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Note

Structure of the acidic exopolysaccharide secreted by *Rhizobium leguminosarum* biovar. *phaseoli* CFN42

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The structures of the exopolysaccharides secreted by strains of *Rhizobium* have been studied in relation to their role in the specificity of the infection of the legumes[1–6]. The patterns of acyl substitution as determinants of these specificities have been studied[7,8]. We now report on the structure and pattern of acylation of the exopolysaccharide secreted by *Rhizobium leguminosarum* biovar. *phaseoli* CFN42.

The exopolysaccharide, isolated from the culture fluid by precipitation with acetone and cetyltrimethylammonium bromide, contained glucose, galactose, glucuronic acid, pyruvic acid, and acetic acid in the molar ratios 5:1:2:2:1 (the ratios of the sugars were identical to those reported[9]). The ^{1}H-n.m.r. spectrum of the exopolysaccharide contained three signals for acetyl groups and one signal for the methyl group of a pyruvic acid acetal. The ^{1}H-n.m.r. spectrum of the methylated polysaccharide contained signals for anomeric protons, of both β- and α-glycosidic units.

The results of the methylation analysis of the exopolysaccharide (Table I) revealed 4- (3 mol per repeating unit), 4,6- (1 mol), and 3,4,6-substituted Glc (1 mol), together with 4,6-substituted Gal (1 mol). Methylation analysis (Table I) of the partially depyruvated exopolysaccharide revealed terminal non-reducing Gal and 3-substituted Glc, and decreased proportions of 4,6-substituted Gal and 3,4,6-substituted Glc. Thus, the terminal non-reducing Gal and 3-substituted Glc must carry pyruvic acid as a 4,6-acetal. Methylation analysis of the methylated, carboxyl-reduced exopolysaccharide (Table I) showed an increase in the proportion of 4,6-substituted Glc, and the mass spectrum showed an increase of the ratio *m/z* 261/263, which indicated the presence of 6,6-dideuterio-4-substituted Glc formed by reduction of 4-substituted GlcA.

When the exopolysaccharide was methylated by the method of Prehm[10], methylation analysis then revealed (Table I) 2,6-di-*O*-methylglucose and 2-*O*-methylgalactose,

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TABLE I

Methylation analysis data for the exopolysaccharide from *R. leguminosarum* var. *phaseoli* CFN42 and for the modified polysaccharide and oligosaccharides

Methylated sugars (as alditol acetates)	T[a]	*Molar ratio*									
		A	B	C	D	A-1	A-2	A-3	A-4	L-1	H-1
2,3,4,6-Me_4-Glc[b]	1.00	–	–	–	–	1.0	–	1.0	–	1.1	2.0
2,3,4,6-Me_4-Gal	1.05	–	0.3	–	–	–	1.0	–	1.0	0.6	–
2,4,6-Me_3-Glc	1.18	–	0.8	–	–	–	1.1	–	1.1	0.6	–
2,3,6-Me_3-Glc	1.21	3.0	3.0	3.0	0.6	1.2	–	2.2	1.6	2.0	2.0
2,6-Me_2-Glc	1.32	–	–	–	1.3		–	–	–	–	–
2,3-Me_2-Glc	1.41	0.9	1.2	1.5	1.9	–	–	–	–	–	1.0
2,3-Me_2-Gal	1.42	1.2	0.6	1.0	0.4	–	–	–	–	0.6	–
2-Me-Glc	1.52	1.2	0.2	1.3	0.9	–	–	–	–	–	–
2-Me-Gal	1.53	–	–	–	0.7	–	–	–	–	–	–
1,2,3,5-Me_4-Glc	0.99	–	–	–	–	–	–	–	–	0.5	–

[a] Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on a W.C.O.T. column of CP-Sil5CB. [b] 2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* Key: *A*, exopolysaccharide; *B*, depyruvated *A*; *C*, methylated and carboxyl-reduced *A*; *D*, methylated *A* obtained by the method of Prehm; *A-1/4*, oligosaccharides obtained by acetolysis of *A*; *L-1*, oligosaccharide obtained by degradation of *A* with lithium–ethylenediamine; *H-1*, oligosaccharide obtained by partial hydrolysis of *A*.

