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WATER-SOLUBLE POLYSACCHARIDES OF ANTARCTIC AND CULTURED *PHORMIDIUM* SPECIES OF THE CYANOPHYCEAE

CHRISTINA M MATULEWICZ,* ELIZABETH E PERCIVAL and HELMUT WEIGEL

The Bourne Laboratory, Royal Holloway College (University of London), Egham, Surrey, TW20 0EX, U K

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Abstract—Hot water extraction of a *Phormidium* species from Antarctica and of a sample of *Phormidium foveolarum* which had been cultured axenically led to the isolation of a water-soluble polysaccharide from both materials Acidic hydrolysis of each gave a similar pattern of monosaccharides comprising arabinose, xylose, rhamnose, fucose, galactose, mannose and glucose, and both contained uronic acid All attempts by a variety of methods to fractionate the Antarctic polysaccharide into more than a single entity were unsuccessful Periodate oxidation, partial hydrolysis and methylation studies on this polysaccharide supported a highly branched molecule with 1,3-linked glucose and 1,4-linked galactose as dominant features

INTRODUCTION

Although the constituent sugars of heteropolysaccharides present in a number of species of the Cyanophyceae have been determined [1], few structural studies have been carried out The envelopes of the spores and heterocysts of *Anabaena cylindrica* [2, 3] contain identical branched heteropolysaccharides consisting of a backbone comprising a repeating unit of three 1,3-linked β-D-glucose residues units linked to C-3 of a single mannose residue with side chains of glucose, mannose, galactose and xylose residues

The blue-green alga *Phormidium*, harvested by Dr J H Johnson from Blue Lake, Cape Royds, Ross Island, Antarctica, was initially placed in ethanol and stored at −15° before drying It was supplied to us in the form of dry powdered white/grey flakes mixed with small stones and powdered rock Dr Hilary Belcher of the Culture Centre of Algae and Protozoa (Cambridge) characterized this alga as a *Phormidium* species and supplied us with an axenic dormant culture of *Phormidium foveolarum* This was cultured axenically, and its water-soluble polysaccharide compared with that of the Antarctic material This was to ensure that the Antarctic species was free from bacterial contamination and that the polysaccharide was derived solely from the alga As far as we are aware, neither species has been investigated chemically previously

RESULTS AND DISCUSSION

The water-soluble polysaccharides from both species of alga, isolated as white powders, each contained *ca* 60% carbohydrate and comprised uronic acid and the same neutral monosaccharides rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose in the approximate molar proportions of 2 5 2 0 1 0 1 5 2 0 4 0 The only significant differences between the Antarctic material and the cultured *P foveolarum* were the higher molar proportions of galactose (4 0) and glucose (8 0) in the latter The essential similarity of the two polysaccharides in our opinion rules out any major contamination of the Antarctic specimen It is probable that the additional glucose in the cultured material is present as a glycogen-type polysaccharide, a common feature of a number of species of the Cyanophyceae Although the residual material remaining after extraction of the algae still contained carbohydrate, hydrolyses of aliquots of both whole algae gave the same pattern of monosaccharides

The polysaccharide from the *Phormidium* sp (hereafter known as the polysaccharide) contained *ca* 9% uronic acid, *ca* 13% protein, 4% ash and was devoid of half ester sulphate GC/MS (CI) of the acetates of glucitol and galactitol derived from the acid fragments of the polysaccharide by esterification/glycosidation, followed by reduction with borodeuteride, hydrolysis and the usual derivatization, did not reveal the $[M+1]^+$ ions, but ions with *m/z* 375 and 377 were revealed in each case These correspond to $[M+1-MeCO_2H]^+$ where M is, respectively, 434 (hexa-*O*-acetylhexitol) and 436 (d_2-hexa-*O*-acetylhexitol) Therefore the polysaccharide contains glucuronic and galacturonic acids The presence of both acids together with the same neutral monosaccharides has previously been reported [4] in the blue-green alga *Nostoc commune*

Because of the possibility of incomplete acid hydrolysis of uronic acid containing polysaccharides and of degradation if complete hydrolysis is achieved, the polysaccharide from the *Phormidium* sp was hydrolysed with 90% formic acid, with 72% sulphuric acid, and with 2 M trifluoroacetic acid The approximate percentages of the neutral sugars in each of the hydrolysates were essentially the same, from which it can be assumed that the hydrolysates give a reasonably true picture of the composition of the polysaccharide

*Present address Departmento de Química Orgánica Facultad de Ciencias Exatus y Naturales, Universidad de Buenos Aires, Pab 2, 1428 Buenos Aires, Argentina

Elution of the polysaccharide from columns of Sephadex G100 and G200 gave single, very broad peaks, indicating the material to be polydisperse and heterogeneous This was confirmed by elution with water followed by increasing concentration of potassium chloride from a DE52 cellulose column Although *ca* 90% of the polysaccharide was recovered from the column and four fractions were separated, the only significant difference in the respective fractions was an increase in the uronic acid content with increase in the concentration of the potassium chloride eluant Although part of the polysaccharide complexed with cetrimide (hexadecyltrimethylammonium bromide), no significant difference emerged in the composition of the complexed material with that which remained in solution

