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STRUCTURAL STUDIES ON A NEW POLYSACCHARIDE, CONTAINING D-RIBURONIC ACID, FROM *Rhizobium meliloti* IFO 13336

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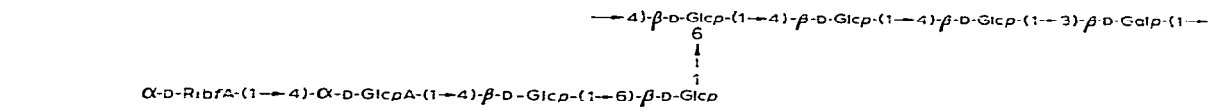
ABSTRACT

The structure of an extracellular, acidic polysaccharide from *Rhizobium meliloti* IFO 13336 was studied by a method involving successive fragmentation with specific β-D-glycanases of *Flavobacterium* M64. The polysaccharide is composed of repeating units of the octasaccharide shown. An acidic component was identified as D-riburonic acid.



INTRODUCTION

We have elucidated the structures[1,2] of polysaccharides from *Alcaligenes faecalis* var. *myxogenes* 10C3 and several strains of *Agrobacterium* and *Rhizobium* by their successive fragmentation, with an extracellular β-D-glycanase (succinoglycan depolymerase) and an intracellular endo-(1→6)-β-D-glucanase of *Flavobacterium* M64, into two tetrasaccharides *via* the octasaccharide repeating-unit, and then methylation analysis and enzymic hydrolysis of the products.

The polysaccharide from *Rhizobium meliloti* IFO 13336 contained D-glucose, D-galactose, D-glucuronic acid, acetic acid, and an unidentified acidic component[3]. This polysaccharide was also hydrolyzed successively by the two enzymes of *Flavobacterium* M64 (ref. 3). We now report structural studies on this polysaccharide by essentially the procedures employed in the previous studies on polysaccharides.

EXPERIMENTAL

General methods. — Paper chromatography (p.c.) was conducted by the descending method, using Toyo filter paper No. 50 with the following solvent systems (v/v): (*A*) 6:4:3 1-butanol–pyridine–water; (*B*) 4:1:5 1-butanol–acetic acid–water;

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(*C*) 7:0.7:23 1-butanol–acetic acid–water; and (*D*) 28:4:13 1-propanol–ethyl acetate–water. Sugars were detected on the paper chromatograms with silver nitrate.

Gas–liquid chromatography (g.l.c.) was performed with a Shimadzu GC7A gas chromatograph fitted with a flame ionization detector. Methylations were performed, as described by Hakomori[4], with sodium methylsulfinyl methanide and methyl iodide in dimethyl sulfoxide. The methylated samples were hydrolyzed, and analyzed as the alditol acetates in a column (3 mm × 2 m) of 0.3% of OV275–0.4% of GEXF 1150 on Shimalite W, as described previously[2].

Carboxyl-reduction of the polysaccharide was performed by the procedure of Taylor and Conrad[5]. D-Glucuronic acid in the polysaccharide was degraded selectively by the method of Lindberg *et al.*[6].

^{1}H-N.m.r. spectra were recorded with a JEOL JNM-FX100 instrument (99.7 MHz) for solutions in deuterium oxide or deuteriochloroform. Samples in deuterium oxide were studied at 85°.

Organism. — *Rhizobium meliloti* IFO 13336 was obtained from the Institute for Fermentation, Osaka.

Preparation of extracellular polysaccharide, and products of enzymic hydrolysis of the polysaccharide. — The extracellular polysaccharide was prepared from the supernatant liquor of cultures grown in a synthetic medium containing 4% of D-glucose, as described previously[7].

The oligosaccharide constituting the repeating unit of the polysaccharide was prepared as follows. The deacylated polysaccharide (100 mg) was extensively hydrolyzed with succinoglycan depolymerase (200 units) for 24 h at 30° in 50mM acetate buffer, pH 5.8 (5 mL), with a few drops of toluene to prevent microbial growth. After hydrolysis, the mixture was dialyzed by vacuum filtration through a collodion membrane. The dialyzable fraction was concentrated to ~1 mL, and salts were removed by chromatography on a column of Sephadex G-10. Fractions in the void volume containing the product were concentrated to a small volume, and mixed with 6 volumes of ethanol. The resultant precipitate was collected by centrifugation, washed with ethanol, and dried *in vacuo*, to yield 87 mg of dried material.

