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STRUCTURAL STUDIES ON EXTRACELLULAR ACIDIC POLYSACCHARIDES SECRETED BY THREE NON-NODULATING RHIZOBIA

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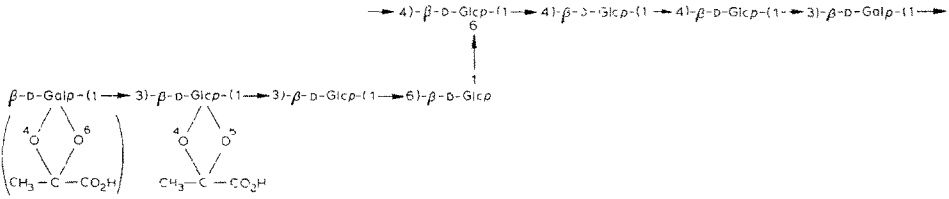
ABSTRACT

The structures of extracellular, acidic polysaccharides from three non-nodulating rhizobia, *Rhizobium trifolii* AHU 1134, *Rhizobium phaseoli* AHU 1133, and *Rhizobium lupini* KLU were studied by a method involving successive fragmentation with specific two β-D-glycanases of *Flavobacterium* M64. These three polysaccharides are composed of repeating units of the octasaccharide shown. Half of the terminal D-galactose residues are substituted by pyruvic acid acetal groups.



INTRODUCTION

One useful method for studying the structures of complicated polysaccharides is to hydrolyze them with specific enzymes. We[1–3] have elucidated the structures of polysaccharides from *Alcaligenes faecalis* var. *myxogenes* 10C3 and several strains of *Agrobacterium* and *Rhizobium* by 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 comprising the octasaccharide repeating-unit, and then by methylation analysis and enzymic hydrolysis of the products.

The polysaccharides from *Rhizobium trifolii* AHU 1134, *R. phaseoli* AHU 1133, and *R. lupini* KLU were also hydrolyzed successively by the two enzymes of *Flavobacterium* M64, but the structures of these polysaccharides appear different from those of other rhizobial polysaccharides studied previously. Here we report structural studies on these polysaccharides.

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EXPERIMENTAL

General methods. – Paper chromatography (p.c.) was conducted by the descending method on Toyo filter paper No. 50 with 6:4:3 (v/v) 1-butanol–pyridine–water as the solvent system. Sugars were detected 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 methylsulfinylmethanide and methyl iodide in dimethyl sulfoxide. The methylated samples were hydrolyzed, and the products analyzed as alditol acetates by g.l.c. on a column (3 mm × 2 m) of 0.3%, OV275–0.4%, GEXF 1150 on Shimalite W, as described previously[1]. Methylated sugars were identified by comparison with methylated sugars derived from the polysaccharide of *Alcaligenes faecalis* var. *myxogenes* 10C3 and by mass spectrometry.

Quantitative analysis of sugars was performed by g.l.c. on a column (3 mm × 2 m) of 3% ECNSS-M on Gas-chrom Q at 180° as described previously[5]. Pyruvic acid was assayed by the method of Koepsel and Sharpe[6].

Total acyl groups were assayed by the method of McComb and McCready[7]. *O*-Acetyl groups and *O*-succinyl groups were identified by ^{1}H-n.m.r. analysis. The amounts of *O*-acetyl and *O*-succinyl groups were calculated from the ratio of signals for these two groups in the spectra. ^{1}H-N.m.r. spectra of solutions in deuterium oxide were recorded with a JEOL JNM-FX100 instrument (99.7 MHz) at 85° with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard.

Organisms. – *Rhizobium trifolii* AHU 1134, *Rhizobium phaseoli* AHU 1133, and *Rhizobium lupini* KLU were obtained from Prof. S. Higashi, Kagoshima University. These strains are non-infectious to their host plants[8]. Their infectious and nodulating abilities were lost during storage.

Preparation of extracellular acidic polysaccharide, and products of enzymic hydrolysis of the polysaccharide. – The extracellular, acidic polysaccharide was prepared from the supernatant liquor of cultures grown in a synthetic medium[3] containing 4% of D-glucose by a method involving precipitation with acetone and cetylpyridinium chloride as described previously[9]. The yields of polysaccharides from *R. trifolii* AHU 1134, *R. phaseoli* AHU 1133, and *R. lupini* KLU were 560 mg, 270 mg, and 800 mg, respectively, per 100 mL of culture medium. The polysaccharide thus obtained was dissolved in water, treated with Amberlite IR-120 (H^+) resin, and freeze-dried. The H^+-form polysaccharide was used for analyses.

