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Structure of the extracellular polysaccharide secreted by *Rhizobium leguminosarum* var. *phaseoli* CIAT 899*

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ABSTRACT

The structure of the extracellular polysaccharide secreted by *Rhizobium leguminosarum* var. *phaseoli* CIAT 899 has been studied by methylation analysis, ^{1}H-n.m.r. spectroscopy, and partial acid hydrolysis. The repeating unit is an octasaccharide made up of D-glucose, D-galactose, pyruvic acid, and acetic acid in the molar ratios 6:2:1.5:1.5. Half of the terminal Gal groups are 4,6-substituted by pyruvic acid acetal groups and the other half by *O*-acetyl groups at position 3. Also, one of the 3-linked glucosyl residues carries a pyruvic acid 4,6-acetal group and one of the 4-linked glucosyl residues is acetylated at position 6.

INTRODUCTION

The exopolysaccharide secreted by the strains of *Rhizobium* play an important role in the infection of legumes and *Rhizobium*–legume symbiosis. The structures of some polysaccharides secreted by strains of *R. leguminosarum* var. *phaseoli* have been described[1–4]; most polysaccharides reported contain glucuronic acid except those described by Amemura and Harada[5]. We now report on the structure of the exopolysaccharide secreted by *R. leguminosarum* var. phaseoli CIAT 899, which is similar to that proposed by Amemura and Harada[5].

EXPERIMENTAL

General methods. — Descending p.c. was performed on Whatman No. 3MM paper, using 1-butanol–ethanol–water (2:1:1). ^{1}H-N.m.r. (200 MHz) spectra (internal 4,4-dimethyl-4-silapentane-1-sulfonate) were recorded at 75° with a Varian XL-200 instrument. A solution of a sample of the polysaccharide in D_2O was freeze-dried and the process was repeated prior to ^{1}H-n.m.r. spectroscopy. G.l.c. was performed with a Hewlett–Packard 5710 A chromatograph fitted with a flame-ionisation detector, a glass

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column (2 m x 0.6 cm) packed with 3% of SP-2340 on Supereloport (100–120 mesh) at 235° for alditol acetates, and a stainless steel column (2 m × 0.3 cm) packed with 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 170° for partially methylated alditol acetates. G.l.c.–m.s. was performed with a Kratos MS-80 RFA instrument fitted with a CP-SIL 5 W.C.O.T. column (25 m × 0.32 mm), using the temperature programme 100→250° at 5°·min^{-1}. The ionisation potential was 70 eV.

H.p.l.c. was performed with a Waters Sugar Analyzer I fitted with a stainless steel column (30 cm × 7.8 cm) packed with HPX-87 H (Bio-Rad), using 7.5mM sulfuric acid at 65° and 0.3 mL · min^{-1}. Gel permeation chromatography (g.p.c.) was performed on a column (90 cm x 2.6 cm) of Bio-Gel P-2 by elution with water. The eluate was monitored by t.l.c. (silica gel; 2:1:1 1-butanol–acetic acid–water; detection with orcinol–sulfuric acid).

Pyruvic acid was determined by the method of Sutherland[6], and *O*-acetyl by the method of Downs and Pigman[7]. Routine polysaccharide hydrolysis was performed with 4M trifluoroacetic acid for 4 h at 100°. After evaporation of the acid, the products in the hydrolysate were reduced and acetylated by the method of Blakeney *et al.*[8].

Isolation and purification of the polysaccharide. — A 7-day old culture of *R. leguminosarum* var. *phaseoli* CIAT 899, grown in Allen 79 medium, was centrifuged, and acetone (2 vol.) was added to the solution. The precipitate was purified by dialysis against water, lyophilization, and precipitation with cetyltrimethylammonium bromide.

Methylation. — The polysaccharide was methylated twice with potassium methylsulfinylmethanide[9] and methyl iodide. The product was purified by chromatography (1:1 dichloromethane–ethanol) on Sephadex LH-20. Further hydrolysis was conducted with aqueous 88% formic acid and 2M trifluoroacetic acid. The products in the hydrolysates were reduced and acetylated by the method of Blakeney *et al.*[8].