The two oligosaccharides produced by hydrolysis of the repeating-unit oligosaccharide with the intracellular, endo-(1→6)-β-D-glucanase of *Flavobacterium* M64 were prepared as follows. The repeating-unit oligosaccharide (50 mg) was digested extensively with intracellular endo-(1→6)-β-D-glucanase (40 units) for 24 h at 30° in 50mM sodium acetate buffer, pH 5.8 (1.5 mL), with a few drops of toluene. After digestion, the mixture was boiled for 1 min, and centrifuged to remove denatured protein. The supernatant liquor was passed through a column of Amberlite IR-120 (H^+) resin, and the effluent was concentrated to a small volume, and the concentrate applied to Toyo filter paper No. 50, developed with solvent *D* (to separate the two oligosaccharide products). The products were extracted from appropriate sections of the paper, and the extracts were evaporated to dryness *in vacuo*. The rapidly and slowly migrating oligosaccharides (yield: 23.8 and 20.5 mg, respectively) were termed oligosaccharide A and oligosaccharide B.

Preparation of succinoglycan depolymerase and intracellular endo-(1→6)-β-D-glucanase. — Succinoglycan depolymerase and intracellular endo-(1→6)-β-D-glucanase were prepared as described previously[8,9]. One unit of enzyme activity is defined as the amount of the enzyme liberating 1 μmole of aldehyde groups as glucose per h.

Preparation of deacylated polysaccharide. — The deacylated polysaccharide was obtained by hydrolyzing a 0.1% solution of the native polysaccharide in 10mM potassium hydroxide for 5 h at 20° under nitrogen. The solution was made neutral with M hydrochloric acid, dialyzed against water, and lyophilized.

Isolation of acidic component. — The pH of a solution of the native polysaccharide (500 mg/500 mL) was adjusted to 2 with sulfuric acid, and the solution was heated for 4 h at 100°, and cooled. Acetone (2 vol.) was added to precipitate the residual polysaccharide, and the supernatant liquor was concentrated to 200 mL, and made neutral with barium carbonate. The resulting precipitate was removed by centrifugation, and the supernatant liquor was passed through a column of Amberlite IR-120 (H^+) resin. The effluent was concentrated to a small volume, and the concentrate was applied to paper, and developed with solvent *D*. The product was extracted from an appropriate section (R_F 0.37) of the paper with water, and the extract was evaporated to dryness *in vacuo*, yielding 38 mg of dried material.

Preparation of authentic D-*riburonic acid.* — D-Riburonic acid was prepared from D-ribose by the method of Heyns and Lenz[10].

RESULTS AND DISCUSSION

The polysaccharide was composed of D-glucose, D-galactose, D-glucuronic acid, and acetic acid in the molar ratios of 5 : 1 : 1 : 2, together with an unidentified, acidic component[3]. On mild hydrolysis at pH 2 for 4 h at 100°, the unidentified, acidic component was liberated completely, and no other component was released during this treatment. The color reaction with the carbazole–sulfuric acid reagent was characteristic of a uronic acid. In p.c., the R_F values of 0.08, 0.30, 0.20, and 0.37 in solvents A, B, C, and D, respectively, coincided with those of authentic D-riburonic acid. The value of $[\alpha]_D^{20}$ +24.3° (*c* 0.48 water) was very similar to that (+24.8°) of D-riburonic acid.

Carboxyl reduction converted the acid into D-ribose, which was identified by g.l.c. (as the alditol acetate) on an ECNSS-M column at 180° (T_{Glc} 0.26), and by p.c. with solvent systems *A* (R_F 0.60) and *B* (R_F 0.31). When the polysaccharide was carboxyl-reduced, the product methylated, and the ether hydrolyzed, and the methylated sugars were analyzed as the alditol acetates by g.l.c. on a column of 0.3% of OV275–0.4% of GEXF 1150, 2,3,5-tri-*O*-methyl-D-ribose (*T* 0.45, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol), corresponding to a nonreducing (terminal) D-ribofuranosyl group, appeared.

From these results, the acid was identified as D-riburonic acid.

