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CYCLIC (1→2)-β-D-GLUCAN AND THE OCTASACCHARIDE REPEATING-UNITS OF EXTRACELLULAR ACIDIC POLYSACCHARIDES PRODUCED BY *Rhizobium**

AKINORI AMEMURA**, MAKOTO HISAMATSU, HIDEKI MITANI,
Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567 (Japan)
AND TOKUYA HARADA
Kobe Women's University, Suma-ku, Kobe 654 (Japan)

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ABSTRACT

Ten strains of *Rhizobium* tested produced extracellular, cyclic (1→2)-β-D-glucan, and five of ten strains produced the linear octasaccharide repeating-units of the extracellular, acidic polysaccharides.

INTRODUCTION

Previously, Hisamatsu *et al.*[1] found that many strains of *Agrobacterium* and *Alcaligenes faecalis* var. *myxogenes* (taxonomically close to *Agrobacterium*) produce extracellular, cyclic (1→2)-β-D-glucan, that most of the strains also produce an octasaccharide repeating-unit of succinoglycan (extracellular, acidic polysaccharide produced by these strains), and that the (1→2)-β-D-glucan bearing some other linked D-glucosyl residues that was reported by Putman *et al.*[2], Gorin *et al.*[3], and Barreto-Berger *et al.*[4] may be due to the presence of the octasaccharide repeating-unit of succinoglycan in their preparations. Dedonder and Hassid[5] and York *et al.*[6] also reported the production of (1→2)-β-D-glucan having other glucosidic linkages by several strains of *Rhizobium*, which is taxonomically related to *Agrobacterium*. Recently, cyclic (1→2)-β-D-glucan lacking minor glucosidic linkages was found in cells of *Rhizobium* by Zevenhuizen[7], and later by Abe *et al.*[8]. We also examined extracellular compounds of low molecular weight produced by *Rhizobium* strains, and found cyclic (1→2)-β-D-glucan and octasaccharide repeating-units not only of succinoglycan but also of other acidic polysaccharides.

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**To whom requests for reprints should be sent.

EXPERIMENTAL

Organisms. — *Rhizobium meliloti* IFO 13336, *R. trifolii* IFO 13337, and *R. japonicum* IFO 13338 were obtained from the Institute for Fermentation, Osaka, Japan. *R. meliloti* J7017 and *R. trifolii* J60 were obtained by courtesy of Prof. Y. Maruyama, the University of Tokyo, Japan; *R. trifolii* 4S, *R. trifolii* AHU 1134, *R. phaseoli* AHU 1133, and *R. lupini* KLU were from Prof. S. Higashi, Kagoshima University, Japan; and *R. leguminosarum* 303 was provided by Dr. Tsuru, the National Institute of Agricultural Science, Japan.

Preparation of extracellular, acidic polysaccharides and low-molecular-weight fractions. — Synthetic medium containing 4% of glucose was supplemented with 0.1% yeast extract, as described previously[9]. The medium (95 mL) in 500-mL, conical flasks was inoculated with a culture (5 mL) grown in the same medium. Six-day cultures were centrifuged at 56,000*g* for 30 min. The supernatant liquor was mixed with two volumes of ethanol, and centrifuged to remove an extracellular acidic polysaccharide. The supernatant liquor was then concentrated to a small volume, again mixed with two volumes of ethanol, and centrifuged. The supernatant liquor was mixed with four volumes of ethanol, and the resultant precipitate was collected by centrifugation, dissolved in water, and the solution subjected to ultrafiltration through Amicon PM10. The dialyzable fraction was concentrated to a small volume, and subjected to chromatography on Sephadex G-10, to remove salt. Fractions in the void volume were collected, and lyophilized (low-molecular-weight fraction).

Chromatography of the low-molecular-weight fraction on DEAE-cellulose. A sample of the low-molecular-weight fraction was applied to a column (3 × 12 cm) of DEAE-cellulose equilibrated with mM potassium chloride. Material was eluted with the first 200 mL of mM potassium chloride, and then with 700 mL of a linear gradient of 1 to 100mM potassium chloride. Fractions (10 mL) were collected, and their sugar content was measured by the phenol–sulfuric acid method[10]. Fractionated materials were appropriately pooled, desalted by chromatography on a column of Sephadex G-10, and the desalted materials lyophilized.