The oligosaccharide comprising the repeating unit of the polysaccharide was obtained as follows. The polysaccharide (100 mg) was extensively hydrolyzed with succinoglycan depolymerase (200 units) for 24 h at 30° in 50mM sodium acetate buffer, pH 5.8 (5 mL), containing 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 low volume, and mixed with

6 volumes of ethanol. The resultant precipitate was collected by centrifugation, washed with ethanol, and dried *in vacuo*, to yield 88, 87, and 79 mg of dried material, respectively, from the polysaccharides of *R. trifolii* AHU 1134, *R. phaseoli* AHU 1133, and *R. lupini* KLU.

The 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 deacylated. The deacylated oligosaccharide 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), containing 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 subjected to p.c. The products were separated into three oligosaccharides, A, B-I, and B-II, having R_{Glc} values of 0.28, 0.17, and 0.10, respectively. These oligosaccharides were extracted from appropriate sections of the paper, and the extracts evaporated to dryness *in vacuo*. The yields of oligosaccharides A, B-I, and B-II from the repeating-unit oligosaccharides of three polysaccharides were 15.6–19.4, 6.3–11.3, and 8.0–12.9 mg, respectively.

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[10,11]. One unit of enzyme activity is defined as the amount of enzyme liberating 1 μmol of aldehyde group as glucose per h.

Preparation of depyruvylated polysaccharide and oligosaccharide. — A solution of 0.1 % of native polysaccharide or oligosaccharide B-I was heated in 10mM hydrochloric acid for 90 min at 100°. Under these conditions, pyruvic acid was liberated completely, and no other components were released. The acid-treated solution was passed through a column of Amberlite IR-45 (OH^-) resin and the effluent was freeze-dried.

Preparation of deacylated repeating-unit oligosaccharide. — A solution of 0.1 % of the sample was stirred in 10mM potassium hydroxide for 5 h at 20° under nitrogen. The solution was made neutral, concentrated to low volume, passed through a column of Sephadex G-10 to remove salts, and the acyl groups were liberated. The oligosaccharide fractions in the effluent were concentrated and freeze-dried.

Smith degradation. — Depyruvylated oligosaccharide B-I (1 mg) was oxidized with 40mM sodium periodate (0.4 mL) for 78 h at 4° and the oxidized oligosaccharide was passed through a mixed bed of Amberlite IR-120 (H^+) resin and Amberlite IR-45 (OH^-) resin. The effluent was evaporated. The dried material was reduced with sodium borohydride and treated with Amberlite IR-120 (H^+) resin. Boric acid was removed by distillation of methanol from the residue. The resulting polyalcohol was heated with 4M trifluoroacetic acid for 4 h at 100°. The hydrolyzate was reduced with sodium borohydride, acetylated with a mixture of pyridine and acetic anhydride, and analyzed by g.l.c. on a column of 3 % ECNSS-M on Gas-chrom Q at 180°.

Preparation of β-D-galactosidase-digested oligosaccharide B-I. — Oligosaccharide B-I (5 mg) was incubated with 0.2 unit of β-D-galactosidase (β-D-galactoside galactohydrolase, from *Aspergillus niger*, Grade V, Sigma Chem. Co.) for 20 h at 30° in 1 mL of sodium acetate buffer (50mM, pH 4.0). The mixture was subjected to p.c. The digested oligosaccharide was separated from liberated galactose by extracting the appropriate section of the paper. The extract was evaporated to dryness.

Digestion of the side chain of polysaccharide with β-D-galactosidase and β-D-glucosidase. — Depyruvylated, deacylated polysaccharide (10 mg) was incubated with 2 units of β-D-galactosidase for 20 h at 30° in 1 mL of sodium acetate buffer (50mM, pH 4.0). After incubation, the mixture was boiled for 1 min and the denatured protein removed by centrifugation. The supernatant was dialyzed against sodium acetate buffer (50mM, pH 5.0) and incubated with 5 mg of β-D-glucosidase (β-D-glucoside glucohydrolase from almond, Sigma Chem. Co.) for 3 days at 30°. The mixture was then boiled for 1 min, the resulting precipitate was removed, and the supernatant solution was dialyzed against water. The dialyzed material was freeze-dried.

