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STRUCTURAL STUDY OF THE EXTRACELLULAR POLYSACCHARIDE GUM FROM *Rhizobium* STRAIN CB744

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ABSTRACT

The structure of the extracellular polysaccharide gum from nitrogen-fixing *Rhizobium* sp. strain CB744 (a member of the slow-growing Cowpea group) has been investigated. Gas-chromatographic analysis of the alditol acetates of the acid hydrolysate showed the gum to be composed of galactose, 4-*O*-methylgalactose, mannose, and glucose in the molar ratio of 1:2.5:3.5:7.0. The polysaccharide is unusual in that it contains no carbonyl substituent, although such substituents are common amongst polysaccharides produced by the slow-growing group. The native and debranched polysaccharides were examined by methylation analysis. The anomeric configurations were determined by ^{13}C-n.m.r. and oxidation by chromium trioxide. It is concluded that there are two β-(1→4)-linked glucopyranosyl residues for each α-(1→4)-linked mannopyranosyl residue, and that each mannose is substituted at O-6 by a β-galactopyranosyl residue, with 71% of the galactose groups being present as 4-*O*-methylgalactose.

INTRODUCTION

The symbiotic interaction between *Rhizobium* bacteria and their host plants is of fundamental importance in agriculture. As part of an ongoing research programme in New Zealand, the composition and structure of rhizobial extracellular polysaccharides are being studied. In addition to classification schemes based on plant–host specificity, the genus *Rhizobium* is also divisible into two distinct groups, the fast-growing, acid producers and the slow-growing, non-acid producers[1]. In contrast to rhizobia belonging to the fast-growing group, which produce extracellular polysaccharides that do not appear to vary greatly in composition within each species, the polysaccharides of slow-growing rhizobia have very diverse compositions[1] and contain a number of unusual sugars[2–6]. Recent structural studies[7,8] on the extra-

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cellular polysaccharides from some strains of *Rhizobium japonicum*, a member of the slow-growing group, have confirmed this diversity. We report here what we believe to be the first structural studies conducted on the extracellular polysaccharide gum produced by a slow-growing *Rhizobium* from the Cowpea group.

EXPERIMENTAL

General methods. — Evaporations were conducted under diminished pressure on a rotary evaporator with a water-bath temperature not exceeding 40°. Optical rotations were measured with a Perkin–Elmer 141 polarimeter and a cell of 10-cm path-length. A Perkin–Elmer 421 grating spectrophotometer was used for recording i.r. spectra. Ash, hydrogen, carbon, nitrogen, and sulphur, were determined by the Micro-analytical Laboratory, University of Otago. Paper chromatographic (p.c.) separation of monosaccharides was achieved by using Whatman No. 1 paper, developed with 8:2:2:1 butyl acetate–pyridine–ethanol–water[1] and detected by silver nitrate–sodium hydroxide[9]. Circular dichroism (c.d.) and optical rotatory dispersion (o.r.d.) spectra were recorded at temperatures between 0 and 92° on a JASCO ORD/UV5 spectropolarimeter (400–200 nm) with cells of 0.1-, 1-, and 2-cm path length and a sample concentration of 0.1 $mg.mL^{-1}$.

Total hydrolysis of native and the debranched polysaccharides was accomplished by boiling for 16 h under reflux with M sulphuric acid. Concentrations of polysaccharide did not exceed 3%. The polysaccharide was debranched by partial acid hydrolysis, the native polysaccharide (26 mg) being refluxed with 0.1M sulphuric acid for 2 h. The reaction was stopped by immersion of the flask in ice–water and adjustment of the pH to 7 with 0.1M sodium carbonate. The products were dialysed against distilled water for 3 days, yielding 15.2 mg of non-diffusible material.

^{13}C-Nuclear magnetic resonance. — Proton-decoupled ^{13}C-n.m.r. spectra were obtained with a JEOL FX-60 spectrometer operating at 15.04 MHz in the pulsed, Fourier-transform mode. Free-induction decays were accumulated with a 45° pulse (8 μs) and a repetition time of 0.6s. Spectra were recorded with 8k data points and a spectral width of 5.0 kHz.