Preparation of depyruvylated polysaccharide. — A solution of a sample in 5mM sulfuric acid was kept for 135 min at 90°, then dialysed, and freeze-dried.

Location of the O*-acetyl groups.* — The polysaccharide was methylated by the method of Prehm[10]. The sample (5 mg) was suspended in trimethyl phosphate (1 mL) by sonication. 2,6-Di-*tert*-butylpyridine (150 μL) and methyl trifluoromethanesulfonate (100 μL) were added. The mixture was kept for 2 h at 50°, then partitioned between chloroform (5 mL) and water (20 mL). The chloroform layer was filtered through Sephadex LH-20. One portion of the methylated polysaccharide was converted into partially methylated alditols, and the other was remethylated conventionally (see above), but using trideuteriomethyl iodide, then hydrolysed, and the products were reduced and acetylated.

Partial hydrolysis. — A 1% solution of the polysaccharide in 0.5M trifluoroacetic acid was heated for 1 h at 100°, then dialysed against water. The diffusate was concentrated and the residue was chromatographed on Bio-Gel P-2. The effluent was monitored by t.l.c. and fractionated accordingly, and each fraction was subjected to preparative p.c. The non-diffusable fraction was lyophilized, and a 1% solution in 0.5M trifluoroacetic acid was kept at 100° for 1 h. The procedure was repeated with heating

for 2 h. Seven oligosaccharides were isolated and were (*a*) methylated, hydrolysed, reduced, acetylated, and subjected to g.l.c.–m.s., and (*b*) reduced, methylated, hydrolysed, reduced, acetylated, and subjected to g.l.c.–m.s.

RESULTS AND DISCUSSION

The extracellular polysaccharide secreted by *R. leguminosarum* var. *phaseoli* CIAT 899, and isolated from the culture fluid by precipitation with acetone and cetyltrimethylammonium bromide, contained glucose, galactose, pyruvic acid, and acetic acid in the molar ratios 6:2:1.5:1.5. The ^{1}H-n.m.r. spectrum contained signals at δ 1.45 and 2.10 which were assigned to the methyl groups of pyruvic acid and acetic acid, respectively. The signals for anomeric protons were in the range δ 4.5–4.8 and, therefore, the glycosidic linkages were β.

Methylation analysis of the polysaccharide gave the results shown in Table I, which revealed non-reducing terminal galactose (0.4 mol per repeating unit of eight sugars), 4,6-disubstituted galactose (0.6 mol), 3,4,6-trisubstituted glucose, 4,6-disubstituted glucose, 3-substituted glucose and galactose, and 4- and 6-substituted glucose residues. Methylation analysis of the depyruvylated polysaccharide showed increases in terminal galactose and 3-substituted glucose, and decreases in 4,6-disubstituted galactose and 3,4,6-trisubstituted glucose. Thus, about half of the terminal non-reducing galactosyl groups and one of two 3-substituted glucosyl residues carried pyruvic acid as a 4,6-acetal group. The branch point of the polysaccharide was indicated by the 4,6-disubstituted glucosyl residue.

The native polysaccharide was methylated by the method of Prehm[10], and methylation analysis then showed (Table I) increases in 2,4,6-tri-*O*-methyl-D-galactose and 2,3-di-*O*-methyl-D-glucose, and decreases in 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose. Thus, the terminal non-reducing non-pyruvylated galactosyl group was acetylated at O-3 and one of the two 4-substituted glucosyl residues was acetylated at O-6.