RESULTS AND DISCUSSION

^{1}H-N.m.r. analysis of the polysaccharides from *R. trifolii* AHU 1134, *R. phaseoli* AHU 1133, and *R. lupini* KLU showed that there was a signal at δ 1.47, assigned to the methyl protons of pyruvic acid residues, and signals at δ 2.13 and 2.68, assigned to the methyl protons of *O*-acetyl groups and methylene protons of *O*-succinyl groups, respectively, in all of the preparations. The molar ratios of *O*-acetyl to *O*-succinyl groups in these polysaccharides were 1:0.11, 1:0.03, and 1:0.11, respectively. Analysis also showed that all of the glycosidic linkages in the polysaccharides are β, because all the signals assigned to anomeric protons were located in the β-anomeric region at δ 4.4-4.8.

The components of the polysaccharides are shown in Table I. All three polysaccharides contained D-glucose, D-galactose, pyruvic acid, and acetic acid in the approximate molar ratios of 6:2·1.5:2, together with small amounts of succinic acid.

TABLE I

COMPONENTS OF EXTRACELLULAR, ACIDIC POLYSACCHARIDES OF *R. trifolii* AHU 1134, *R. phaseoli* AHU 1133, AND *R. lupini* KLU

Strain	D-*Glucose*	D-*Galactose*	*Pyruvic acid* Wt % *(molar ratio)*	*Acetic acid*	*Succinic acid*
R. trifolii AHU 1134	67.8 (6.3)	21.6 (2)	7.0 (1.3)	5.6 (1.5)	1.2 (0.2)
R. phaseoli AHU 1133	65.0 (6.3)	20.8 (2)	6.9 (1.4)	5.7 (1.7)	0.4 (0.1)
R. lupini KLU	68.6 (6.1)	22.4 (2)	6.5 (1.2)	7.8 (2.1)	1.7 (0.2)

TABLE II

METHYLATION ANALYSIS OF NATIVE AND MODIFIED POLYSACCHARIDES AND OLIGOSACCHARIDES OF *R. trifolii* AHU 1134

Compound	*Methylated sugar (relative proportions)*								
	2,3,4,6-Glc (T 0.81[a])	*2,3,4,6-Gal (T 1.00[a])*	*2,4,6-Glc (T 1.35[a])*	*2,4,6-Gal (T 1.48[a])*	*2,3,4-Glc (T 1.62[a])*	*2,3,6-Glc (T 1.75[a])*	*2,3-Glc (T 2.60[a])*	*2,3-Gal (T 2.68[a])*	*2-Glc (T 3.11[a])*
Native polysaccharide	0.0	0.5	1	1.1	0.8	1.9	0.9	0.5	1.0
Depyruvylated polysaccharide	0.0	0.9	2	1.0	0.9	2.2	0.9	0.0	0.0
Repeating-unit oligosaccharide	0.0	0.5	1	0.8	1.7	1.9	0.0	0.5	0.8
Oligosaccharide A	1	0.0	0.0	0.8	0.0	2.1	0.0	0.0	0.0
Oligosaccharide B-I	0.0	0.9	1	0.0	1.2	0.1	0.1	0.0	1.0
Oligosaccharide B-II	0.0	0.0	1	0.1	0.9	0.0	0.0	0.9	1.0
β-D-Galactosidase-digested oligosaccharide B-I	0.0	0.1	1	0.0	1.1	0.0	0.7	0.0	0.2
β-D-Galactosidase, β-D-glucosidase-digested, depyruvylated, deacylated, polysaccharide	1	0.0	0.2	1.0	0.3	2.2	1.0	0.0	0.0

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

The polysaccharides were hydrolyzed completely into their repeating-unit oligosaccharides with succinoglycan depolymerase. The oligosaccharides were a mixture of two different forms of oligosaccharide whose terminal galactose residues were linked with or without pyruvic acid, as shown later. The repeating unit (deacylated) was hydrolyzed completely with the intracellular endo-(1→6)-β-D-glucanase of *Flavobacterium* M64 into three smaller oligosaccharides (oligosaccharides A, B-I, and B-II). The native polysaccharides, the depyruvylated polysaccharides, the repeating-unit oligosaccharides, and oligosaccharides A, B-I, and B-II were methylated, the products were hydrolyzed, and the methylated sugars were analyzed as alditol acetates by g.l.c. on a column of 0.3% OV275 0.4% GEXF 1150. Results for preparations from the polysaccharide of *R. trifolii* AHU 1134 are shown in Table II. Preparations from the polysaccharides of the two other strains gave identical results, within the limits of experimental error.

