Pergamon

PII: S0031-9422(96)00366-4

Phytochemistry, Vol. 43, No. 4, pp. 731–734, 1996

0031-9422/96 $15.00 + 0.00

THE RESISTANT 'BIOPOLYMER' IN CELL WALLS OF *COELASTRUM SPHAERICUM*

MARÍA C. RODRÍGUEZ* and ALBERTO S. CEREZO†‡§

Departments of *Biology and †Organic Chemistry (CIHIDECAR-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires (1428), Argentina

(*Received in revised form* 3 *May* 1996)

Key Word Index—*Coelastrum sphaericum*; Chlorococcales; trilaminar layer; algaenans; resistant 'biopolymer'; FT-IR; CP-MAS ^{13}C NMR.

Abstract—The resistant 'polymer' in the trilaminar sheath of *Coelastrum sphaericum* is a complex formed by three different types of biopolymers, a hydrophobic aliphatic network mainly composed of polymethylenic chains with amido groups, together with smaller amounts of cellulose and proteins. The composition of the hydrophobic polymer is close to that of algaenan of other trilaminar layer-containing species. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Algaenans, i.e. macromolecular, non-hydrolysable compounds, are reported in the outer trilaminar layer (TLS) of the cell walls in several marine and freshwater chlorophycean microalgae [1–13], as well as in ultralaminae-containing lacustrine and marine kerogens [14–18]. These highly resistant materials consist of a macramolecular network based on long polymethylenic chains associated with amide groups and minor amounts of *N*-alkyl substituted pyrroles [18]. The present paper presents an analysis of the resistant biopolymer in cell walls of *Coelastrum sphaericum*, which appears to be closely associated with fibrillar polysaccharides in the trilaminar layer. In addition, a small amount of protein could be detected within the macromolecular domain.

RESULTS AND DISCUSSION

Solid state ^{13}C NMR spectroscopy was carried out on the residue resistant to phosphoric acid hydrolysis (PARR) and the subsequently obtained acetolysis-resistant material (ARM) (Fig. 1). PARR and ARM, together with the insoluble residue obtained after extraction of the cell walls with 4-methylmorpholine *N*-oxide (MMNO) (MOIR) and its hydrolysed residue (MOIR-AH), were also submitted to FT-IR spectroscopy.

Major peaks appeared in PARR (Fig. 1A) at δ 102.3, 85.8, 81.6, 72.0, 62.1, 59.8 and 27.4. Resonances between δ 105 and 60 usually derive from anomeric plus ring carbohydrate carbons in polysaccharides, while the one at δ 27.4 would correspond to methylenic

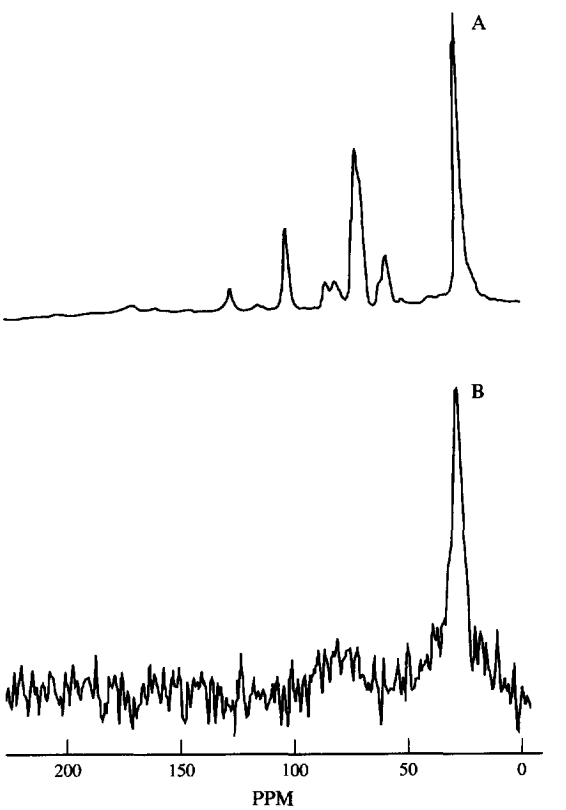

Fig. 1. CP-MAS ^{13}C NMR spectra (A) residue resistant to phosphonic acid hydrolysis and (B) acetolysis-resistant material from cell walls of *Coelastrum sphaericum*.

