4, but also at C-6 of some of the β-D-galactose units and pyruvate was forming a five-membered ketal with *O*-3 and *O*-4 of nonreducing terminal galactose residues (Bilan, Vinogradova, Shashkov, & Usov, 2007; Ciancia et al., 2007; Estevez et al., 2009; Farias et al., 2008; Ohta, Lee, Hayashi, & Hayashi, 2009). In some cases, it was thought that this polymer could be an arabinogalactan, as different quantities of arabinose were detected. However, information about the arabinan moiety is scarce (Ciancia et al., 2007; Estevez et al., 2009; Siddhanta, Shanmugam, Mody, Goswami, & Ramabat, 1999). It has been shown that it is constituted by β-(1 → 3)-arabinopyranose units, and it is highly sulfated. Besides, small quantities of hydroxyproline rich glycoproteins (HRGPs) were also detected in these cell walls (Estevez et al., 2009; Fernández et al., 2010).

In conclusion, this study completes the determination of the structures of the cell wall polysaccharide system of *C. vermilara*, which is composed by major amounts of fibrillar β-(1 → 4)-mannans and three different soluble sulfated polysaccharide structures: (1) highly ramified sulfated and pyruvylated β-(1 → 3)-galactans (Ciancia et al., 2007); (2) linear highly sulfated β-(1 → 3)-arabinans, with the arabinose units in the pyranose form (Fernandez et al. in prep.); and (3) linear β-(1 → 4)-mannans sulfated on C-2 and with possible side chains of single stubs of β-D-mannopyranose and/or xylopyranose on C-6 on some of the mannose units.

Acknowledgements

This work was supported with grants from National Research Council of Argentina, CONICET (PIP 559-2009 to M.C. and PIP 071-2010 to J.M.E.), and the National Agency for Promotion of Science and Technology, ANPCYT (PICT 2008-0500 to M.C. and PICT 2010-0658 to J.M.E.), and University of Buenos Aires (UBACyT 2010-2012, G058).

References

Bilan, M. I., Vinogradova, E. V., Shashkov, A. S., & Usov, A. I. (2007). Structure of a highly pyruvylated galactan sulfate from the pacific green alga *Codium yezoense* (Bryopsidales, Chlorophyta). *Carbohydrate Research*, *342*, 586–596.

Cardoso, M. A., Noseda, M. D., Fujii, M. T., Zibetti, R. G. M., & Duarte, M. E. R. (2007). Sulfated xylomannans isolated from red seaweeds *Chondrophycus papillosus* and *C. flagelliferus* (Ceramiales) from Brazil. *Carbohydrate Research*, *342*, 2766–2775.

Carlberg, G. E., & Percival, E. (1977). Carbohydrates of the seaweeds *Urospora wormskioldii* and *Codiolum pusillum*. *Carbohydrate Research*, *57*, 223–234.

Chanzy, H. D., Grosrenaud, A., Vuong, R., & Mackie, W. (1984). The crystalline polymorphism of mannan in plant cell walls and after recrystallisation. *Planta*, *161*, 320–329.

Ciancia, M., Quintana, I., Vizcargüénaga, M. I., Kasulin, L., de Dios, A., Estevez, J. M., et al. (2007). Polysaccharides from the green seaweeds *Codium fragile* and *C. vermilara* with controversial effects on hemostasis. *International Journal of Biological Macromolecules*, *41*, 641–649.

Ciucanu, I., & Kerek, K. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *134*, 209–217.

Davis, A. L., Hoffmann, R. A., Russell, A. L., & Debet, M. (1995). ^{1}H- and ^{13}C-NMR characterization of the digalactosylmannopentaose liberated from legume seed galactomannan by β-mannanase action. *Carbohydrate Research*, *271*, 43–54.

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemistry Journal*, *84*, 106–110.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method of determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Dunn, E. K., Shoue, D. A., Huang, X., Kline, R. E., MacKay, A. L., Carpita, N. C., et al. (2007). Spectroscopic and biochemical analysis of regions of the cell wall of the unicellular 'mannan weed' *Acetabularia acetabulum*. *Plant Cell Physiology*, *48*, 122–133.

