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FRAGMENTATION ANALYSIS OF EXTRACELLULAR ACID POLYSACCHARIDES FROM SEVEN *Rhizobium* STRAINS

PART I. D-GLUCURONIC ACID-CONTAINING OLIGOSACCHARIDES

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ABSTRACT

The extracellular, bacterial polysaccharides from seven *Rhizobium* strains have been submitted to partial hydrolysis with acid. Several neutral oligosaccharides, some containing pyruvic acid, were isolated together with D-glucuronic acid-containing oligosaccharides. The polysaccharide from *Rh. meliloti* did not contain glucuronic acid. For the other six strains, the following components were characterized: 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucuronic acid, 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucose, and *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→4)-D-glucose. These results indicate the presence of chains containing two β-(1→4)-linked D-glucuronic acid residues, β-linked to D-glucose at position 4.

INTRODUCTION

The importance of the bacteria–host interaction between bacteria of the genus *Rhizobium* and plants of the family Leguminosae has long been known[1]. The possible role of the extracellular bacterial polysaccharides in this interaction has been discussed, and results from detailed, chemical analysis of seven strains belonging to different bacteria–host groups have been reported[2]. D-Galactose, D-glucose, D-glucuronic acid, pyruvic acid, and acetyl groups are common constituents of the polysaccharides examined, an exception being the polysaccharide produced by *Rh. meliloti*, which is known to lack uronic acids. In studying the structure of these polysaccharides, partial hydrolysis with acid has been used. From the hydrolysates, neutral oligosaccharides, some containing pyruvic acid, have been isolated and characterized, together with hitherto undescribed oligosaccharides containing D-glucuronic acid. Partial hydrolysis of the methylated extracellular polysaccharide of *Rh. radicolum* gave a methylated aldobiouronic acid, which appeared to be cellobiouronic acid[3].

We now report on the oligosaccharides containing D-glucuronic acid.

EXPERIMENTAL

General methods. — The organisms, cultivation technique, and the preparation of the polysaccharides were as reported earlier[2]. Either Whatman No. 1 or 3MM paper was used for analytical and preparative, high-voltage electrophoresis, and for preparative p.c. High-voltage electrophoresis was performed with pyridine–acetic acid–water (5:2:43, pH 5.3) and strips (22 × 47 cm) of paper at 1.6 kV for 3 h (No. 1) and 0.8 kV for 6 h (No. 3MM). P.c. was performed by the descending method, using ethyl acetate–acetic acid–pyridine–water (5:1:5:3). T.l.c. was carried out on Machery Nagel cellulose, using the foregoing solvent system. Detection was effected with *p*-anisidine hydrochloride and aniline hydrogen phthalate.

Partial, acid hydrolysis of the polysaccharide. — The optimum conditions were as follows. The polysaccharide (200 mg) was soaked in water overnight, and trifluoroacetic acid was added to give a highly viscous solution (0.5% in M acid), which was incubated at 100° for 2 h in a sealed flask. The acid was removed by evaporation under diminished pressure below 50°.

A solution of the residue in water (2 ml) was subjected to preparative high-voltage electrophoresis. The neutral components have no mobility in this system and partial fractionation of the acid components occurred. Four fractions (*A*–*D*) were obtained, with *D* having the highest mobility. Fractions *C* and *D* were homogeneous and were identified as D-glucuronic acid and pyruvic acid, respectively. When fractions *A* and *B* were subjected to preparative p.c. and repeated, preparative high-voltage electrophoresis, the D-glucuronic acid-containing oligomers **2**, **3**, and **4** were separated from other acidic carbohydrate material containing pyruvic acid as the only acidic constituent.

Because of the tedious isolation and purification procedures, the yields of **2**–**4** were low (~2 mg), and their mobilities were as follows:

Component	**1**	**2**	**3**	**4**
R_{GLC}	0.54	0.23	0.46	0.17
M_{GLCA}	1.0	1.21	0.65	0.85

Hydrolysis of the acidic oligosaccharides. — Hydrolysis was carried out at 120° for 2 h with M trifluoroacetic acid for partial degradation, and 2M acid for total degradation.