Native polysaccharide (85 mg) was dispersed in 1 mL of 18mM sodium hydroxide in D_2O. 1,4-Dioxane was used as the internal standard, and the chemical shifts are relative to external tetramethylsilane.

Material of low molecular weight resulting from partial acid hydrolysis of the polysaccharide was prepared from ^{13}C-n.m.r. spectroscopy by boiling under refluxing 186 mg of native polysaccharide in 0.1M sulphuric acid for 2 h. The solution was made neutral by adding barium carbonate and solids were removed by filtration. The filtrate was evaporated to dryness and the residue (59 mg) resuspended in 7 mL of methanol. This mixture was filtered and the filtrate evaporated to dryness (41 mg). A ^{13}C-n.m.r. spectrum was obtained by dissolving this methanol-soluble fraction in D_2O (1 mL). The ^{13}C-n.m.r. conditions previously described were used to record the spectrum at 40°.

Gas–liquid chromatography. — A Shimadzu G.C. 5A chromatograph fitted with a flame-ionisation detector was used for g.l.c. Separation of peracetylated alditols was achieved with a glass column (180 × 0.2 cm) containing 3% ECNSS on 100–120-mesh Gas Chrom Q (Supelco, Inc.). Partially methylated, peracetylated alditols were separated by the same method and also separated with 3% OV-225 (Applied Science Laboratories) on the same support.

G.l.c. was conducted isothermally at 180° and the flow rate of carrier gas (nitrogen) was 35 mL.min^{-1}. For the peracetylated alditols, g.l.c. was also performed at 160° in order to achieve better separation of galactose and 4-*O*-methylgalactose.

Gas–Liquid chromatography–mass spectrometry. — G.l.c.–m.s. was conducted with a Varian 1400 gas chromatograph fitted with a 180 × 0.2-cm stainless-steel column (3% ECNSS on 100–120 Gas Chrom Q) operated isothermally at 160°. The carrier gas was helium at a flow rate of 15 mL.min^{-1}.

This g.l.c. instrument was linked to a Varian MAT CH7 mass spectrometer. Spectra were recorded at an ionising potential of 70 eV, an accelerating voltage of 3 kV, and an ion-source temperature of 250°.

The organism. — *Rhizobium* sp. strain CB744 was from the Applied Biochemistry Divisional collection. It was sustained on slopes of yeast extract–mannitol medium[10]. Prior to production of polysaccharide, single colonies of *Rhizobium* CB744 were removed from plates of the same medium.

Isolation of the polysaccharide. — For polysaccharide production, the organism was shaken in yeast extract-mannitol medium (500 mL in 1-L flasks) for 21 days at 28°. Bacterial cells were removed by centrifuging at 3000*g* for 20 min and the polysaccharide was isolated by ethanolic precipitation[1]. The polysaccharide was dialysed against distilled water for 4 days at 3°. Approximately 0.6 g of polysaccharide was obtained from 1 L of growth medium.

Preparation of peracetylated alditols. — Peracetylated alditols were prepared from 21 mg of polysaccharide by the method described by Jansson *et al.*[11]. *myo*-Inositol (5 mg) was used as the internal standard.

Attempted reduction of the polysaccharide. — The procedure of Taylor and Conrad[12] was used with 3 mg of polysaccharide. The yield after dialysis and freeze drying was 2.96 mg. The polysaccharide having *myo*-inositol (0.8 mg) added was successively hydrolysed, reduced, and acetylated for g.l.c. identification of components. The experiment was repeated with 1 mg of galacturonan.

Methylation analysis. — Polysaccharide samples (before or after acetylation) could not be dissolved in dimethyl sulphoxide. Therefore the native polysaccharide and partially acid-hydrolysed polysaccharides were methylated according to the procedure described by Haworth and modified by Hirst and Percival[13]. The native polysaccharide and partially acid-hydrolysed polysaccharides (250 mg) were dissolved in 30% sodium hydroxide (15 mL) and dimethyl sulphate (3 mL) was added to the ice-cooled reaction vessels every for 5 h. Further aliquots of 30% sodium hydroxide (15 mL) and dimethyl sulphate (15 mL) were added daily for 5 days. The mixtures were dialysed against running tap water for 24 h and distilled water for 48 h. During

