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## Note

# Structural characterisation of the exocellular polysaccharide from *Cyanospira capsulata*

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*Cyanospira capsulata*, a heterocystous cyanobacterium isolated[1] from Magadi lake (Kenya) in 1982, synthesises an exocellular polysaccharide. Some compositional data on this macromolecule, together with its production under laboratory conditions and its isolation from the bacterial slime, have been reported[2]. A better characterisation of this hydrocolloid became desirable in view of its interesting rheological properties[3].

We now report preliminary results on the purification, composition, and structure of the polysaccharide.

The crude polysaccharide contained[4] 6% of protein; when an aqueous solution was kept for 1 h at 100° and then centrifuged, the protein content of the material in the supernatant solution was reduced to 2%. This figure remained constant on further treatment with isoamyl alcohol–chloroform or after gel-filtration chromatography on Sephadex G-100. The presence of proteins in purified preparations of the polysaccharide was confirmed by polyacrylamide gel electrophoresis with sodium dodecyl sulphate, which showed a broad major band with $M_r$ 66,000 and minor bands of lower $M_r$. The association of proteins or peptides with several polysaccharides has been reported[5,6], together with some indications in favour of a functional and structural role for these components.

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TABLE I

THE AMINO ACID COMPOSITION OF THE PROTEINACEOUS COMPONENTS IN PURIFIED *Cyanospira capsulata* POLYSACCHARIDE

| *Amino acid* | *Residues per 1000 residues* | *Amino acid* | *Residues per 1000 residues* |
|---|---|---|---|
| Aspartic acid | 117 | Methionine | 0 |
| Threonine | 86 | Isoleucine | 41 |
| Serine | 62 | Leucine | 57 |
| Glutamic acid | 96 | Tyrosine | 0 |
| Proline | 27 | Phenylalanine | 41 |
| Glycine | 120 | Histidine | 14 |
| Alanine | 120 | Lysine | 5 |
| Cysteine | 0 | Arginine | 24 |
| Valine | 53 | | |

Amino acid analysis of the purified polysaccharide (Table I) revealed a high proportion of aspartic acid, glutamic acid, glycine, and alanine, and the absence of cysteine, methionine, and tyrosine. The protein content calculated from amino acid analysis agrees with that measured by the Lowry procedure. The composition reported in Table I is similar to that of extracellular polysaccharides from other cyanobacteria[7,8] and is reminiscent of those of pectins from higher plants[5].

Acid hydrolysis of the purified polysaccharide, followed by h.p.l.c. of sugars derivatised with 5-dimethylaminonaphthalene-1-sulphonylhydrazine (Dns-hydrazine), revealed glucose, mannose, arabinose, fucose, and galacturonic acid. For quantitative determination, the best conditions for acid hydrolysis were found to be treatment with 2M trifluoroacetic acid for 180 min at 120°. The figures obtained (Table II) after extrapolation of the observed values to zero showed the polysaccharide to contain equimolar proportions of the component monosaccharides. These data were confirmed also by g.l.c.–m.s. of the sugars as the acetylated aldononitriles prepared after carboxyl reduction ($NaBD_4$) and hydrolysis of the polysaccharide. Analysis of the derivatives revealed galactose-*6,6-*$d_2$, arabinose, fucose, mannose, and glucose in equimolar proportions.

Methylation analyses (Table III) revealed (1→3)-linked glucose and (1→4)-linked mannose. The arabinose and half of the galacturonic acid residues occurred as non-reducing end groups, and branching involved the fucose and the remainder of the galacturonic acid residues.

Smith degradation, followed by acid hydrolysis and h.p.l.c. of the Dns-sugars (Table II) revealed loss of the arabinose and mannose; the remaining sugars were still present in equimolar proportions. These results agreed with the formation of 2,3,4-tri-*O*-methylarabinose and 2,3,6-tri-*O*-methylmannose in the methylation analysis.

Partial acid hydrolysis (2M trifluoroacetic acid, 1 h, 100°) of the polysaccharide and ion-exchange chromatography of the products gave neutral and acidic com-

TABLE II

SUGAR COMPOSITION OF *Cyanospira capsulata* POLYSACCHARIDE

| *Treatment* | *Component sugar (%)*[a] | | | | |
|---|---|---|---|---|---|
| | *Glc* | *Ara* | *Man* | *Fuc* | *GalA* |
| (*1*) Hydrolysed in 2M $CF_3COOH$ at 120° for 3 h | 20 ±3[b] | 20 ±3 | 19 ±2 | 16 ±2 | 25 ±4 |
| (*2*) As in (*1*) after Smith degradation | 34 ±3 | — | — | 31 ±2 | 34 ±4 |

[a]Values extrapolated at zero time. [b]Errors calculated from three different experiments.