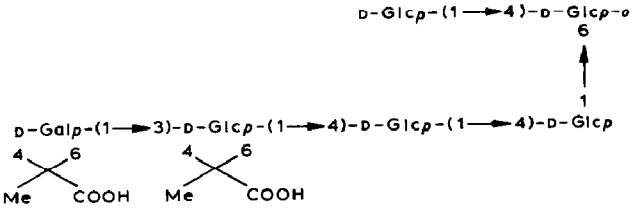
an increased proportion of 2,3-di-*O*-methylglucose, and decreased proportions of 2,3,6-tri-*O*-methylglucose and 2,3-di-*O*-methylgalactose. Thus, the terminal non-reducing pyruvated Gal must be 3-acetylated and the 4-substituted Glc must be 3- or 6-acetylated.

These findings were confirmed by the methodology described[6] for *Rhiz. leguminosarum* var. *phaseoli* CIAT899. The polysaccharide was methylated (Prehm), then remethylated in a basic medium using trideuteriomethyl iodide. The results of methylation analysis were identical to those for the exopolysaccharide, except for the products derived from the non-reducing pyruvated Gal and for the 4-substituted Glc. The mass spectrum (*m/z* 43, 101, 117, 261, and 264) for the former product showed the fragmentation expected for a 1,4,5,6-tetra-*O*-acetyl-2-*O*-methyl-3-*O*-trideuteriomethylhexitol and that (*m/z* 45, 48, 87, 99, 101, 113, 117, 233, and 236) of the latter product was consistent with a mixture of a 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-6-*O*-trideuteriomethylhexitol and/or 1,4,5-tri-*O*-acetyl-2,6-di-*O*-methyl-3-*O*-trideuteriomethylhexitol. These results were in agreement with the three signals for OAc in the ^{1}H-n.m.r. spectrum. Since 1 mol of acetate was indicated by colorimetric determination, non-stoichiometric acetylation of the repeating unit of the exopolysaccharide at O-3 of non-reducing pyruvated Gal and O-3 or O-6 of 4-linked Glc is proposed.

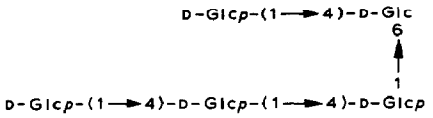
Acetolysis of the exopolysaccharide, followed by *O*-deacylation, yielded a mixture of di- and oligo-saccharides and a degraded polysaccharide, which was fractionated by dialysis. The low-molecular-weight products were isolated by gel-permeation chromatography and preparative p.c., and shown by methylation analysis (Table I) to be D-Glc*p*-(1→4)-D-Glc (A-1), D-Gal*p*-(1→3)-D-Glc (A-2), D-Glc*p*-(1→4)-D-Glc*p*-(1→4)-D-Glc (A-3), and D-Gal*p*-(1→3)-D-Glc*p*-(1→4)-D-Glc*p*-(1→4)-D-Glc (A-4).

Methylation analysis of the degraded polysaccharide (Table I) revealed the major component to be 4-linked Glc.

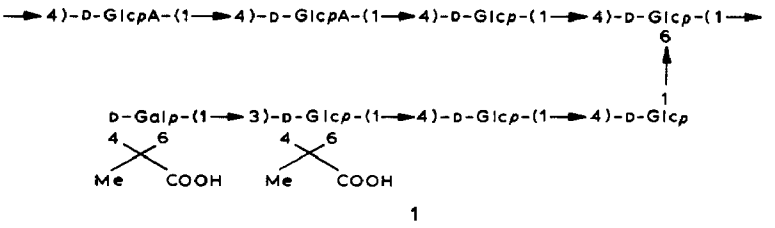
The exopolysaccharide was degraded with lithium–ethylenediamine. Methylation analysis of the resulting oligosaccharide L-1 (Table I) indicated the following structure.



Partial acid hydrolysis of the exopolysaccharide gave four oligosaccharides, methylation analysis of which (Table I) indicated that three were identical to A-1/3, and the fourth (H-1) was identified on the basis of the structure of L-1 as



On the basis of the above results, the octasaccharide repeating unit **1** is proposed tentatively for the polysaccharide.