Alditol formation. — A solution of each oligosaccharide (50 μg) in water (1 ml) was treated with $NaBH_4$ (2–3 mg) at room temperature for 18 h. The pH of the mixture was adjusted to ~3 with Amberlite IR-120(H^+) resin before removal of the borate ions by repeated evaporations with methanol (3 × 5 ml).

Methyl glycoside and methyl ester methyl glycoside formation. — A solution of each neutral or acidic sugar (50 μg) in methanol (1 ml) containing Dowex-50W x8

(H^+) resin (100–200 mesh) was left at 67° for 24 h, and then subjected to microfiltration and concentration.

Methyl ester methyl glycosides of **2**, **3**, and **4** were reduced to methyl glycosides, using $NaBH_4$ as described for the alditol formation.

Methylation analysis. — A solution of the methyl glycoside of each reduced oligosaccharide (2–3 mg) in *N*,*N*-dimethylformamide (3 ml) was shaken and, at 30-min intervals, sodium hydride (15 mg) and methyl iodide (1 ml) were added. After 5 h, more sodium hydride (15 mg) and methyl iodide (2 ml) were added and the mixture was shaken overnight. The product was dissolved in chloroform and extracted with water (6 × 5 ml). The combined extracts were concentrated to dryness and the residue was hydrolysed with 0.5M H_2SO_4 for 4 h at 100°. The hydrolysate was neutralized with Dowex-1(HCO_3^-) resin, filtered, and concentrated to dryness.

A solution of the residue in pyridine (0.5 ml) was stored for 30 min, and then treated with hexamethyldisilazane (0.1 ml) and chlorotrimethylsilane (0.05 ml). After 10 min, the solvents were removed by evaporation, and the residue was dissolved in tetrachloromethane and subjected to g.l.c. with 2,3,4,6-tetra-*O*-methyl-D-glucose as internal standard, using a Perkin–Elmer F-11 gas chromatograph equipped with a flame-ionization detector and a stainless-steel column (2 m × 3 mm) packed with 3% of OV-17 on Gas Chrom Q (80–100 mesh), a nitrogen flow-rate of 20 ml/min, and a temperature programme of 3°/min from 120° to 180°.

The methyl glycosides of the reduced disaccharides and the methyl glycosides of reference disaccharides were transformed into their Me_3Si derivatives by treating each sugar (100 μg) with a mixture (1 ml) of pyridine–hexamethyldisilazane–chlorotrimethylsilane (10:2:1) at 70° for 2 h. The solvents were removed by evaporation, and a solution of the residue in tetrachloromethane was subjected to g.l.c.[4] at 175° using a column of 3% of XE-60 on Gas Chrom Q (100–200 mesh), and a nitrogen flow-rate of 20 ml/min.

RESULTS AND DISCUSSION

The acidic components isolated after partial hydrolysis of the *Rhizobium* polysaccharides were identified as follows.

Component **1** was D-glucuronic acid. The chromatographic and electrophoretic mobilities were identical with those of authentic material. The acid was transformed into D-glucurono-6,3-lactone in acid solution, and the glucose formed on hydrolysis of the reduced methyl ester methyl glycoside was oxidized by D-glucose oxidase.

Component **2** was 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucuronic acid. Total, acid hydrolysis of **2** gave D-glucuronic acid and its lactone, whereas transformation into the methyl ester methyl glycosides and subsequent reduction and acid hydrolysis gave glucose.

Methylation of the reduced methyl ester methyl glycosides followed by hydrolysis yielded two *O*-methyl sugars. The retention times of their Me_3Si derivatives in g.l.c. corresponded to those of the anomers of 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-glucoses (Table I). Thus, the D-glucuronic acid residues are (1→4)-linked.