The polysaccharide was hydrolyzed completely to the repeating-unit oligo-

TABLE I

METHYLATION ANALYSIS OF NATIVE AND MODIFIED POLYSACCHARIDES AND OLIGOSACCHARIDES OF *Rhizobium meliloti* IFO 13336

Compound	*Methylated sugar (relative proportions)*					
	2,3,5-Rib (T 0.45[a])	*2,3,4,6-Glc* (T 1.00[a])	*2,4,6-Gal* (T 1.48[a])	*2,3,4-Glc* (T 1.56[a])	*2,3,6-Glc* (T 1.64[a])	*2,3-Glc* (T 2.11[a])
Native polysaccharide	0	0	1.0	0.9	3.3	1
Reduced polysaccharide	0.8	0	1.0	0.9	4.2	1
Native-pH 2 treated polysaccharide	0	0	1.1	1.0	3.2	1
Reduced-pH 2 treated polysaccharide	0	0.8	1.1	1.0	3.3	1
Repeating unit oligosaccharide	0	0	0.8	2	3.2	0
Oligosaccharide A	0	0	0	1	1.3	0
Oligosaccharide B	0	1	0.8	0	2.2	0
Partially hydrolyzed oligosaccharide A	0	0.9	0	0.1	1	0

[a]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Column: 0.3% of OV275–0.4% of GEXF 1150 on Shimalite W.

saccharide with succinoglycan depolymerase. The repeating unit was further hydrolyzed completely with the intracellular endo-(1→6)-β-D-glucanase of *Flavobacterium* M64 into two smaller oligosaccharides (oligosaccharides A and B). The native polysaccharide, the carboxyl-reduced polysaccharide, the repeating-unit oligosaccharide, and oligosaccharides A and B were methylated, and the products hydrolyzed, and the methylated sugars were analyzed as the alditol acetates by g.l.c. on a column of 0.3% of OV275–0.4% of GEXF 1150, giving the results shown in Table I.

The sugars of the methylated native polysaccharide were 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucose in the molar ratios of 1:1:3:1. When the polysaccharide was carboxyl-reduced, the methylated sugars showed increases of about 1 mole of 2,3,6-tri-*O*-methyl-D-glucose and 1 mole of 2,3,5-tri-*O*-methyl-D-ribose per mole. These increases were due to reduction of the carboxyl groups in (1→4)-linked D-glucuronic acid residues and terminal D-ribosyluronic acid groups. When the carboxyl-reduced polysaccharide was treated with dilute acid, and the terminal D-ribosyl groups were removed, there was an increase of about 1 mole of 2,3,4,6-tetra-*O*-methyl-D-glucose and a decrease of 1 mole of 2,3,6-tri-*O*-methyl-D-glucose per mole. However, when the native polysaccharide was treated with dilute acid, there was no increase of the terminal D-glucose and no decrease of 2,3,6-tri-*O*-methyl-D-glucose; this indicates that the D-ribosyluronic acid group is linked to the D-glucuronic acid residue through a (1→4) linkage.

Methylation analysis of the repeating-unit oligosaccharide (see Table I) in-

dicated that the oligosaccharide has no branching residues. Thus, succinoglycan depolymerase hydrolyzes the linkage adjacent to branching D-glucose residues, to produce linear repeating-units. These linkages must be (1→4), because there was an increase of 1 mole of 2,3,4-tri-*O*-methyl-D-glucose per mole, corresponding to (1→6)-linked D-glucose, on hydrolysis with succinoglycan depolymerase.

The reducing-terminal sugar of the repeating unit was determined to be D-galactose by the method described previously[2]: (*1*) reduction of the reducing-terminal sugar with sodium borohydride, (*2*) hydrolysis of the reduced oligosaccharide, (*3*) separation of the sugar and alditol by g.l.c. on a column of Silicone OV-17, and (*4*) identification of the alditol by g.l.c. on an ECNSS-M column.

The methylated sugars from methylated oligosaccharide A were 2,3,4-tri-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratio of 1:1 (see Table I). There was no methylated sugar corresponding to the terminal sugar, indicating that the terminal of oligosaccharide A was D-riburonic acid, followed by D-glucuronic acid. When oligosaccharide A was partially hydrolyzed with 4M trifluoroacetic acid for 4 h at 100°, and the resulting hydrolyzate, containing D-riburonic acid, D-glucose, and (D-glucosyluronic acid)-D-glucose, was methylated, 1 mole of 2,3,4,6-tetra-*O*-methyl-D-glucose and 1 mole of 2,3,6-tri-*O*-methyl-D-glucose were obtained per mole, indicating that the D-glucose residue is linked to D-glucuronic acid through a (1→4) linkage. These results indicate that the structure of oligosaccharide A is

D-Rib*f*A-(1→4)-D-Glc*p*A-(1→4)-D-Glc*p*-(1→6)-D-Glc*p*.