‡Member of the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

§Author to whom correspondence should be addressed.

carbon atoms. A small signal also appeared at δ 127.3 (olefinic and/or aromatic carbon atoms), together with a weak peak from the carbonyl in amide groups (δ 172–174), in agreement with the low intensity resonance of carbon atoms linked to nitrogen [19]. The spectrum of ARM was characterized by a lower signal-to-noise ratio and only showed a peak at δ 27.4 (Fig. 1B). This spectrum was identical to those obtained for the non-hydrolysable constituents of *Chlorella fusca*, *Nanochlorum eucaryotum* [11, 16] and *Scenedesmus quadricauda* [15].

The CP-MAS spectrum of cellulose IV showed bands at δ 103.3 (C-1), 72.3 (C-2, C-3, C-5), 82.2 (C-4) and 62.3 (C-6) [20]; anomeric carbon resonances shifted to δ 105.0 (cellulose I) and δ 107.9 and 106.2 (cellulose II) [20]. When cellulose was dissolved in dimethyl sulphoxide containing 20% MMNO, the anomeric carbon peak appeared at δ 102.5 [21]. The C-1 resonance for a $(1\rightarrow4)$ linked β-D-glucopyranose residue in a xyloglucan backbone was observed at δ 102.7 [22]. It has been reported [13] that strong acid treatment in alghumin eliminated NMR signals beyond δ 40, in agreement with the assignation of these bands to non-crystalline cellulose. Signals at δ 101.7 were also assigned to the anomeric carbon in $(1\rightarrow4)$-β-D-mannan [23], while those at δ 69.9, 72.1, 81.0, 75.9 and 61.9 would correspond to C-2, C-3, C-4, C-5 and C-6, respectively.

The FT-IR spectrum of PARR showed a broad and strong band centred at 3439 cm^{-1} with a shoulder at 3231 cm^{-1} (OH with polymeric intermolecular hydrogen bonds). Sharp and strong peaks at 2924 and 2853 cm^{-1} (ν_{as} CH_2 and $\nu_s CH_2$), together with a wide 720 cm^{-1} band, indicate the presence of polymethylenic chains with $n \geq 4$. The lack of significant amounts of methyl groups was shown by the absence of peaks or shoulders at 2960 and 2870 cm^{-1}; besides, no absorptions were found at 1340 and 1255 cm^{-1} indicative of tertiary or quaternary carbon atoms. Neither aromatic nor unsaturated groups were detected. A strong band at 1160–970 cm^{-1} was present (δ OH and νC–O), corresponding to non-localized, highly coupled vibrational modes of polysaccharide backbones, in the fingerprint region for these macromolecules [24]. A wide band at 1180 cm^{-1} (P–O–C bond, alkyl substituents) suggested the formation of organic phosphates during phosphoric acid treatment [25]. The FT-IR spectrum after direct acetolysis of the original cells lacked the 1180 cm^{-1} band.

The IR spectrum of ARM showed the above mentioned absorptions at 3445, 2926, 2853, 1098 and 724 cm^{-1}, together with that corresponding to organic phosphates (1180 cm^{-1}). New bands could be assigned to the partial acetylation of hydroxyl groups (–O–CO–CH_3), i.e. 1735, 1460 and 1380 cm^{-1} [25]. The intensity of bands in the fingerprint region for carbohydrates (1160–970 cm^{-1}) decreased and wide amide I (1657 cm^{-1}) and amide II (1547 cm^{-1}) bands appeared.