The sugars of the methylated native polysaccharide were 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-glucose, 2,3-di-*O*-methyl-D-galactose, and 2-*O*-methyl-D-glucose in the molar ratios of 0.5:1:1:1 2:1:0.5:1. When the polysaccharide was depyruvylated, the methylated sugars showed increases of 0.5 mol of terminal D-galactose and one mol of 2,4,6-tri-*O*-methyl-D-glucose per mol, and decreases of 0.5 mol of 2,3-di-*O*-methyl-D-galactose and one mol of 2-*O*-methyl-D-glucose per mol. These results show that half of the non-reducing, terminal D-galactose residues and one of two, (1→3)-linked D-glucose residues of this polysaccharide are substituted with pyruvic acid at O-4 and O-6. This polysaccharide has D-glucose residues branching through a (1→4)- or (1→6)-linkage.

Methylation analysis of the repeating-unit oligosaccharide indicated that the oligosaccharide has no branching residues. Thus succinoglycan depolymerase hydrolyzed the linkage adjacent to branching D-glucose residues, producing linear repeating-units. These linkages are (1→4), because hydrolysis with succinoglycan depolymerase led to an increase of one mol of 2,3,4-tri-*O*-methyl-D-glucose per mol, corresponding to a (1→6)-linked D-glucose.

The reducing-terminal sugar of the repeating-unit oligosaccharide obtained from the polysaccharides of the three strains was found to be D galactose, and the degree of polymerization of these repeating units was found to be 8 by the method described previously[1].

The methylated sugars from methylated oligosaccharide A were 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-galactose in the molar ratios of 1:2:1 (see Table II), indicating that the oligosaccharide is composed of one non-reducing terminal D-glucose, two (1→4)-linked D-glucose residues, and one (1→3)-linked D-galactose residue. As the D-galactose residue should be located at the reducing end, oligosaccharide A must have the sequence D-Glc*p*-(1→4)-D-Glc*p*-(1→4)-D-Glc*p*-(1→3)-D-Gal*p*.

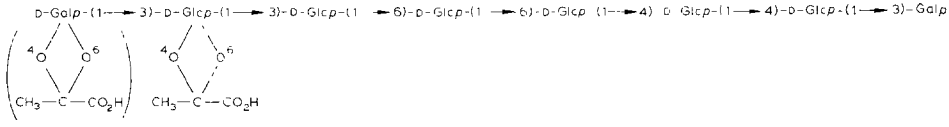
Judging from the methylated sugars of methylated oligosaccharides B-I and

B-II, oligosaccharide B-I is composed of one, non-reducing terminal D-galactose residue, one (1→3)-linked D-glucose residue linked with pyruvic acid at O-4 and O-6, one (1→3)-linked D-glucose residue lacking pyruvic acid, and one (1→6)-linked D-glucose residue. Oligosaccharide B-II has the same sugar linkages as those of oligosaccharide B-I, except that the non-reducing terminal D-galactose residues are substituted with pyruvic acid.

To determine whether a (1→3)- or (1→6)-linked D-glucose residue is located at the reducing terminal, we subjected depyruvylated oligosaccharides B-I and B-II to Smith degradation. The products were D-glucitol and D-glycerol in approximately 1:1 molar ratio. Therefore, the reducing-terminal D-glucose residue of these oligosaccharides has a (1→6) linkage, because D-arabinitol should have been produced if (1→3)-linked D-glucose were located at the reducing terminal. Consequently the backbone sequence of oligosaccharides B-I and B-II is D-Gal*p*-(1→3)-D-Glc*p*-(1→3)-D-Glc*p*-(1→6)-D-Glc*p*. One of the (1→3)-linked D-glucose residues is substituted with pyruvic acid at O-4 and O-6, and the non-reducing terminal D-galactose residue of oligosaccharide B-II is also substituted with pyruvic acid at the same position.

When oligosaccharide B-I was treated with β-D-galactosidase, D-galactose was liberated from the non-reducing terminal. The methylated sugars from the enzyme-digested oligosaccharide (Table II) showed decreases of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2-*O*-methyl-D-glucose, and increase of 2,3-di-*O*-methyl-D-glucose, indicating that the new terminal of the oligosaccharide is a D-glucose residue linked with pyruvic acid at O-4 and O-6. Thus, pyruvic acid is located at the (1→3)-linked D-glucose residue close to the non-reducing terminal of oligosaccharide B-I and probably of oligosaccharide B-II.