Periodate oxidation of the polysaccharide resulted in the reduction of 0 4 mol periodate per anhydro unit A second oxidation of the polyalcohol failed to reduce any further periodate, indicating that acetal formation had not prevented complete oxidation The polyalcohol material contained 40% carbohydrate of which 13% was uronic acid Analysis of the hydrolysate of the polyalcohol gave 24% glycerol, 9% erythritol, 5% threitol and a reduction in the percentages of the sugars with the exception of mannose which was the same as that in the initial polysaccharide Glycerol is derived from hexose units which are 1,6-linked or are non-reducing end units, and from interchain 1,4-linked pentose units Erythritol is derived from 1,4-linked glucose and/or mannose units, and threitol from 1,4-linked galactose units

Partial hydrolysis studies with different acids and a variety of conditions failed to yield oligosaccharides in sufficient quantity for characterization, or any evidence of a repeating unit in the polysaccharide After hydrolysis with 0 025 M oxalic acid for 4 hr at 95°, 30% of the starting material was recovered as soluble polymeric material In this, the uronic acid content had increased to 18% and the neutral sugars consisted mainly of glucose and mannose with appreciable amounts of galactose and very much smaller proportions of the other sugars than found in the initial polysaccharide

Methylation of the polysaccharide gave rise to a chloroform-soluble fraction and a water-soluble fraction, the latter containing 62% carbohydrate of which 11 7% was uronic acid The nitrogen content was nil After hydrolysis, the methylated sugars in both fractions were characterized by GC/MS (Table 1) There were no significant differences in the proportions of the various methylated sugars in the two materials A re-methylation by the Hakomori method or by the Purdie method failed to alter significantly the composition of the methylated sugars The only significant change in the proportion of the methylated sugars resulted from methanolysis of the water-soluble material before hydrolysis, when a con-

Table 1 Composition (approximate %) of sugars in the polysaccharide after one methylation step

Sugar*	T†	A‡	B§	C‖	Linkages
2,3,5-Ara	0 38	1 0	0 7	0 5	End group
2,3,4-Rha	0 45	2 6	3 9	t	End group
2,3,4-Ara and/or 2,3,4-Xyl	0 57	7 9¶	7 9¶	2 0**	End group
2,3,4-Fuc				0 8	End group
2,4-Rha	0 92	3 4	4 6	2 3	1,3-
2,3,4,6-Glc and/or 2,3,4,6-Man				4 2**	End group
2,4-Fuc	1 0	19 1¶	16 9¶	5 9	1,3-
2,4-Xyl and/or 2,4-Ara				1 9	1,3-
2,3,4,6-Gal	1 17	7 1	7 2	2 1**	End group
2,3- and/or 3,4-Xyl				2 7	1,4- and/or 1,2-
2-Fuc	1 42	4 4††	3 5††	4 9	1,3,4-
2,4,6-Glc	1 79	18 0	18 9	22 9	1,3-
2,3,6-Man	1 97	2 9	2 4	3 7	1,4-
2,3,6-Gal	2 23	12 7	11 9	13 8	1,4-
4,6-Man	2 88	4 2	4 3	6 9	1,2,3-
2,6-Glc	3 29	6 3	6 9	4 1	1,3,4-
3,6-Man	3 54	3 0	1 9	15 0	1,2,4-
Not identified‡‡	3 80	1 1	1 6	—	
2,4-Glc	4 27	4 6	4 8	4 9	1,3,6-
6-Glc	4 80	1 6	2 5	1 4	1,2,3,4-

*Numbers indicate the carbon atoms with methoxyl groups

†Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol

‡Hydrolysis of water-soluble fraction with 2 M trifluoroacetic acid

§Hydrolysis of the chloroform-soluble fraction with 2 M trifluoroacetic acid

‖Methanolysis followed by hydrolysis with 90% formic acid

¶No CIMS were run and so an average response factor (0 79) was used for this peak An average MW was used when the calculation was carried out by dividing the area by the MW

**Calculated from the heights of the GC/MS For 2,3- and/or 3,4-pentose an average response factor (0 68) was used

††Calculated using the molar response factor for 1,2,3,5-tetra-*O*-acetyl-4-*O*-methyl-6-deoxyhexitol

‡‡Di-*O*-methylhexose

siderable enhancement (3 → 15%) of 3,6-di-*O*-methylmannose was found on subsequent hydrolysis Because of the overlap of peaks by a number of the methylated sugars (Table 1), it is impossible to determine the exact amount of each methylated sugar Nevertheless, it can be calculated that the reduction of periodate is in reasonable agreement with the linkages found by methylation

From the various results it can be concluded that the water-soluble polysaccharide from the *Phormidium* sp is a highly branched molecule The dominant interchain units are 1,3-linked glucose and 1,4-linked galactose Some of the glucose units carry branches at C-2 and C-4 Galactose appears to be unbranched, which is surprising in view of the periodate oxidation results Mannose is mainly 1,4-linked but carries branches at C-2 and C-4, and fucose is branched at C-4