Though much has been discussed about the alterations introduced in the resistant polymers by acetylation [7–9], CP-MAS and FT-IR spectra demonstrate that a product richer in the hydrophobic polymer was obtained after acetolysis. Transmission electron microscope (TEM) observations revealed that the decrease in carbohydrate content after acetolysis was associated with the disappearance of the electronlucent band in the trilaminar layer.

MOIR, obtained after a milder treatment, showed a similar FT-IR spectrum, namely, absorptions at 3441 (hydrogen-bonded OH), 2924 and 2853 (ν_{as} CH_2 and $\nu_s CH_2$), 1377 (δ CH) and 1316 (CH_2-wagging), together with bands at 1156, 1065 and 1026 cm^{-1}, arising from the fingerprint region for polysaccharides. After acid hydrolysis of MOIR (MOIR-AH), the hydroxyl absorption sharpened and the polysaccharide fingerprint overlapped bands (1160–970 cm^{-1}) were reduced in intensity by 50%.

PARR accounted for *ca* 8–10% of the dry weight of the algal cells, while MOIR comprised *ca* 2%. ARM (obtained after acetolysis of PARR) yielded 0.1–0.2% of algaenan. The trilaminar layer was completely degraded with CrO_3, but remained unaffected by ethanolamine and was insoluble in dioxane. No significant addition of bromine was observed.

PARR, ARM and MOIR were shown to contain 20, 1.5 and 14 wt% of carbohydrates, respectively. The decrease in carbohydrate contents from PARR (20%) to ARM (1.5%), show that only after acetolysis could the bulk of the remaining polysaccharides be eliminated. Moreover, the hydrophobic network also reduced the efficiency of extraction with MMNO. This is one of the few indicated solvents for cellulose; in fact, it has been used to solubilize entire cell walls in TLS-devoid species [26]. Sulphuric acid hydrolysis of ARM yielded only glucose. For PARR, a mixture of mannose and glucose (molar ratio 5.4:94.6) was obtained, while MOIR-AH afforded a mixture of glucose, mannose and galactose (molar ratio 70.5:21.3:8.2). It is interesting to note that, during autospore formation in TLS-containing Chlorococcales, the internal microfibrillar layer is degraded [27] and the resulting remains of the mother cell walls left in the culture media only consist of the TLS. When comparing the monosaccharide composition of mother cell walls (TLS), which have undergone no extraction procedure [28], a higher molar percentage of mannose is obtained. Cell walls of Chlorococcales contain β-mannan [28–30]. Moreover, in PARR, for instance, the monosaccharide composition and some NMR signals suggest the presence of mannan, together with cellulose. According to monosaccharide analyses of PARR, MOIR and ARM, mannan is more readily removed. This might suggest that the hydrophobic polymer is more closely related to cellulose than to mannan.

Colorimetric measurement for protein contents of 1.3, 1.6 and 2.5% were obtained for PARR, ARM and MOIR, respectively. Nitrogen microanalyses gave values of 6.30% (PARR), 1.90% (ARM) and 7.02% (MOIR), which would correspond to significantly higher percentages of proteins than those determined colorimetrically, viz. 39.4% (PARR), 11.9% (ARM)

and 44.0% (MOIR). These results suggest that there might be other sources of nitrogen, different from protein. High nitrogen contents have been frequently observed for TLS-containing material [13]. It is highly probable that faint FT-IR amide I ($1657\,cm^{-1}$) or amide II ($1550\,cm^{-1}$) bands in ARM and MOIR are due to non-protein amide groups, such as those recently described for algaenan from *S. quadricauda* [18] and *C. fusca* [16]. The existence of hydrophobic protection also explains why the amide groups could not be removed completely by the alkaline–acid treatment [18].