Methylation analysis. — Samples (2 mg) were methylated as described by Hakomori[11]. The methylated samples were hydrolyzed with 4M trifluoroacetic acid for 6 h at 100°, and the sugars analyzed as alditol acetates in a column (3 mm × 3 m) of 0.3% of OV275–0.4% of GEXF1150 on Shimalite W (Wako Pure Chem., Osaka, Japan) by g.l.c. as described previously[12]. The column temperature was held for 4 min at 140°, and then raised to 180° at 0.5° per min.

G.l.c. — G.l.c. was performed in a GC7A gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a flame-ionization detector.

^{1}H-N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra of 0.5% solutions in deuterium oxide were recorded with a JEOL JNM-FX100 instrument (JEOL, Tokyo, Japan) at 85°, with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as an internal standard.

Quantitative analysis of sugars. — Samples (2 mg) were hydrolyzed in a sealed tube with 4M trifluoroacetic acid for 6 h at 100°. Each hydrolyzate was converted into

a mixture of alditol acetates, and this was analyzed by g.l.c. in a column (3 mm × 2 m) of 3% of ECNSS-M on Gas-chrom Q (Applied Science Laboratories).

Uronic acids were determined by the modified carbazole reaction described by Galambos[13]. D-Glucuronic acid and D-riburonic acid were identified, after reduction of the carboxyl group by the method of Taylor and Conrad[14], as D-glucose and D-ribose, respectively, by g.l.c.

Quantitative analysis of organic acids. — Pyruvic acid was analyzed by the method of Koepsell and Sharpe[15]. Total acyl groups were assayed by the method of McComb and McCready[16]. *O*-Acetyl and *O*-succinyl groups were identified by ^{1}H-n.m.r. analysis. The proportions of *O*-acetyl and *O*-succinyl groups were calculated from the ratio of the signals of these groups in the spectra.

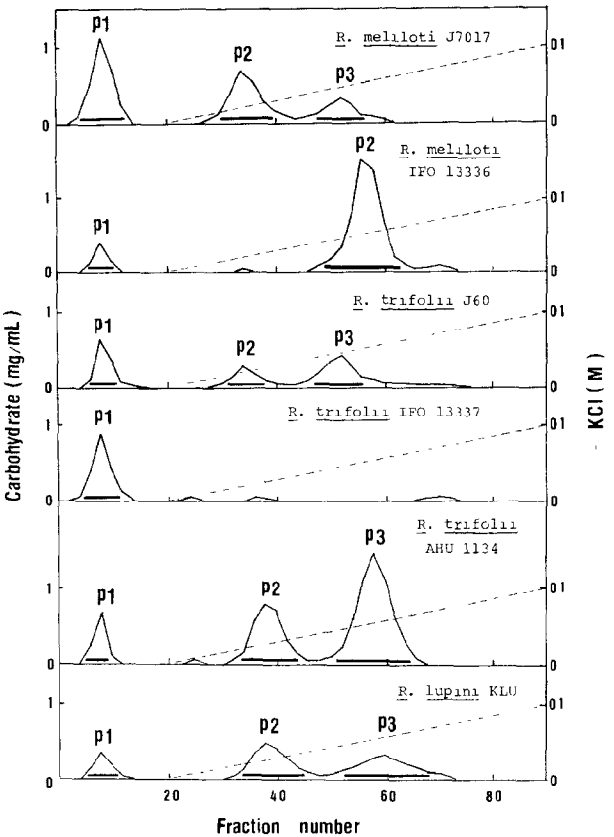


Fig. 1. DEAE-cellulose chromatography of the low-molecular-weight fractions of *Rhizobium* strains. [The sample was applied to a column (3 × 12 cm) of DEAE-cellulose equilibrated with mM potassium chloride, and material was eluted with a linear gradient of 1 to 100mM potassium chloride. Fractions were analyzed for carbohydrate by the phenol–sulfuric acid method. Solid bars indicate pooled fractions.]