subsequent evaporation to low volume, the mixtures were periodically checked for neutrality by using pH paper. The polysaccharides were dried overnight in a vacuum oven, redissolved in 30% sodium hydroxide, and the cycle repeated. After dialysis of the products of the sixth cycle of both reactions, a chloroform extract of the permethylated polysaccharide was found to produce no peak in the i.r. region 3500–3250 cm^{-1}. To the permethylated native polysaccharide (130 mg) and the permethylated, partially acid-hydrolysed polysaccharide (168 mg) was added *myo*-inositol (20 and 25 mg respectively) and the permethylated polysaccharides were successively hydrolysed, reduced, and acetylated as previously described for g.l.c. and g.l.c.–m.s. identification of components. Standard compounds for comparison of retention times in g.l.c. were obtained by methylating guar gum and cellulose by the same method.

Oxidation of the polysaccharide by chromium trioxide. — The method described by Angyal and James[14] was used for oxidation of the acetylated polysaccharide.

Polysaccharide (55 mg) was dissolved in formamide (20 mL), 1:1 acetic anhydride–pyridine (20 mL) was added, and the resulting mixture was stirred for 18 h at 20°. After dialysis against distilled water for 2 days, the mixture was vacuum dried, dissolved in anhydrous chloroform, and examined by i.r. spectroscopy. After three cycles, no peak was observed in the region 3500–3250 cm^{-1}.

Acetylated polysaccharide (19.5 mg) was dissolved in acetic acid (5 mL) and finely powdered chromium trioxide (80 mg) was added. The suspension was agitated vigorously for 1 h at 50°. The mixture was dialysed against distilled water for 2 days. The non-diffusible material was vacuum dried (5 mg), and then successively hydrolysed, reduced, and acetylated for g.l.c. analysis as previously described.

RESULTS AND DISCUSSION

Light microscopy of a wet Indian-ink film-preparation showed that the extracellular polysaccharide from *Rhizobium* sp. strain CB744 is not capsular but is a loose-slime type. Microanalysis of the polysaccharide showed C, 37.47%; H, 5.57; N, 1.62%; ash, 10.85%; sulphur and phosphorus were absent. The polysaccharide had $[\alpha]_D^{20}$ +20.8°, suggesting that most of the anomeric linkages are β-D. The ^{13}C-n.m.r. spectrum of the native polysaccharide showed, *inter alia*, signals at 104.63, 104.30, 104.06, and 100.65 p.p.m., suggesting the presence of three β-anomeric linkages and one α linkage, respectively[15–18]. No signal was observed downfield from the anomeric signals. The absence of chromophores in the c.d. and o.r.d. spectra[19–21] and the absence of i.r. absorption at 1700–1725 cm^{-1}, in conjunction with the absence of a ^{13}C-n.m.r. signal at 175 ±5 p.p.m. indicates[22] that carbonyl groups are absent from the polysaccharide. After attempted carboxyl-reduction[12] and complete acid-hydrolysis of the product, g.l.c. analysis showed no change in the ratio of peracetylated alditols, confirming that uronic acid is not present in the polysaccharide. Under identical conditions, reduction of galacturonan was achieved. The production of a neutral extracellular polysaccharide is unusual as compared to other slow-growing

TABLE I

SUGAR ANALYSIS OF NATIVE POLYSACCHARIDE GUM, PARTIALLY ACID-HYDROLYSED GUM, AND OXIDISED, PERACETYLATED GUM

Sugar (as alditol acetate)	*Molar ratio*		
	A[a]	*B*[b]	*C*[c]
Galactose	1.0		0.4
4-*O*-methylgalactose	2.5		0.3
Mannose	3.5	3.5	3.5
Glucose	7.0	7.0	0.5

[a]Alditol acetates obtained from acid hydrolysate of native polysaccharide gum. [b]Alditol acetates obtained from acid hydrolysate of partially acid-hydrolysed gum. [c]Alditol acetates obtained from acid hydrolysate of chromium trioxide-oxidised, peracetylated gum.

Rhizobia, of which a large number have been found to produce polysaccharides containing carboxylic substituents[1,2,7,8].