Microscopical examination revealed that Aniline Blue was completely excluded from the algal cells. Neither phloroglucinol nor Sudan IV stained the cell wall significantly. Both native cells, as well as PARR and ARM, showed autofluorescence when illuminated with UV light; secondary fluorescence was also evident after application of primuline. Neutral red produced a bright red fluorescence in the cell walls, especially noticeable at the species-typical cell-wall protuberances. In order to quench the orange autofluorescence of the cells, these were pretreated with Toluidine Blue [31]. Even so, cell walls still showed fluorescence with Neutral red [31], indicating the presence of hydrophobic domains within it. Untreated material, as well as PARR and MOIR, showed fluorescence in the cell walls with Calcofluor White, a fluorescent brightener which binds preferentially to β-1,4-hexapyranosyl polysaccharides [32], showing the presence of fibrillar polysaccharides coexisting with the hydrophobic polymer in the cell wall. TEM observations indicated that the TLS (20–30 nm) withstands saponification and phosphoric acid digestion, though trilaminar morphology became less distinct in PARR. Only curled electron opaque residues remained visible after acetolysis (ARM) and MMNO extraction (MOIR).

In summary, the hydrophobic polymer of *C. sphaericum* resembles the algaenans of other TLS-comprising species [11–13, 15, 16]. This 'polymer' is neither a unique substance nor a mixture of closely related products, but a complex formed by three different types of biopolymers, with the predominance of a hydrophobic aliphatic one consisting of polymethylenic chains, together with non-hydrolysable amide moieties (algaenan) [18] and smaller quantities of cellulose and possibly mannan and protein. This composition explains the remarkable resistance of the cell walls in TLS-containing algal species. These walls are refractory to natural biodegradation [13], thus contributing through selective preservation to the formation of kerogens [14]. This hydrophobic coating of fibrillar polysaccharides [13] or a network trapping polysaccharides and nitrogen-containing compounds [14] are responsible for the difficulties encountered in the isolation and degradation of these products.

EXPERIMENTAL

Algal material. Coelastrum sphaericum var. *dilatatum* Bohlin et Vischer was grown axenically in Detmer-glucose 1% (w/v) media [33] at room temp. under continuous illumination and agitation. Cells were harvested by filtration.

Isolation of non-hydrolysable residue. Algal cells were extracted in a Soxhlet apparatus with boiling EtOH and Et_2O. The resulting dry residue was resuspended in 6% aq. KOH and heated under reflux for 6 hr with vigorous mechanical stirring. The soln was then neutralized. The insoluble residue was thoroughly washed in H_2O, EtOH and Et_2O until dry. The resulting powder was vigorously and continuously agitated in 85% H_3PO_4 at room temp. for 1 week. The solid residue was isolated by centrifugation and repeatedly washed with H_2O, EtOH and Et_2O. A light grey material was obtained (PARR).

Acetolysis. This procedure was carried out both on lyophilized cells and the residue after H_3PO_4 digestion. In both cases, the material was first washed in HOAc and the pellet, after centrifugation, resuspended in Ac_2O–H_2SO_4 (9:1) and heated at 100° for 10 min [3]. The solid residue was washed with HOAc, EtOH and Et_2O and kept under vacuum in a desiccator until dry (ARM).

MMNO treatment [26]. Cells were sonicated in 0.4 ml 60% MMNO and heated at 120° during 30 min; this procedure was repeated ×3. After centrifugation, soluble and insoluble frs were sepd. MMNO was eliminated by repeated washing of the insoluble fr. (MOIR). Part of MOIR was hydrolysed first in 72% H_2SO_4 at room temp. for 30 min. The acid was then diluted to 0.5 M and the material heated at 100° during 6 hr. The acid was pptd with $BaSO_4$ and eliminated by centrifugation, obtaining MOIR-AH.