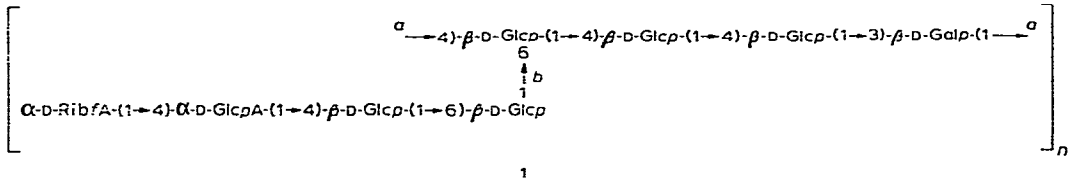
Oligosaccharide B was composed of two (1→4)-linked, D-glucose residues, one (1→3)-linked D-galactose residue, and one nonreducing (terminal) D-glucosyl group (see Table I). As the D-galactose residue should be located at the reducing end, oligosaccharide B must have the sequence

D-Glc*p*-(1→4)-D-Glc*p*-(1→4)-D-Glc*p*-(1→3)-D-Gal*p*.

The repeating-unit oligosaccharide is an octasaccharide composed of one terminal D-ribosyluronic acid group, one (1→4)-linked D-glucosyluronic acid residue, three (1→4)- and two (1→6)-linked D-glucosyl residues, and one (1→3)-linked D-galactose residue. Consequently, oligosaccharides A and B are linked through a (1→6)-D-glucosidic linkage, to form the following octasaccharide as the repeating unit.

DRib*f*A(1→4)DGlc*p*A(1→4)DGlc*p*(1→6)DGlc*p*(1→6)DGlc*p*(1→4)DGlc*p*(1→4)DGlc*p*-(1→3)DGal*p*

The repeating unit should be polymerized between D-galactose and (1→6)-linked D-glucose residues, through an intermolecular, (1→4) linkage, to form a branched structure. Thus, a possible structure for the polysaccharide (deacylated) is as shown in formula **1**. An alternative structure is that in which another (1→6)-linked D-glucose in the repeating unit is linked to the D-galactose residue, to form a branching structure. In this case, (1→6) linkages should be present in the backbone.



To determine which (1→6)-linked D-glucose participates in the branch point, we removed the D-riburonic acid, and reduced the D-glucuronic acid to D-glucose, and then attempted to hydrolyze a possible side-chain with α-D-glucosidase (Sigma Chemical Co.) and β-D-glucosidase (Sigma Chemical Co.). However, the modified polysaccharide was resistant to hydrolysis with these D-glucosidases. The structure of the backbone of the polysaccharide shown in **1** is the same as that of the polysaccharides from *Alcaligenes faecalis* var. *myxogenes* 10C3 and some strains of *Agrobacterium* and *Rhizobium* studied previously[1,2]. Thus, structure **1** seems more plausible than the alternative structure. In **1**, linkages denoted *a* are hydrolyzed by succinoglycan depolymerase, and *b* linkages are hydrolyzed by the intracellular endo-(1→6)-β-D-glucanase.

The anomeric configuration of the polysaccharide shown in **1** was determined by ^{1}H-n.m.r. spectroscopy. The spectrum of the native polysaccharide showed the presence of two anomeric protons, corresponding to α-linkages (δ 5.30 and 5.09). Resolution of signals corresponding to β-linkages was poor. When D-riburonic acid was removed by treatment with dilute acid, the spectrum showed only one signal (δ 5.32) for the α-linkage. The resolution was improved by this treatment, probably owing to lessening of the viscosity, and distinct signals were obtained for β-linkages (δ 4.72, 4.67, 4.55, and 4.47). When the D-glucuronic acid was subsequently degraded, another signal, assigned to the α-linkage, disappeared. Therefore, the linkages of D-Rib*f*A-(1→4)-D-Glc*p*A and D-Glc*p*A-(1→4)-D-Glc*p* must be α-linkages, and the other linkages must be β-linkages.

The polysaccharide of *R. meliloti* IFO 13336 is a new polysaccharide containing D-riburonic acid at the terminal of the side chains. The present report appears to be the first to show the presence of D-riburonic acid in a polysaccharide. The structure of the backbone seems to be identical to that of the principal polysaccharide of *R. meliloti*, but the side chain is different. Extracellular, acidic polysaccharides of *Rhizobium* may participate in the specificity of the symbiotic relationship between the bacteria and their legume-plant hosts, as some workers[11,12] have suggested. It would be of interest to know whether the difference in the structure of the side chain has any influence on the relationship between the organisms and their host plants.

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