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STRUCTURAL STUDIES ON CYCLIC (1→2)-β-D-GLUCANS (CYCLOSOPHORAOSES) PRODUCED BY *Agrobacterium* AND *Rhizobium**

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ABSTRACT

Eight cyclic (1→2)-β-D-glucans (cyclosophoraoses) of different molecular weights were isolated from culture filtrates of *Agrobacterium* and *Rhizobium*. By 6.2-MPa, liquid chromatography of their partial hydrolyzates, it was concluded that they were cyclosophoroheptadecaose, cyclosophoro-octadecaose, cyclosophorononadecaose, cyclosophoroeicosaose, cyclosophoroheneicosaose, cyclosophorodocosaose, cyclosophorotricosaose, and cyclosophorotetracosaose. Cyclic (1→2)-β-D-glucans from 19 strains of *Agrobacterium* and *Rhizobium* tested were divided into four classes on the basis of differences in the distribution patterns of the eight cyclosophoraoses.

INTRODUCTION

In 1942, McIntire *et al.*[1] found a low-molecular-weight glucan different from an extracellular acidic polysaccharide in culture filtrates of *Agrobacterium tumefaciens*. Putman *et al.*[2] showed that this glucan consisted mainly of (1→2)-linked β-D-glucopyranosyl residues. Subsequently, the culture filtrates of many strains of *Agrobacterium*[3,4] and *Rhizobium*[5,6] were found to contain (1→2)-β-D-glucans having some other D-glucosidic linkages. Hisamatsu *et al.*[7] and Amemura *et al.*[8] recently reported that many strains of *Agrobacterium* and *Rhizobium* produce extracellular, cyclic (1→2)-β-D-glucan, and that most of the strains also produce the linear octasaccharide repeating-unit of extracellular acidic polysaccharides.

Cyclic (1→2)-β-D-glucans were also found in cells of *Rhizobium* by Zevenhuizen[9,10], and, later, by Abe *et al.*[11]. Abe *et al.*[11] also reported that addition

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of the cyclic (1→2)-β-D-glucan isolated from the cells of *R. trifolii* 4S to seedlings of white clover, which is the host plant, promoted infection-thread formation and nodule formation. On gel chromatography on a column of Sephadex G-50, (1→2)-β-D-glucan gave a narrow, symmetrical peak[6,10], but in paper chromatography, it gave two spots[7]. McNeil *et al.*[12] very recently showed by f.a.b.-m.s. that (1→2)-β-D-glucans from *Agrobacterium tumefaciens* and *Rhizobium* species were mixtures of circular molecules containing 17 to 23 D-glucosyl residues. In the present work, we separated cyclic (1→2)-β-D-glucans obtained from *Agrobacterium* and *Rhizobium* into materials of homogeneous molecular size, and examined their properties.

EXPERIMENTAL

General methods. — Paper chromatography (p.c.) was conducted on Toyo No. 50 filter paper (Toyo, Tokyo, Japan) by the descending method in 1:1:1 (v/v) 1-butanol–pyridine–water. Spots were detected by spraying the paper with the sodium periodate–silver nitrate reagent[13].

Gas–liquid chromatography (g.l.c.) was performed in a Shimadzu GC7A gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a flame-ionization detector. Methylations were conducted as described by Hakomori[14]. The methylated samples were hydrolyzed, and analyzed as the alditol acetates on a column (2 m × 3 mm) of 0.3% of OV275–0.4% of GEXF1150 on Shimalite W (Wako Pure Chem., Osaka, Japan), as already described[15].

Liquid chromatography (l.c.) was conducted at room temperature with a model 6000A solvent-delivery system, a model U6K injector, and a column of μBondapak carbohydrate (all from Waters Associates, Milford, Mass., U.S.A.), and a model SE-31 differential refractometer (Showa Denko, Tokyo, Japan), using 3:2 acetonitrile–water as the solvent system at a flow rate of 2.0 mL/min, and a pressure of 11 MPa.

^{13}C-N.m.r. spectra of solutions (2–3%) in D_2O were recorded at room temperature with a JEOL JNM-FX 200 instrument (JEOL, Tokyo, Japan) at 50.10 MHz in the pulsed, Fourier-transform mode, with complete proton-decoupling. Chemical shifts are expressed in p.p.m. downfield from that of tetramethylsilane, using 1,4-dioxane (67.40 p.p.m.) as the internal standard.

Organisms. — *Agrobacterium radiobacter* IFO 12607, IFO 12664, IFO 12665, IFO 13127, IFO 13256, IFO 13532, IFO 13533, *A. rhizogenes* IFO 13259, *A. tumefaciens* IFO 3058, *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. phaseoli* AHU 1133, *R. trifolii* AHU 1134, and *R. lupini* KLU were from Prof. S. Higashi, Kagoshima University, Japan; and *R. leguminosarum* 303 was provided by Dr. S. Tsuru, the National Institute of Agricultural Science, Japan.