Microscopical analyses. Phloroglucinol test for lignin: samples were suspended in 2% phloroglucinol in EtOH and drops of 50% HCl were added after several min. The lignin-containing ref. stained red–violet [34]. Aniline Blue staining: cells were mounted in Aniline Blue (0.05% aq. soln) and observed after 5–10 min and again after 1 hr [9]. Sudan IV was prepd in 70% EtOH [34]. Epifluorescence microscopy was carried out using UV excitation at *ca* 365 nm. For the study of secondary fluorescence, samples were first treated for 5 min in an aq. soln of primuline (0.005%) [10]. Calcofluor White 0.01% was prepd in 80% EtOH. Toluidine Blue (0.05%) was prepd in 0.1 M NaOAc buffer (pH 4) and 0.1% Neutral Red in 0.1 M K-Pi buffer (pH 6.5–7) [31]. Toluidine Blue treatment was carried out for at least 45 min before observation. Samples after each extraction step were fixed in 1% glutaraldehyde for TEM observation. After embedding in Spurr's resin, ultramicrotome sections were stained with uranyl acetate and lead citrate [34].

Analytical methods. Total carbohydrates were determined by the PhOH–H_2SO_4 method [35] and proteins by the procedure of ref. [36]. Determination of N was performed as per ref. [37] and protein content was calculated after multiplication by a factor of 6.25.

Bromination of algaenan [8]. Extracted product (100 mg) was resuspended in 8 ml CCl_4. Br_2 (2 g) was added and the soln kept in the dark at room temp. for 6

days. The reaction mixt. was dild with Et_2O, the algaenan sepd, washed by centrifugation and dried *in vacuo* to constant wt.

Insolubility in dioxane and ethanolamine [8]. These were observed after stirring under reflux during at least 24 hr.

Oxidative degradation [8]. Both entire cells and extracted product were completely degraded after 7 days at room temp. in a soln of CrO_3 (2 g in 4 ml H_2O–HOAc, 1:1).

Sugar analysis. Products were hydrolysed with H_2SO_4 [26] and the acetylated alditols analysed by GC on a Supelco SP 2330 capillary column, run isothermally at 220° (FID and injection temps 240°) using N_2 as carrier gas at 1 ml min^{-1} with a split ratio of 100:1.

Spectroscopic analyses. FTIR spectra were obtained from KBr discs between 4000 and 400 cm^{-1}. Solid state ^{13}C NMR [11–13] were recorded at 75 MHz. All spectra were acquired using cross-polarization, dipolar decoupling and magic-angle spinning (CP-MAS). Samples (50–200 mg) were spun in 7 mm zirconia rotor tubes at 3 kHz. ^{13}C signals were generated by single 1 msec contact cross-polarization from protons and high-power ^1H decoupling was carried out during acquisition of the ^{13}C free induction decay. Pulse 4.5 μsec ^1H 90°; the recycle time for H relaxation was 1 sec. Spectra were accumulated for 8–24 hr. TMS was used as int. standard.

Acknowledgements—This work was funded by the Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET) and the Organización de los Estados Americanos (OEA).