1

The repeating unit is non-stoichiometrically acetylated at O-3 of the non-reducing pyruvated Gal and at O-3 or O-6 of 4-linked Glc.

EXPERIMENTAL

Isolation and purification of the exopolysaccharide. — A 7-day old culture of *R. leguminosarum* biovar. *phaseoli* CFN42, grown in Allen 79 medium, was centrifuged, then diluted with acetone (2 vol.). The precipitate was dialysed against water, the retentate was lyophilised, and the exopolysaccharide was precipitated with cetyltrimethylammonium bromide[11].

General methods[6]. — Uronic acid was determined by the carbazole method[12]. The solvents used in chromatography were *A*, BuOH–EtOH–H_2O (2:1:1); and *B*, BuOH–Py–H_2O (6:4:3). The ^{1}H-n.m.r. spectrum of the exopolysaccharide showed signals at δ 2.13, 2.20, and 2.24, which were assigned to methyl groups of acetates, and δ 1.47 assigned to methyl groups of pyruvic acid.

Methylation analysis. — The exopolysaccharide was methylated, hydrolysed, reduced, and acetylated as described[6]. The ^{1}H-n.m.r. spectrum of the methylated exopolysaccharide showed signals for anomeric protons: three doublets at δ 4.44 (J 8.2 Hz) and 4.71 (J 8.2 Hz), both assigned to the anomeric proton in β-glycosidic units, and 4.85 (J 3.5 Hz), which was assigned to that in α-glycosidic units.

Reduction of the methylated exopolysaccharide[1]. — Glucuronic acid methyl ester groups were reduced with $LiAlD_4$ to give the 6,6-dideuteriohydroxymethyl derivative.

Preparation of the depyruvated exopolysaccharide. — A solution of the exopolysaccharide (3.5 mg) in 5mM sulfuric acid (6 mL) was kept for 135 min at 90°, then dialysed, and freeze-dried.

Location of the O*-acetyl-groups.* — The exopolysaccharide was methylated by the method of Prehm[10]. The product was subjected to hydrolysis, reduction, and acetylation before and after remethylation in a basic medium, using trideuteriomethyl iodide[6].

Acetolysis[13]. — The exopolysaccharide (300 mg) was stirred with acetic acid–acetic anhydride–conc. sulfuric acid (10:10:1, 40 mL) for 18 h at room temperature. The products were *O*-deacetylated with methanolic M sodium methoxide and dialysed against water. The diffusate was concentrated and then eluted from Bio-Gel P-2 with water, the eluate was monitored by t.l.c., and each fraction was subjected to preparative p.c. (solvents *A* and/or *B*). Four oligosaccharides were isolated and subjected to methylation analysis. The product in the retentate was subjected to methylation analysis.

Partial hydrolysis. — A solution of the exopolysaccharide (300 mg) in 0.5M trifluoroacetic acid was heated for 90 min at 100°, then dialysed against water. The retentate was lyophilised and the product was hydrolysed with 2M trifluoroacetic acid for 2 h at 100°, then dialysed. The two diffusates were concentrated and the components were separated into neutral and acidic sugars by chromatography on Amberlite IR-120 (H^+) and IRA-400 (AcO^-) resins. Four neutral sugars were isolated as described above.

Reaction with lithium in ethylenediamine[14]. — The exopolysaccharide (13 mg), dried *in vacuo* overnight at 40°, was suspended in ethylenediamine (2 mL) and dried over 4A molecular sieve, and pieces (2–5 mm) of lithium wire (45 mg/cm) were added to maintain the deep-blue color for 1 h. The reaction was terminated by adding water to the cooled mixture. Toluene was then added to the solution, the solvents were evaporated, and the process was repeated several times. A solution of the residue in water was acidified with acetic acid to pH 4.5 and purified by elution from Dowex AG-50W-X12 (H^+) resin with water, and from Bio-Gel P-2 with water. The material eluted in the partially included volume was subjected to preparative p.c. (solvent *B*). A pure oligosaccharide was isolated and subjected to methylation analysis.

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