Preparation of cyclic (1→2)-β-D-glucan. — Synthetic medium[16] containing 4% of D-glucose was supplemented with 0.1% of yeast extract or traces of thiamine and biotin. 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 mixed with ethanol (2 vol.), and centrifuged at 19,000*g* for 30 min, to remove extracellular acidic polysaccharide and cells. The supernatant liquor was then concentrated to a small volume, again mixed with ethanol (2 vol.), and centrifuged. The supernatant liquor was mixed with ethanol (4 vol.), and the resultant precipitate was collected by centrifugation, dissolved in water, and subjected to ultrafiltration through Amicon PM 10. The dialyzable fraction was concentrated to a small volume, and subjected to chromatography on Sephadex G-10, to remove salts. Fractions in the void volume were collected, concentrated to a small volume, and applied to a column (3 × 12 cm) of DEAE-cellulose equilibrated with mM potassium chloride, to remove an octasaccharide repeating-unit of extracellular, acidic polysaccharide. A neutral material [cyclic (1→2)-β-D-glucan] was eluted with mM potassium chloride (150 mL) without adsorption to the column. An acidic material (octasaccharide repeating-unit) was adsorbed to the column. The fractions containing the cyclic (1→2)-β-D-glucan were collected, 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.

Estimation of molecular weight of cyclic (1→2)-β-D-glucan. — A cyclic (1→2)-β-D-glucan (2.5 mg) was hydrolyzed in 0.1M trifluoroacetic acid (1 mL) for 60–120 min at 100°. A solution of the partial hydrolyzate was analyzed by l.c. on a column (25 cm × 4.6 mm) of Finepak SIL NH_2-10 (Japan Spectroscopy, Tokyo, Japan), with 11:9 acetonitrile–water as the solvent system at a flow rate of 1.0 mL/min and a pressure of 6.2 MPa, with refractometric detection, according to the method of Koizumi *et al.*[17].

RESULTS AND DISCUSSION

Components of cyclic (1→2)-β-D-glucans of *Agrobacterium* and *Rhizobium* were separated by paper chromatography, with development for three days (see Fig. 1). The cyclic (1→2)-β-D-glucans of all nine strains of *Agrobacterium* tested gave the same separation pattern, whereas those of ten strains of *Rhizobium* tested gave four kinds of separation pattern. At least five spots having different R_F values were observed. For isolation of these compounds, large amounts of cyclic (1→2)-β-D-glucan were applied to papers and developed for five or six days. The products were extracted with water from appropriate sections of the paper, and the extracts concentrated *in vacuo* at 40°. The products in the first and second spots (**a** and **b**) were prepared from the cyclic (1→2)-β-D-glucan of *R. trifolii* AHU 1134, whereas those in the third, fourth, and fifth spots (**c, d,** and **e**) were prepared from the cyclic (1→2)-β-D-glucan of *A. radiobacter* IFO 12665. These products were purified by rechromatography. On methylation analysis of each product, only 3,4,6-tri-*O*-methylglucose was found.

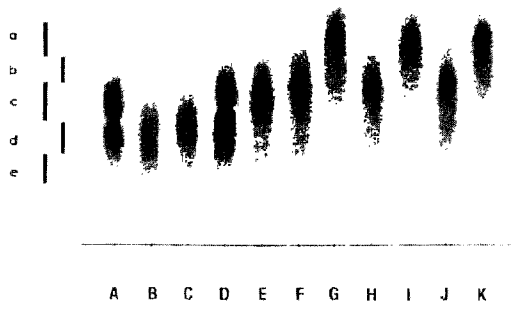

Fig. 1. Paper chromatogram of cyclic (1→2)-β-D-glucans produced by *Agrobacterium* and *Rhizobium*. [A–K: cyclic (1→2)-β-D-glucans of *A. radiobacter* IFO 12665, *R. meliloti* J7017, *R. meliloti* IFO 13336, *R. trifolii* J60, *R. trifolii* IFO 13337, *R. trifolii* 4S, *R. trifolii* AHU 1134, *R. leguminosarum* 303, *R. phaseoli* AHU 1133, *R. japonicum* IFO 13338, and *R. lupini* KLU. Solid bars indicate the sections extracted with water, and **a**, **b**, **c**, **d**, and **e** indicate the products extracted from each section.]