TABLE I

G.L.C. DATA OF THE Me_3Si DERIVATIVES OF THE HYDROLYZED, CARBOXYL-REDUCED, METHYL ESTER METHYL GLYCOSIDES OF OLIGOSACCHARIDES 2–4

Substances	T *values*[b]			
	Major anomer		*Minor anomer*	
Reference compounds				
2,3,4,6-Me_4-glucose[a]	1.00		0.92	
2,3,6-Me_3-glucose	1.23		0.10	
2,3,4-Me_3-glucose	1.06			
3,4,6-Me_3-glucose	1.18		0.96	
2,4,6-Me_3-glucose	1.18		1.29	
Products of methylation analysis of				
2	1.21	1.00	1.06	0.92
3	1.20	1.00	1.07	0.92
4	1.22	1.00	1.07	0.91

[a]2,3,4,6-Me_4-glucose stands for 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [b]Elution time relative to that of the Me_3Si derivative of 2,3,4,6-tetra-*O*-methyl-D-glucose.

Establishment of the β configuration of the glycosidic bond and additional proof of the (1→4)-linkage was gained by g.l.c. of the Me_3Si derivatives of the reduced methyl ester methyl glycosides. The retention times (1.00, 1.18) were the same as those of the Me_3Si derivatives of the cellobiose methyl glycosides and different from those (0.98, 1.06) of the corresponding maltose derivatives.

Component **3** was cellobiouronic acid, 4-*O*-(β-D-glucopyranosyluronic acid)-D-glucose. Total, acid hydrolysis liberated D-glucose, and D-glucuronic acid and its lactone, whereas hydrolysis of the alditol derivative gave D-glucuronic acid and D-glucitol. Therefore, D-glucose is the reducing residue of **3**.

The carboxyl-reduced methyl ester methyl glycoside of **3** was indistinguishable [g.l.c. of Me_3Si derivative and methylation analysis (Table I)] from the corresponding derivative of **2**.

Component **4** was *O*-(β-D-glucopyranosyluronic acid)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→4)-D-glucose. Total, acid hydrolysis gave D-glucose and D-glucuronic acid. Hydrolysis of the carboxyl-reduced methyl ester methyl glycoside gave D-glucose only. The presence of D-glucose in the terminal, reducing position was verified by the formation of glucitol on hydrolysis of the alditol derivative. The R_{GLC} and M_{GLCA} values indicate that **4** is a trisaccharide, and the methylation data (Table I) indicate only (1→4)-linkages. Partial, acid hydrolysis gave D-glucose, D-glucuronic acid, and two oligosaccharides indistinguishable from **2** and **3** (p.c. and electrophoresis).

In control experiments, in which the polysaccharide was simulated by a mixture of the monomer constituents in the correct molecular proportions, none of the products **2**–**4** was formed during heating with trifluoroacetic acid. The saccharides **2**–**4** therefore retain structural details of the native polysaccharides. The same D-glucuronic

acid-containing oligosaccharides were formed on partial hydrolysates of the six *Rhizobium* polysaccharides, namely[2], from *Rh. trifolii* U226, *Rh. leguminosarum* U311, *Rh. phaseoli* U453 and U458 (infective and co-operative, *i.e.*, nitrogen fixing in the host), Coryn (infective and non-co-operative, *i.e.*, merely parasitic), and Bart A (non-infective).

Methylation analysis of some *Rhizobium* polysaccharides[5,6] has indicated that the D-glucopyranosyluronic acid residues are linked through positions 1 and 4. The results reported herein confirm these conclusions, as they prove that the D-glucopyranosyluronic acid residues are linked to D-glucose residues by β-(1→4)-linkages.

The identification of the saccharides **2** and **4** shows that all the polysaccharides examined contain two β-(1→4)-linked D-glucuronic acid residues. Two uronic acid residues in neighbouring positions are thus proved to contribute to the structures of six different *Rhizobium* polysaccharides. Earlier work indicated the presence of single residues of D-glucuronic acid as in the repeating unit suggested[6] for the polysaccharide produced by *Rh. trifolii* TA1.

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