REFERENCES

1. Atkinson, A., Gunning, B. and John, P. (1972) *Planta* **107**, 1.
2. Staehelin, L. and Pickett-Heaps, J. (1975) *J. Phycol.* **11**, 163.
3. Marchant, H. (1977) *J. Phycol.* **13**, 102.
4. Good, B. and Chapman, R. (1978) *Am. J. Botany* **65**, 27.
5. Dempsey, G., Lawrence, D. and Cassie, V. (1980) *Phycologia* **19**, 13.
6. Burczyk, J., Szkawran, H., Zontek, I. and Czygan, F. (1981) *Planta* **151** 247.
7. Honegger, R. and Brunner, U. (1981) *Can. J. Botany* **59**, 2713.
8. Berkaloff, C., Casadevall, E., Largeau, C., Metzger, P., Peracca, S. and Virlet, J. (1983) *Phytochemistry* **22**, 389.
9. Brunner, U. and Honegger, R. (1985) *Can. J. Botany* **63**, 2221.
10. Puel, F., Largeau, C. and Giraud, G. (1987) *J. Phycol.* **23**, 649.
11. Derenne, S., Largeau, C., Berkaloff, C., Rousseau, B., Wilhelm, C. and Hatcher, P. (1992) *Phytochemistry* **31**, 1923.
12. Guilford, W., Schneider, D., Labovitz, J. and Opella, S. (1988) *Plant Physiol.* **86**, 134.
13. Zelibor, J., Romankiw, L., Hatcher, P. and Colwell, R. (1988) *Appl. Environ. Microbiol.* **54**, 1051.
14. Tegelaar, E., de Leeuw, J., Derenne, S. and Largeau, C. (1989) *Geochim. Cosmochim. Acta* **53**, 3103.
15. Derenne, S., Largeau, C., Casadevall, E., Berkaloff, C. and Rousseau, B. (1991) *Geochim. Cosmochim. Acta* **55**, 1041.
16. Derenne, S., Largeau, C. and Hatcher, P. (1992) *Org. Geochem.* **18**, 417.
17. Derenne, S., Le Berre, F., Largeau, C., Hatcher, P., Connan, J. and Raynaud, J. (1992) *Org. Geochem.* **19**, 345.
18. Derenne, S., Largeau, C. and Taulelle, F. (1993) *Geochim. Cosmochim. Acta* **57**, 851.
19. Farrar, T. and Becker, E. (1971) *Pulse and Fourier Transform NMR*, p. 155. Academic Press, New York.
20. Dudley, R., Fyfe, C., Stephenson, P., Deslandes, Y., Deslandes, Y., Hamer, G. and Marchessault, L. (1983) *J. Am. Chem. Soc.* **105**, 2469.
21. Gagnaire, D. and Vincendon, M. (1977) *Bull. Soc. Chem. Fr.* 479.
22. Gil-Serrano, A. and Tejero-Mateo, P. (1988) *Carbohydr. Res.* **181**, 278.
23. Jarvis, M. (1990) *Carbohydr. Res.* **197**, 276.
24. Séné, C., McCann, M., Wilson, R. and Grinter, R. (1994) *Plant Physiol.* **106**, 1623.
25. Nakanishi, K. (1964) *Infrared Absorption Spectroscopy*, p. 233. Holden Day, San Francisco, CA.
26. Joseleau, J., Chambat, G. and Chumpitazi-Hermoza, B. (1981) *Carbohydr. Res.* **90**, 339.
27. Pickett-Heaps, J. (1975) *Green Algae: Structure, Reproduction and Evolution in Selected Genera*, p. 606. Sinauer Associates, Sunderland, MA.
28. Burczyk, J., Terminska-Pabis, K. and Smietana, B. (1995) *Phytochemistry* **38**, 837.
29. Loos, E. and Meindl, D. (1982) *Planta* **156**, 270.
30. Takeda, H. (1993) *J. Plant Res.* **106**, 195.
31. Lulai, E. and Morgan, W. (1992) *Biotech. Histochem.* **67**, 185.
32. Maeda, H. and Ishida, N. (1967) *J. Biochem.* **62**, 1967.
33. Rodríguez, M., Accorinti, J. and Cerezo, A. (1989) *Anal. Asoc. Qca. Arg.* **77**, 27.
34. O'Brien, T. and McCully, M. (1981) *The Study of Plant Structure*, p. 357. Termocarphi, Melbourne, Australia.
35. Dubois, M., Gills, K., Hamilton, J., Rebers, P. and Smith, F. (1956) *Analyt. Chem.* **28**, 350.
36. Lowry, O., Rosebrough, N., Farr, L. and Randall, R. (1951) *J. Biol. Chem.* **193**, 265.
37. Steyermark, A. (1951) *Quantitative Organic Microanalysis*, p. 389. Blakiston, New York.