Estevez, J. M., Fernández, P. V., Kasulin, L., Dupree, P., & Ciancia, M. (2009). Chemical and *in situ* characterization of macromolecular components of the cell walls from the green seaweed *Codium fragile*. *Glycobiology*, *19*, 212–228.

Farias, E. H. C., Pomin, V. H., Valente, A. P., Nader, H. B., Rocha, H. A. O., & Mourão, P. A. S. (2008). A preponderantly 4-sulfated, 3-linked galactan from the green alga *Codium isthmocladum*. *Glycobiology*, *18*, 250–259.

Fernández, P. V., Ciancia, M., Miravalles, A. B., & Estevez, J. E. (2010). Cell wall polymer mapping in the coenocytic macroalga *Codium vermilara* (Bryopsidales, Chlorophyta). *Journal of Phycology*, *46*, 556–565.

Huizing, H. J., & Rietema, H. (1975). Xylan and mannan as cell wall constituents of different stages in the life-histories of some siphoneous green algae. *British Phycological Journal*, *10*, 13–16.

Huizing, H. J., Rietema, H., & Sietsma, J. H. (1979). Cell wall constituents of several siphoneous green algae in relation to morphology and taxonomy. *British Phycological Journal*, *14*, 25–32.

Kaihou, S., Hayashi, T., Otsuru, O., & Maeda, M. (1993). Studies on the cell-wall mannan of the siphonous green algae *Codium latum*. *Carbohydrate Research*, *240*, 207–218.

Lechat, H., Amat, M., Mazoyer, J., Buléon, A., & Lahaye, M. (2000). Structure and distribution of glucomannan and sulfated glucan in the cell walls of the red alga *Kappaphycus alvarezii* (Gigartinales, Rhodophyta). *Journal of Phycology*, *36*, 891–902.

Love, J., & Percival, E. J. (1964). The polysaccharides of the green seaweed *Codium fragile*: Part III. A β-1,4-linked mannan. *Journal of the Chemical Society*, 3345–3349.

Lowry, O. H., Rosenbrough, N. J., & Farr, A. L. (1951). Protein measurements with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Mackie, W., & Preston, R. D. (1968). The occurrence of mannan microfibrils in the green algae *Codium fragile* and *Acetabularia crenulata*. *Planta*, *79*, 249–253.

Ohta, Y., Lee, J.-B., Hayashi, K., & Hayashi, T. (2009). Isolation of sulfated galactan from *Codium fragile* and its antiviral effect. *Biological and Pharmaceutical Bulletin*, *32*, 892–898.

Park, J. T., & Johnson, M. J. (1949). A sub microdetermination of glucose. *Journal of Biological Chemistry*, *181*, 149–151.

Percival, E., & McDowell, R. H. (1981). Algal walls. Composition and biosynthesis. In W. Tanner, & F. A. Loewus (Eds.), *Encyclopedia of plant physiology* (pp. 277–316). Berlin: Springer.

Pérez Recalde, M., Noseda, M. D., Pujol, C. A., Carlucci, M. J., & Matulewicz, M. C. (2009). Sulfated mannans from the red seaweed *Nemalion helminthoides* of the South Atlantic. *Phytochemistry*, *70*, 1062–1068.

Popper, Z. A., & Tuohy, M. C. (2010). Beyond the green: Understanding the evolutionary puzzle of plant and algal cell walls. *Plant Physiology*, *153*, 373–383.

Siddhanta, A. K., Shanmugam, M., Mody, K. H., Goswami, A. M., & Ramabat, B. K. (1999). Sulphated polysaccharides of *Codium dwarkense* Boergs from the west coast of India: Chemical composition and blood anticoagulant activity. *International Journal of Biological Macromolecules*, *26*, 151–154.

Stevenson, T. T., & Furneaux, R. H. (1991). Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydrate Research*, *210*, 277–298.

Stortz, C. A., Cases, M. R., & Cerezo, A. S. (1997). The system of agaroids and carrageenans from the soluble fraction of the tetrasporic stage of the red seaweed *Iridaea undulosa*. *Carbohydrate Polymers*, *34*, 61–65.

Wutz, M., & Zetsche, K. (1976). Zur Biochimie und Regulation des Heteromorphen Generationswechsels der Grünalge. *Planta*, *129*, 211–216.