TABLE I

COMPOSITION OF ACIDIC OLIGOSACCHARIDES OBTAINED BY DEAE-CELLULOSE CHROMATOGRAPHY OF LOW-MOLECULAR-WEIGHT FRACTIONS AND EXTRACELLULAR ACIDIC POLYSACCHARIDES OF *Rhizobium*

Compound	*Component (molar ratio)*							*References*
	Glucose	*Galactose*	*Glucuronic acid*	*Riburonic acid*	*Pyruvic acid*	*Succinic acid*	*Acetic acid*	
R. meliloti J7017								
PS[a]	7.0	1	0.0	0.0	1.3	0.3	0.6	17
P2[b]	10.5	1	0.0	0.0	0.7	0.0	0.0	
P3[b]	7.1	1	0.0	0.0	1.0	0.8	0.8	
R. meliloti IFO 13336								
PS	5.2	1	1.1	0.8	0.0	0.0	2.1	17,18
P2	5.4	1	1.1	0.8	0.0	0.0	1.8	
R. trifolu J60								
PS	6.8	1	0.0	0.0	1.2	0.2	0.2	17
P2	9.2	1	0.0	0.0	0.8	0.0	0.2	
P3	7.0	1	0.0	0.0	1.0	0.8	0.2	
R. trifolu AHU 1134								
PS	6.3	2	0.0	0.0	1.3	0.2	1.5	19
P2	7.4	2	0.0	0.0	0.9	0.0	1.7	
P3	6.1	2	0.0	0.0	1.8	0.0	1.9	
R. lupini KLU								
PS	6.1	2	0.0	0.0	1.2	0.2	2.1	19
P2	6.9	2	0.0	0.0	1.0	0.0	1.7	
P3	6.0	2	0.0	0.0	1.8	0.1	1.8	

[a] PS: extracellular acidic polysaccharide. [b] P2,P3 cf. Fig. 1.

TABLE II

METHYLATION ANALYSIS OF ACIDIC OLIGOSACCHARIDES OBTAINED BY DEAE-CELLULOSE CHROMATOGRAPHY OF LOW-MOLECULAR-WEIGHT FRACTIONS AND EXTRACELLULAR ACIDIC POLYSACCHARIDES OF *Rhizobium*

Compound	*Methylated sugar (molar ratio)*										*References*
	2,3,4,6-Glc	*2,3,4,6-Gal*	*2,4,6-Glc*	*3,4,6-Glc*	*2,4,6-Gal*	*2,3,4-Glc*	*2,3,6-Glc*	*2,3-Glc*	*2,3-Gal*	*2-Glc*	
R. meliloti J7017											
PS[a]	0.0	0.0	2.1	0.0	1.0	0.9	2.3	2	0.0	0.0	17
P2[b]	0.0	0.0	2.3	1.9	1.1	2.3	2.3	1	0.0	0.0	
P3[b]	0.0	0.0	1.8	0.0	0.8	2.1	2.2	1	0.0	0.0	
R. meliloti IFO 13336											
PS	0.0	0.0	0.0	0.0	1.0	0.9	3.3	1	0.0	0.0	17,18
P2	0.0	0.0	0.1	0.3	0.7	2	3.2	0.0	0.0	0.0	
R. trifolii J60											
PS	0.0	0.0	1.8	0.0	1.2	1.1	2.3	2	0.0	0.0	17
P2	0.0	0.0	2.0	1.9	1.0	1.9	2.0	1	0.0	0.0	
P3	0.0	0.0	1.9	0.4	0.9	2.1	2.2	1	0.0	0.0	
R. trifolii AHU 1134											
PS	0.0	0.5	1	0.0	1.1	0.8	1.9	0.8	0.5	1.0	19
P2	0.0	0.8	1	1.4	0.8	1.6	1.9	0.0	0.0	0.8	
P3	0.0	0.0	1	0.1	0.8	1.7	2.1	0.0	0.7	0.9	
R. lupini KLU											
PS	0.0	0.5	1	0.0	1.0	0.8	2.2	0.9	0.5	1.0	19
P2	0.0	1.0	1	0.4	0.8	1.8	2.1	0.0	0.0	0.8	
P3	0.0	0.2	1	0.0	0.7	2.0	2.2	0.0	0.7	0.8	

[a]PS: extracellular acidic polysaccharide. [b]P2,P3: *cf.*, Fig. 1.