EXPERIMENTAL

General methods The general methods have been described previously [5] GC was performed on glass columns (a) 3% OV-225 on Gas-Chrom Q (100–120) mesh (2 m × 5 mm) and (b) SE 30 WCOT (25 m × 0 35 mm) A Pye 104 gas chromatograph with a flame ionization detector was used for column (a), and chromatography was performed at (i) 190° isothermally for the alditol acetates, (ii) 170° isothermally for the methylated alditol acetates and (iii) programmed from 130° to 190° at 4°/min and afterwards isothermally, for the polyol after derivatization as the alditol acetates The N_2 flow rate was 40 ml/min A Varian 3700 gas chromatograph with a flame ionization detector and a CDS 111 data system was used with column (b) at an operating temp (iv) 170° isothermally for the alditol acetates and (v) programmed from 100° to 170° at 4°/min and afterwards isothermally for the derivatized polyols The He flow rate was 1 0 ml/min and the split ratio 1 30 GC/MS was performed on a Pye 104 gas chromatograph coupled to a VG Micromass 12 F mass spectrometer using column (a) For EIMS the mass spectrometer was operated at 70 eV, for CIMS the reagent gas was 2-methylpropane at a pressure of 0 1 torr Reductions were carried out with $NaBH_4$ in aq soln or with $NaBD_4$ in D_2O or in 1 1 oxolane–ethanol Excess borohydride or borodeuteride was destroyed, sodium ions were removed with Amberlite IR-120 (H^+) resin, and boric acid by distillation with MeOH Hydrolyses were carried out in sealed tubes with 90% (w/w) HCO_2H [5], 72% (w/w) H_2SO_4 [6] or with 2 M TFA for 16 hr at 95° Uronic acid was analysed by the modified uronic acid carbazole reaction of Bitter and Muir [7] Values were read from a standard graph of glucuronolactone Sulphate was determined by the method of Jones and Letham [8] after digestion of the polysaccharide [9] The protein content was calculated by multiplying the nitrogen content by 6 25

Extraction of the Phormidium *sp and hydrolyses of the derived polysaccharide* The alga + powdered rock (123 g) was extracted with H_2O (3 × 1 l) with stirring for 3 hr on a boiling water bath The polysaccharide was isolated from the combined aq solns, after dialysis, by freeze-drying (5 48 g) It was analysed for carbohydrate, uronic acid, sulphate, protein and ash Separate portions (50 mg each) were hydrolysed by the three acids detailed under general methods and the percentage composition of the neutral monosaccharides in each of the hydrolysates was determined

Characterization of galacturonic and glucuronic acids The polysaccharide (320 mg of carbohydrate) was hydrolysed with 90% HCO_2H and the acidic fragments (60 mg carbohydrate), after separation from the neutral monosaccharides on De-Acidite FF (1P) ($HCOO^-$ form) resin, were converted into their ester glycosides with 3% MeOH–HCl Reduction with $NaBD_4$ followed by hydrolysis and reduction with $NaBH_4$ gave alditols which were characterized as their acetates by GC/MS (EI and CI)

Culture of Phormidium foveolarum *(with June Briggs)* An axenic sample of *P foveolarum* was cultured in Kratz and Myers medium [10] under axenic conditions at 20° After 7 weeks the alga was harvested and freeze-dried (770 mg) The water-soluble polysaccharide (25 mg) was extracted as for the Antarctic alga and analysed for carbohydrate and uronic acid A portion was hydrolysed with HCO_2H and the hydrolysate analysed by PC (solvents B and C [5] and by GC of the derived alditol acetates

Periodate oxidation and reduction of the derived polyaldehyde The polysaccharide (140 mg) was oxidised with 0 015 M $NaIO_4$ in 0 1 M NaOAc buffer (pH 5 0, 200 ml) in the dark Aliquots (0 1 ml) were withdrawn at intervals and the extent of oxidation was measured [11] Reaction was complete in 30 hr and the excess of periodate was destroyed with ethylene glycol The polyaldehyde was reduced to the polyalcohol with $NaBH_4$ and the latter recovered in 75% yield An aliquot was subjected to a second periodate oxidation A second aliquot was hydrolysed with 2 M TFA and the hydrolysate analysed by PC (solvents B and C [5]) and by GC of the derived alditol acetates

Methylation of the polysaccharide A dried sample of the polysaccharide (250 mg) was methylated by the Hakomori method [12] modified by Bjorndal and Lindberg [13] The soln containing the methylated material was poured into H_2O and the aq mixture extracted with $CHCl_3$ (3 × 150 ml) The aq extract was dialysed, concd and freeze-dried (114 mg) The organic extract was evapd to dryness (81 mg) Aliquots of the two products were hydrolysed with 2 M TFA A second aliquot of H_2O-soluble material was methanolysed with 3% MeOH–HCl before hydrolysis with 90% HCO_2H The alditol acetates derived from the three hydrolysates were analysed by GC/MS (EI and CI) The figures given in Table 1 have been adjusted for the response corrections established by Sweet *et al* [14] The H_2O-soluble, methylated material was subjected separately to a second Hakomori and a Purdie methylation

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