These findings were confirmed as follows. The native polysaccharide was methylated (Prehm), then remethylated in a basic medium (see Experimental) using trideuteriomethyl iodide, and subjected to methylation analysis. The chromatogram and the mass spectrum for the product in each peak were identical to those obtained from the native polysaccharide, except for the non-reducing terminal galactosyl group (*m/z* 45, 71, 74, 87, 101, 104, 117, 129, 148, 161, 164, and 208) and for the 4-substituted glucose (*m/z* 45, 48, 87, 99, 101, 113, 117, 233, and 236). The mass spectrum for the product in the peak that corresponded to the non-reducing terminal galactosyl group showed the fragmentation expected for a 1,5-di-*O*-acetyl-2,4,6-tri-*O*-methyl-3-*O*-trideuteriomethylhexitol. The mass spectrum for the product in the peak corresponding to the 4-substituted glucose was consistent with a mixture of a 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol and a 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-6-*O*-trideuteriomethylhexitol.

Partial acid hydrolysis of the native polysaccharide gave seven oligosaccharides that were isolated by g.p.c. and p.c. Methylation analysis of the oligosaccharides before

TABLE I

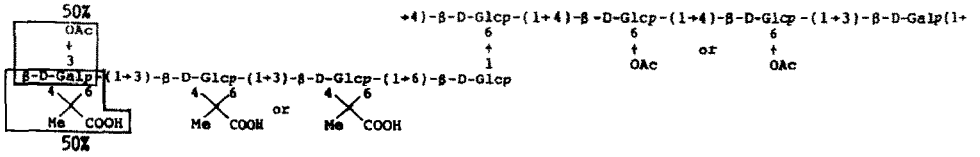
Methylation analysis data for native and modified polysaccharide and oligosaccharides from *R. phaseoli* CIAT 899

Methylated sugars (as alditol acetates)	*Molar ratio*											
	T^a	T^b	*A*	*B*	*C*	*O-I*	*O-II*	*O-III*	*O-IV*	*O-V*	*O-VI*	*O-VII*
2,3,4,6-Me_4-Glc[c]	1.00	1.00	–	–	–	–	1.0	1.0	1.0	1.0	1.0	1.0
2,3,4,6-Me_4-Gal	1.25	1.06	0.4	0.8	–	1.0	–	–	–	–	–	–
2,4,6-Me_3-Glc	1.94	1.17	0.7	1.2	0.9	1.0	1.0	–	–	–	–	0.8
2,4,6-Me_3-Gal	2.24	1.23	0.9	1.0	1.6	–	–	–	–	–	–	–
2,3,4-Me_3-Glc	2.57	1.24	1.1	0.9	1.0	–	–	–	0.7	0.8	1.6	1.7
2,3,6-Me_3-Glc	2.57	1.19	2.0	2.0	1.3	–	–	2.0	0.9	2.2	0.8	1.1
2,3-Me_2-Glc	5.57	1.40	0.9	0.7	2.1	–	–	–	–	–	–	–
2,3-Me_2-Gal	5.97	1.41	0.6	–	0.5	–	–	–	–	–	–	–
2-Me-Glc	8.60	1.50	0.7	0.1	0.7	–	–	–	–	–	–	–

[a] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on an ECNSS-M column.[b] For a W.C.O.T. column of CP-Sil5. [c] 2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* A, native polysaccharide; B, depyruvylated polysaccharide; C, native polysaccharide methylated by the method of Prehm; O-I, oligosaccharide I; *etc.*

and after reduction indicated the oligosaccharides to be β-D-Gal*p*-(1→3)-D-Glc, β-D-Glc*p*-(1→3)-D-Glc, β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc, β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→4)-D-Glc, β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glc, β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→4)-D-Glc, and β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-β-D-Gcl*p*-(1→6)-β-D-Glc*p*-(1→4)-D-Glc.

On the basis of the above results, the octasaccharide repeating-unit **1** is proposed for the polysaccharide. This structure is similar to that proposed by Amemura and Harada[5].



1

Half of the terminal non-reducing galactosyl groups are 4,6-pyruvylated and the other half are 3-acetylated. One of two (1→3)-linked glucosyl residues is 4,6-pyruvylated and one of the two (1→4)-linked glucosyl residues is 6-acetylated. An alternative structure would have the 3-linked galactosyl residues between the terminal non-reducing galactosyl groups and the first 3-linked glucosyl residues.

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