RESULTS AND DISCUSSION

The low-molecular-weight fractions produced by *Rhizobium* strains were subjected to DEAE-cellulose chromatography (see Fig. 1). All of the strains examined produced a neutral material (designated P1) that was eluted without adsorption to the column. *R. meliloti* IFO 13336 afforded one acidic material (P2), whereas *R. meliloti* J7017, *R. trifolii* J60, *R. trifolii* AHU 1134, and *R. lupini* KLU produced two acidic materials (P2 and P3). *R. trifolii* IFO 13337 and the four strains *R. trifolii* 4S, *R. japonicum* IFO 13338, *R. leguminosarum* 303, and *R. phaseoli* AHU 1133 (data on these four strains are not shown in Fig. 1) did not give appreciable amounts of acidic material.

On methylation analysis of the neutral material P1 from each strain, only 3,4,6-tri-*O*-methyl-D-glucose was found. The ^{1}H-n.m.r. spectrum showed a β-anomeric signal at 4.88 p.p.m., with $J_{1,2}$ 7 Hz. Therefore, we concluded that P1 is a cyclic (1→2)-β-D-glucan.

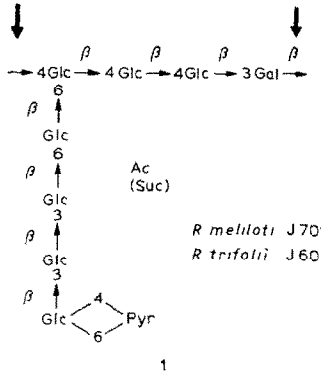
The components, and methylation analysis, of the acidic materials P2 and P3 from each strain are shown in Tables I and II, respectively. The ratios of sugar components of P3 were identical to the ratios of those of the corresponding, extracellular acidic polysaccharides, whereas, although the contents of other sugar components were the same, the D-glucose content of P2 was slightly higher than those of the polysaccharides. These higher values were due to small proportions of cyclic (1→2)-β-D-glucan contaminating P2, as was also indicated by the finding that the methylated sugars from P2 contained 3,4,6-tri-*O*-methyl-D-glucose (see Table II). The contaminating, cyclic (1→2)-β-D-glucan was removed from preparations of P2 by treating them with weak alkali at room temperature. The cyclic (1→2)-β-D-glucan and the acidic material seemed to form a complex. This type of complex has also been found in several strains of *Agrobacterium*[1]. Another difference between the components of P2 and P3 was that P2 from *R. meliloti* J7017 and *R. trifolii* J60 had lesser amounts of acyl groups (*O*-succinyl and *O*-acetyl groups) than P3, and P2 from *R. trifolii* AHU 1134 and *R. lupini* KLU had lesser amounts of pyruvic acid than had P3. The degrees of polymerization of P2 and P3 after removing cyclic (1→2)-β-D-glucan, determined by the method described previously[12], were all 8.

Analysis of the methylated sugars from P2 and P3 indicated that, unlike the extracellular acidic polysaccharides, they did not contain one mol of 2,3-di-*O*-methyl-D-glucose, corresponding to (1→4,1→6)-linked branching D-glucose, but had an additional one mol of 2,3,4-tri-*O*-methyl-D-glucose, corresponding to (1→6)-linked D-glucose (see Table II). The other methylated sugars were in the same ratios as those for the polysaccharides. Therefore, P2 and P3 are linear compounds, and their additional (1→6) linkages seem to be formed by cleaving the (1→4) linkages of branching (1→4,1→6) linkages in the polysaccharides, although P2 and P3 are not considered to be the hydrolysis products of the polysaccharides, as will be described later.

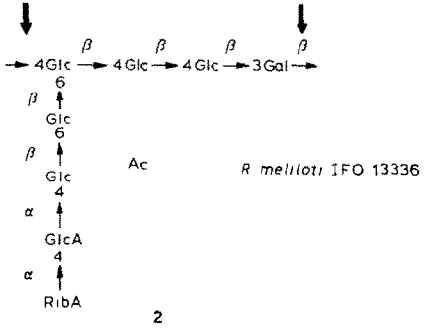
The (nonreducing) terminal D-galactosyl groups of P3 of *R. trifolii* AHU 1134

and *R. lupini* KLU bore a pyruvic acetal group at O-4 and O-6, judging from the results of methylation and component analyses, whereas those of P2 of these strains were devoid of pyruvic acid.