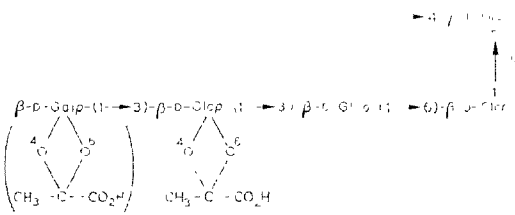
On hydrolysis of the repeating-unit octasaccharide with endo-(1→6)-β-D-glucanase, one mol of 2,3,4-tri-*O*-methyl-D-glucose per mol was lost, as shown in Table II. Therefore oligosaccharide A and oligosaccharide B-I (or B-II) are linked through a (1→6)-D-glucosidic linkage, to form the following octasaccharide as a repeating unit.



Half the non-reducing, terminal D-galactose residue is pyruvylated and there are also ~2 mol of acyl groups per mol. We could separate two types of octasaccharide (with a pyruvylated and a nonpyruvylated terminal residue) by chromatography on DEAE-cellulose.

The repeating unit is presumably polymerized between (1→3)-linked 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**. The anomeric configurations of all

eight glycosidic linkages are β, as described earlier. An alternative structure is that in which another (1→6)-linked D-glucose residue in the repeating unit is linked to the D-galactose residue to form a branched structure. In this case, (1→6) linkages should be present in the backbone. However, this alternative structure was rejected as follows. Depyruvylated, deacylated polysaccharide was digested extensively with β-D-galactosidase, and subsequently with β-D-glucosidase to remove the side chains of the polysaccharide from the non-reducing terminal sugar. The resulting, digested polysaccharide was methylated. As shown in Table I, this polysaccharide lacks 100% of the terminal D-galactose, 90% of the (1→3)-linked D-glucose, and 70% of the (1→6)-linked D-glucose residues of the undigested, depyruvylated polysaccharide. This result indicates that one (1→6)-linked D-glucose residue in the repeating-unit octasaccharide is located at the side chain of the polysaccharide and another (1→6)-linked D-glucose residue is situated at the branching point. In **1**, linkages denoted as *a* are hydrolyzed by succinoglycan depolymerase, whereas *b* linkages are hydrolyzed by intracellular endo-(1→6)-β-D-glucanase.



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The glycosyl-residue sequence and the mode of pyruvylation of the polysaccharides from *R. phaseoli* AHU 1133 and *R. lupini* KLU were identical with those of the polysaccharide of *R. trifolii* AHU 1134, because these three polysaccharides were hydrolyzed successively with the two specific enzymes, and the resulting oligosaccharides were indistinguishable by p.c. and methylation analysis. Moreover, the ^{1}H-n.m.r. spectra of these three polysaccharides were the same.

Structure **1** is very similar to that of the extracellular, acidic polysaccharides (succinoglycan) of *Alcaligenes faecalis* var. *myxogenes*[1,12] 10C3, many strains of *Agrobacterium*[1,4,12], and *R. meliloti*[1,3,12–14], and a strain of *R. trifolii*[3], the major difference being that, in succinoglycan, the non-reducing terminal-sugar residue is D-glucose instead of D-galactose, and only one pyruvyl substituent is present instead of two as in the present polysaccharide. The two terminal sugar residues of the polysaccharide of *R. meliloti* IFO 13336 are changed to D-riburonic acid and D-glucuronic acid[2]. All of these polysaccharides are hydrolyzed successively by succinoglycan depolymerase and endo-(1→6)-β-D-glucanase of *Flavobacterium* M64 into a tetrasaccharide *via* their octasaccharide repeating-unit. The method involving sequential fragmentation with the specific enzymes described here is rather simple and seems very good for elucidating the structure of complicated polysaccharides.

The structures of the polysaccharides of *R. trifolii* AHU 1134, *R. phaseoli* AHU

1133, and *R. lupini* KLU are different not only from those of polysaccharides of *R. meliloti* but also from those of strains of *R. trifolii*[15-17], *R. leguminosarum*[17], *R. japonicum*[18,19], and other species studied previously. The polysaccharides of *R. trifolii* AHU 1134 and *R. phaseoli* AHU 1133 do not seem to be typical polysaccharides of *R. trifolii* and *R. phaseoli*, because most polysaccharides of these species reported thus far contain glucuronic acid[15-22], which is absent from our polysaccharides. As the rhizobial strains used for this study are non-infectious to their host legumes, the assignment of these strains to each species is uncertain. These strains are also non-infectious to the host legume of *R. meliloti*, alfalfa, although the structure of their polysaccharides is very similar to that of polysaccharides of *R. meliloti*.

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