Four components were separated by p.c. from a total acid-hydrolysate. Three of these components were identified by p.c. and g.l.c.–m.s. of the peracetylated alditol derivatives as galactose, mannose, and glucose in the molar ratio of 1:3.5:7.0, (Table I). By p.c., the fourth component co-chromatographed with 4-*O*-methylgalactose, which has a mobility relative to glucose of 1.70 and following demethylation with boron tribromide[23] the product co-chromatographed with galactose. The peracetylated alditol derivative of this fourth component gave a mass spectrum having the major fragments *m/z* 43, 85, 87, 127, 129, 189, 201, and 261, showing that it is either a 3- or 4-*O*-methylated, peracetylated hexitol. Mono-*O*-methyl derivatives of other hexitols have been shown[11] to be well separated from peracetylated 3- or 4-*O*-methylgalactitol, but these two derivatives have identical g.l.c. retention-times.

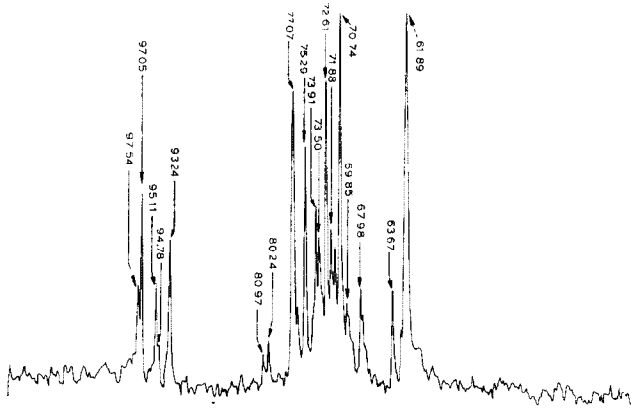

Fig. 1. ^{13}C-N.m.r. spectrum of the low-molecular-weight methanol-soluble material following partial acid hydrolysis.

As an authentic sample of 3-*O*-methylgalactose was not available for comparison, unequivocal identification of this component cannot be made based on this evidence alone.

The ^{13}C-n.m.r. spectrum of the methanol-soluble, low-molecular-weight material removed during partial acid hydrolysis (Fig. 1) shows no peaks downfield from 98 p.p.m., indicating that only monomers are present[16,24,25]. The signals at 93.24 and 97.05 p.p.m. were assigned to C-1 of α-galactopyranose and β-galactopyranose, respectively, on the basis of previously reported values[26,27,28]. Signals in the anomeric region (95.11 and 94.78 p.p.m.) were assigned to β-mannopyranose and α-mannopyranose respectively; these are in agreement with the literature[24,25] not only with respect to chemical shift but also in relative natural abundance.

It is possible that the peak at 97.54 p.p.m. results from the influence that mono-*O*-methyl substitution exerts on the anomeric carbon atom of β-galactopyranose, especially as the relative abundance of the peaks at 97.54 plus 97.05 p.p.m. to the peak at 93.24 p.p.m. is in agreement with that reported for β-galactopyranose and α-galactopyranose, respectively[26]. Glucose is apparently absent from this preparation, as no signals are observed at 96.5, 92.7 (C-1 of β-D-glucopyranose and C-1 of α-D-glucopyranose), 73.4 (C-3 of α-D-glucopyranose), and 70.3 p.p.m. (C-4 of α- and β-D-glucopyranose).

Shaskov and coworkers[29] have demonstrated that the C-4 of methyl 4-*O*-methyl-α-D-galactopyranoside gives a signal at 81.1 p.p.m. On the other hand, Voelter *et al.*[26] reported that C-3 of methyl 3-*O*-methyl-β-D-galactopyranoside gives a signal at 82.0 p.p.m. Furthermore, the chemical shift of the methoxyl carbon atom substituted at C-3 of galactose is[26] 56.2 p.p.m., whereas the methoxyl carbon atom substituted at C-4 of galactose resonates[29] at 62.1 p.p.m. It was therefore concluded that the fourth component separated by g.l.c. is peracetylated 4-*O*-methylgalactitol.