TABLE III

METHYLATION ANALYSES OF CARBOXYL-REDUCED EXOPOLYSACCHARIDE OF *Cyanospira capsulata*

| *Methylated sugar*[a] | *Mole % as* | |
|---|---|---|
| | *Alditol acetate*[b] | *PAAN*[c] |
| 2,3,4,6-$Me_4$-Gal(*6,6-$d_2$*) | 8 | 8 |
| 2,3,4-$Me_3$-Ara | 16 | 17 |
| 2,3,6-$Me_3$-Glc | 30 | 28 |
| 2,3,6-$Me_3$-Man | 15 | 20 |
| 4,6-$Me_2$-Gal(*6,6-$d_2$*) | 11 | 6 |
| 2-Me-Fuc | 20 | 21 |

[a]2,3,4,6-$Me_4$-Gal = 2,3,4,6-tetra-*O*-methyl-D-galactose, *etc.* [b]Hydrolysis with 2M $CF_3COOH$ (120°, 20 min). [c]Acetylated aldononitriles. Sugars were released by sequential formolysis (aq. 89% formic acid, 100°, 2 h) and acetolysis/hydrolysis (80°, 4-h periods). Values corrected for degradation of 2,3,4-$Me_3$-Ara.

ponents. Gel filtration of the neutral fraction gave one disaccharide which yielded equimolar amounts of fucose and mannose on hydrolysis. The $^{13}C$-n.m.r. spectra confirmed the presence of the two monosaccharides (Table IV). Assignment of individual resonances based on differential-isotope-induced shift[10] and comparison with literature data[11–13] indicated that fucose was (1→4)-linked to mannose. The chemical shift difference between the resonances of the $\alpha$ and $\beta$ forms of the mannose residue is close to reported data[14].

The configurations at the anomeric centres in the disaccharide were obtained from the $^1H$-n.m.r. data. That mannose was the reducing residue was inferred from the characteristic[15] H-1 resonances at $\delta$ 4.97 ($J_{1,2}$ 1.4 Hz) and 4.71 ($J_{1,2}$ <1 Hz) for the $\alpha$ and $\beta$ forms, respectively. The signal at $\delta$ 1.16 (d) is characteristic[16,17] of H-6,6,6 of fucose, and that at $\delta$ 4.52 ($J$ ~1 Hz) for H-1 indicated[18] this sugar to be $\alpha$. The results from n.m.r. spectroscopy suggested the disaccharide to have the structure $\alpha$-Fuc-(1→4)-Man.

Gradient elution of the acidic components retained by the ion-exchange column yielded three fractions which could not be further resolved by gel-filtration chromatography. These fractions were heterogeneous, as shown by the occurrence

TABLE IV

$^{13}$C-N.M.R. CHEMICAL SHIFTS (P.P.M.) FOR THE DISACCHARIDE AND THE ASSIGNMENTS

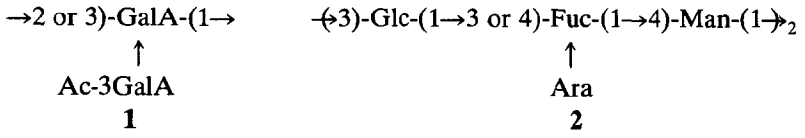
| *Atom* | *α-Fuc* ($D_2O$)[a] | *α-Man* ($D_2O$)[a] | *β-Man* ($D_2O$)[a] | *α-Fuc* ($Me_2SO$)[b] | *α-Man* ($Me_2SO$)[b] |
|---|---|---|---|---|---|
| C-1 | 100.98 | 94.66 | 94.50 | 101.74 | 94.57 |
| | (0.00)[c] | (0.12) | (0.11) | | |
| C-2 | 70.85 | 71.25 | 71.66 | 71.34 | 70.70 |
| | (0.12) | (0.12) | (0.12) | | |
| C-3 | 73.21 | 69.82 | 72.52 | 73.61 | 69.86 |
| | (0.10) | (0.10) | (0.10) | | |
| C-4 | 79.15 | 77.58 | 77.26 | 78.70 | 77.39 |
| | (0.05) | (0.00) | (0.00) | | |
| C-5 | 76.65 | 76.75 | 75.59 | 76.23 | 77.09 |
| | (0.00) | (0.02) | (0.02) | | |
| C-6 | 19.80 | 61.39 | 61.39 | 20.33 | 61.30 |
| | (0.04) | (0.10) | (0.10) | | |

[a]Chemical shifts relative to internal 1,4-dioxane (67.4 p.p.m.). [b]Chemical shifts relative to $Me_2SO$ (40.4 p.p.m.). [c]Deuterium-induced differential isotope shifts.

in their $^1$H-n.m.r. spectra of multiple signals for H-6,6,6. Attempts to purify these mixtures by ion-exchange h.p.l.c. were unsuccessful.