From these findings, we concluded that both P2 and P3 are linear octasaccharide repeating-units of extracellular acidic polysaccharides, as found previously in culture filtrates of strains of *Alcaligenes faecalis* var. *myxogenes* and *Agrobacterium*. Formulas **1**, **2**, and **3** show the structures of *R. meliloti* J7017, *R. trifolii* J60, *R.*



1



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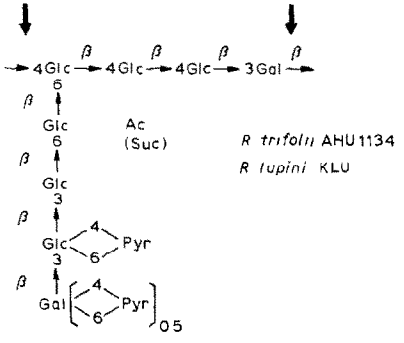

3

meliloti IFO 13336, *R. trifolii* AHU 1134, and *R. lupini* KLU elucidated previously[17–19]. The polysaccharide of *R. meliloti* J7017 and *R. trifolii* J60 is a succinoglycan; *i.e.*, it has the same structure as the polysaccharides of strains of *Alcaligenes faecalis* var. *myxogenes* and *Agrobacterium*. The repeating units found in culture filtrates were linear forms that would be obtained by hydrolysis of the linkages indicated by the arrows. There is a similarity in the structures of these octasaccharide repeating-units: they have the same sequence of glycosyl residues except for two glycosyl residues at the nonreducing terminal.

The contents of acyl groups or pyruvic acetal in the octasaccharides (P2 and P3) were not uniform. Because the contents of these organic acids in the polysaccharides were roughly the average of the contents of those in P2 and P3 (see Table I), two types of repeating unit seem to exist in an approximately equimolar ratio in the polysaccharides.

The amounts of cyclic (1→2)-β-D-glucan, the octasaccharide repeating-unit, and the extracellular acidic polysaccharide per 100 mL of culture medium produced by ten strains of *Rhizobium* are shown in Table III. As already described, all of the strains produced cyclic (1→2)-β-D-glucan in yields of 10–40 mg, and five of the ten strains produced the octasaccharide repeating-unit in yields of 30–160 mg.

We had shown[9] that the linear octasaccharide repeating-unit of an extracellular acidic polysaccharide (succinoglycan) found in the culture of *Alcaligenes faecalis* var. *myxogenes* is not the hydrolysis product of the polysaccharide, but that it seems to be formed from certain intracellular components that are intermediates in the synthesis of succinoglycan. Recently, Tolmasky *et al.*[20] isolated lipid-bound oligosaccharide formed by incubation of uridine 5-(D-glucosyl diphosphate) and a particulate enzyme of *R. meliloti* R41, and reported that the structure of the oligosaccharide moiety seemed to be the same as that of our preparation of the linear octasaccharide

TABLE III

PRODUCTION OF CYCLIC (1→2)-β-D-GLUCANS, THE OCTASACCHARIDE REPEATING-UNITS OF EXTRACELLULAR ACIDIC POLYSACCHARIDES, AND EXTRACELLULAR ACIDIC POLYSACCHARIDES BY *Rhizobium*

Strain	*Cyclic (1→2)-β-D-glucan (mg/100 mL)*	*Octasaccharide repeating-unit (mg/100 mL)*	*Extracellular acidic polysaccharide (mg/100 mL)*
R. meliloti J7017	40	43	220
R. meliloti IFO 13336	11	123	840
R. trifolii J60	19	31	580
R. trifolii IFO 13337	33	0	150
R. trifolii 4S	30	0	240
R. trifolii AHU 1134	25	155	560
R. leguminosarum 303	15	0	220
R. phaseoli AHU 1133	32	0	270
R. japonicum IFO 13338	26	0	600
R. lupini KLU	12	53	800

repeating-unit produced by *Alcaligenes faecalis* var. *myxogenes*. These studies have shown that, in the biosynthesis of the extracellular, acidic polysaccharides of these organisms, the linear octasaccharide preceded by D-galactose is formed first, and then the octasaccharide is polymerized, affording a branching structure.

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