The polysaccharide is thus composed of the neutral hexoses, galactose, 4-*O*-

TABLE II

METHYLATION ANALYSIS OF NATIVE (A) AND PARTIALLY HYDROLYSED[a] (B) POLYSACCHARIDE FROM RHIZOBIUM STRAIN CB744

Methylated sugars[b] *(as alditol acetate)*	*T*[c]	*Ratio of peak areas*	
		A[d]	*B*[e]
2,3,4,6-Gal	1.28	1	
2,3,6-Glc	2.61	2.01	1.96
2,3,6-Man	2.23		1
2,3-Man	5.01	1.08	

[a]Partially hydrolysed by boiling under reflux for 2 h in 0.1M sulphuric acid. [b]2,3,4,6-Gal = 1,5 di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, and so on. [c]Retention time of partially methylated alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. [d]Relative to 2,3,4,6-Gal. [e]Relative to 2,3,6-Man.

methylgalactose, mannose, and glucose in the molar ratio of 1:2.5:3.5:7.0 (Table I, Column A).

Methylation analysis of the native polysaccharide gum showed three components by g.l.c. (Table II, Column A), identified by co-chromatography with known compounds and by g.l.c.–m.s. The result indicates that galactose and 4-*O*-methylgalactose are in the pyranose form and are terminal, and suggests that glucose is in the pyranose form and is (1→4)-linked. Furthermore, mannose appears to be in the pyranose form, linked through O-4 and O-6, and therefore it is a branch point in the backbone.

The fraction of high molecular weight resulting from partial acid hydrolysis was shown by p.c. and g.l.c.–m.s. of the alditol acetate derivatives to be composed of mannose and glucose in the molar ratio of 1:2.0 (Table I, Column B). Methylation analysis of the partially acid-hydrolysed polysaccharide gave two components (Table II, Column B). These components were identified by co-chromatography with known compounds and by g.l.c.–m.s. and show that glucose and mannose are (1→4)-linked. Glycofuranosidic linkages are known[30] to be much more acid labile than glycopyranosidic linkages. As the galactose and 4-*O*-methyl galactose, which are pyranoid, are lost by partial hydrolysis but glucose and mannose are not, it follows that glucose and mannose are in the pyranose form. The ^{13}C-n.m.r. spectrum of the native polysaccharide is consistent with the absence of furanosyl sugars[31]. The loss of galactose and 4-*O*-methylgalactose by partial acid-hydrolysis was coincident with O-6 of the mannose residues becoming available for methylation. This result would indicate that galactose and 4-*O*-methylgalactose are linked to mannose at O-6 and had been removed during partial acid hydrolysis. As glucose and mannose both substituted at O-1 and O-4 are the only species present in the methylated, partially hydrolysed polymer, this structure cannot be branched. The molar ratio of permethylated mannitol and glucitol remains at 1:2.0, and so glucose does not appear in the side chain but is present in the backbone.

The non-diffusible product remaining after dialysis of the chromium trioxide-treated, acetylated polysaccharide was successively hydrolysed, reduced, and acetylated. The g.l.c. pattern (Table I, Column C) shows that mannopyranosyl residues have the α configuration and the glucopyranosyl, galactopyranosyl, and 4-*O*-methylgalactopyranosyl residues have the β configuration. This conclusion is consistent with the observed optical rotation and the interpretation of the ^{13}C-n.m.r. spectrum of the native polysaccharide.

CONCLUSIONS

The polysaccharide isolated from the growth medium of *Rhizobium* sp. strain CB744 is shown to be composed of galactose, 4-*O*-methylgalactose, mannose, and glucose in the molar ratio of 1:2.5:3.5:7.0. An unusual feature of this composition is absence of the carbonyl substituents commonly present in the polysaccharides of other slow-growing *Rhizobia*[1,2,7,8].

Methylation analysis of the native, and partially hydrolysed polysaccharide in conjunction with the data obtained from ^{13}C-n.m.r. spectroscopy and chromium trioxide oxidation shows the polysaccharide to have a branched structure the main chain of which consists of two, β-(1→4)-linked glucopyranosyl residues for each α-(1→4)-linked mannopyranosyl residue. The side chain consists of a single β-galactopyranosyl residue linked to the O-6 of each mannopyranosyl residue in the main chain. Position 4 of 71% of the galactosyl residues are substituted by an *O*-methyl group.

While not definitive, the foregoing data suggest that the polysaccharide is composed of a tetrasaccharide repeating-unit. However, we do not consider that the evidence presented is sufficient to establish the structure with complete confidence.

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