The purified polysaccharide contains acetyl substituents. They were detected ($\delta$ 2.1, s) in the $^1$H-n.m.r. spectra of the acidic fractions. Colorimetric and g.l.c. analyses of the hydrolysed polysaccharide indicated the presence of acetal-linked pyruvate groups (2–3%). As this finding was not confirmed by the lactic dehydrogenase procedure[19], the possibility of misleading results, due to degradation products arising from prolonged hydrolysis, cannot be ruled out.

The results reported above accord with a decasaccharide repeating-unit in which arabinose and galacturonic acid branches are linked to fucose and galacturonic acid residues, respectively, and the main chain galacturonic acid pair (**1**) must occur in a 1:2 ratio with the remaining tetrasaccharide repeat (**2**).




The resistance of the galacturonic acid branches to oxidation with periodate indicates 3-*O*-acetylation.

## EXPERIMENTAL

The crude polysaccharide was prepared[2] at Centro di Studio dei Microorganismi Autotrofi of the Italian Research Council (CNR), Florence.

*Purification of the polysaccharide.* — A solution of the crude polysaccharide in deionised water (10 mg/mL) was heated for 1 h at 100°, then centrifuged at 100 000*g* for 45 min. The supernatant solution was dialysed against water and then lyophilised, to give the purified polysaccharide with a yield of ~70%.

*Protein and amino acid analysis.* — Protein contents of the crude and purified polysaccharides were estimated by the method of Lowry *et al.*[4]. Amino acids were determined with an amino acid analyzer (LKB 4400). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to Laemmli[20] with the use of a discontinuous buffer system.

*Total hydrolysis and sugar analysis.* — Solutions of the polysaccharide (5 mg) in 2M $CF_3COOH$ (1 mL) were heated at 120° in sealed vials for periods of 20 to 240 min. Each solution was then concentrated under reduced pressure, an aliquot (100 μL) of a solution of the residue in water (1 mL) was treated with Dns–hydrazine, and the products were analysed by reverse-phase h.p.l.c., according to the method of Mopper and Johnson[21] with minor modifications. H.p.l.c. was performed on a Perkin–Elmer Series 2 liquid chromatograph equipped with a Perkin–Elmer LC-55 variable-wavelength detector operating at 254 nm, a Perkin-Elmer LCI-100 electronic integrator, and a column (25 × 0.46 cm) of Supelcosil LC18 (Supelco Inc.).

For g.l.c.–m.s. analyses, carboxyl reduction of the polysaccharide was performed with $NaBD_4$ *via* the ethylene glycol ester[22].

*Methylation analyses.* — The carboxyl-reduced polysaccharide was analysed using the procedure described[23,24]. Methyl silicone capillary columns (fused silica, 25 m × 0.2 mm i.d.) were employed for g.l.c. (flame-ionisation detector) and g.l.c.–m.s. For the partially methylated, acetylated aldononitriles, the temperature program was 3 min at 130°, then 5°/min to 185°; for methylated alditol acetates, 3 min at 150°, then 5°/min to 185°. The products were identified by e.i.- and c.i.-m.s. Quantitation was based on the detector responses, which were corrected for differences in molecular weight.

*Smith degradation.* — The polysaccharide (100 mg) was treated under the conditions described by Cardemil and Wolk[25].

*Determination of pyruvic acid.* — A 0.4% solution of the polysaccharide in 2M HCl was heated for 3 h at 100°. Aliquots of this solution were used to determine the pyruvic acid content by the 2,4-dinitrophenylhydrazine method[26].

*Graded acid hydrolysis of the polysaccharide.* — The polysaccharide (600 mg) was hydrolysed in 2M $CF_3COOH$ (60 mL) for 1 h at 100° in sealed vials. The acid was evaporated *in vacuo* and the product was chromatographed on a column (1.5 × 40 cm) of QAE Sephadex A-25 equilibrated with 5mM phosphate buffer (pH 7). Elution with the same buffer (15 mL/h, 120 mL) afforded the unretained fraction, which was concentrated under reduced pressure and chromatographed on a column (15 × 20 cm) of Spectragel HW-40F by elution with formic acid (pH 3.3, 12 mL/h). Fractions (2 mL) were collected and monitored with the phenol–sulphuric acid reagent[27]. The disaccharide fractions, homogeneous by t.l.c.[28], were combined and

freeze-dried. Yields in different preparations were from 3 to 4%. Acidic components were eluted from the ion-exchange column with a linear gradient formed from 5mM phosphate buffer (pH 7, 150 mL) and 300mM NaCl in the same buffer (150 mL). Fractions (2 mL) were collected and tested for the presence of sugars as above. Appropriate fractions were combined, freeze-dried, and chromatographed on the gel-filtration column as described before.

*N.m.r. spectroscopy.* — N.m.r. spectra were recorded with a Bruker AM 200 spectrometer. Samples were deuterium-exchanged by freeze-drying solutions in $D_2O$ (99.7 and 99.96%).

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REFERENCES

1 G. Florenzano, C. Sili, E. Pelosi, and M. Vincenzini, *Arch. Microbiol.*, 140 (1985) 301–306.
2 C. Sili, E. Pelosi, M. Vincenzini, R. Materassi, and G. Florenzano, *Proc. Eur. Congr., Biotechnol., Third,* Vol. 1, Verlag Chemie, Weinheim, 1984, pp. 91–96.
3 L. Navarini, C. Bertocchi, A. Cesaro, R. Lapasin, and V. Crescenzi, *Carbohydr. Polym.*, in press.
4 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–274.
5 D. M. W. Anderson, F. J. McDougall, and C. G. A. McNabb, *Food Hydrocolloids,* 1 (1987) 243–246.
6 D. M. W. Anderson, *Food Addit. Contam.*, 3 (1986) 123–132.
7 M. Nakagawa, Y. Takamura, and O. Yagi, *Agric. Biol. Chem.*, 51 (1987) 329–337.
8 M. Schrader, G. Drews, J. R. Golecki, and J. Weckesser, *J. Gen. Microbiol.*, 128 (1982) 267–272.
9 K. Stellner, H. Saito, and S. Hakomori, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
10 P. E. Pfeffer, K. M. Valentine, and F. W. Parrish, *J. Am. Chem. Soc.*, 101 (1979) 1265–1274.
11 I. Backman, B . Erbing, P.-E. Jansson, and L. Kenne, *J. Chem. Soc., Perkin Trans. 1,* (1988) 889–898.
12 P.-E. Jansson, L. Kenne, and E. Schweda, *J. Chem. Soc., Perkin Trans. 1,* (1988) 2729–2736.
13 M. Forsgren, P.-E. Jansson, and L. Kenne, *J. Chem. Soc., Perkin Trans. 1,* (1985) 2383–2388.
14 M. J. King-Morris and A. S. Serianni, *J. Am. Chem. Soc.*, 109 (1987) 3501–3508.
15 A. J. Mort, J. P. Utille, G. Torri, and A. S. Perlin, *Carbohydr. Res.*, 121 (1988) 221–232.
16 E. F. Hounsell, N. J. Jones, H. C. Gooi, T. Feizi, A. S. R. Donald, and J. Feeney, *Carbohydr. Res.*, 178 (1988) 67–78.
17 L. M. Likhosherstov, O. S. Novikova, V. E. Piskarev, E. E. Trusikhina, V. A. Derevitskaya, and N. K. Kochetkov, *Carbohydr. Res.*, 178 (1988) 155–163.
18 H. M. Flowers, *Carbohydr. Res.*, 119 (1983) 75–84.
19 M. Duckworth and W. Yaphe, *Chem. Ind. (London),* (1970) 747–748.
20 U. K. Laemmli, *Nature (London),* 227 (1970) 680–685.
21 K. Mopper and L. Johnson, *J. Chromatogr.*, 256 (1983) 27–38.
22 I. W. Sutherland, *Biochemistry,* 9 (1970) 2180–2185.
23 M. E. Slodki, R. E. England, R. D. Plattner, and W. E. Dick, Jr., *Carbohydr. Res.*, 156 (1986) 199–206.

24 M. C. CADMUS, M. E. SLODKI, AND J. J. NICHOLSON, *J. Ind. Microbiol.*, 4 (1989) 127–133.
25 L. CARDEMIL AND C. P. WOLK, *J. Biol. Chem.*, 251 (1976) 2967–2975.
26 J. SLONEKER AND D. G. ORENTAS, *Nature (London)*, 194 (1962) 478–479.
27 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
28 K. KOIZUMI, T. UTAMURA, AND Y. OKADA, *J. Chromatogr.*, 321 (1985) 145–157.