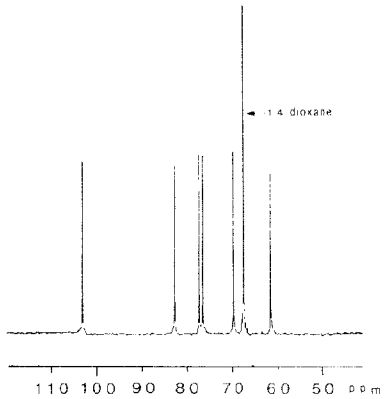

Fig. 2. 50.10-MHz, ^{13}C-n.m.r. spectrum of **a**.

The ^{13}C-n.m.r. spectrum of **a** showed only six signals (see Fig. 2), indicating that the material was homogeneous in molecular size. These six signals, at δ_c 102.79, 82.64, 77.18, 76.28, 69.71, and 61.50, were readily assigned as those of C-1, C-2, C-5, C-3, C-4, and C-6, respectively, by comparison with the spectra[18] of

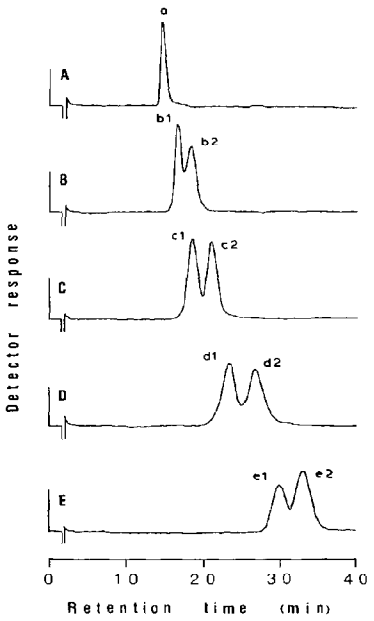

Fig. 3. 11-MPa, liquid chromatograms of **a** (A), **b** (B), **c** (C), **d** (D), and **e** (E) on a column of μBondapak carbohydrate.

sophorose and 2-*O*-methyl-β-D-glucopyranose. The ^{13}C-n.m.r. spectra of **b, c,** and **e,** however, showed two signals for C-1 and C-2, and that of **d** showed two signals for C-2. These results suggested that **b, c, d,** and **e,** were not homogeneous, because cyclic forms of (1→2)-β-D-glucan should show only one chemical shift for each carbon atom when they are homogeneous in molecular size. Therefore, we purified the preparations further by 11-MPa l.c. on a column of μBondapak carbohydrate. As shown in Fig. 3, **a** gave a single peak, but **b, c, d,** and **e** gave two peaks in l.c. The products separated (**b1, b2, c1, c2, d1, d2, e1,** and **e2**) were collected from the effluent, and further purified by rechromatography. A material that was liberated from the carbohydrate column and was slightly water-soluble could be removed by gel chromatography on Sephadex G-10. The resulting preparations were homogeneous, as their ^{13}C-n.m.r. spectra all showed only six signals. D-Glucans **b2** and **c1** were the same cyclosophoraose because their retention times in l.c., and their ^{13}C-n.m.r. spectra, were the same.

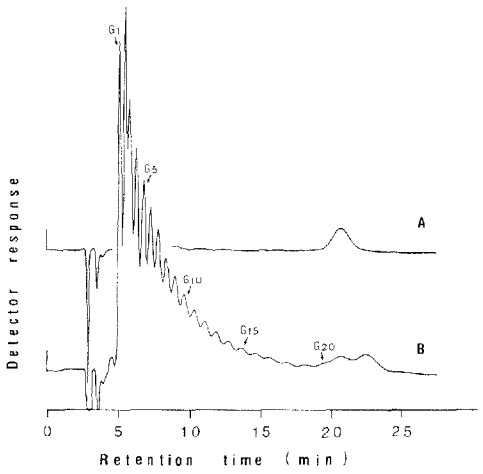

Fig. 4. 6.2-MPa, liquid chromatograms of **d2** (A) and a partial hydrolyzate of **d2** (B), on a column of Finepak SIL NH_2-10. [G1 indicates the position of D-glucose, and G5–G20, those of the respective D-glucose oligomers.]

For estimation of molecular weights, the eight cyclosophoraoses were partially hydrolyzed, and the hydrolyzates were analyzed by l.c. on a column of Finepak SIL NH_2-10. The l.c. elution-profiles of **d2** and of a partial hydrolyzate of **d2** are illustrated in Fig. 4. The last peak of the partial hydrolyzate of **d2** was the 22nd peak when counted from the first peak of D-glucose, and **d2** was eluted at the same position as the 21st peak of the hydrolyzate. Thus, the 22nd peak is considered to be that of a docosaose which is an open, circular form of **d2**; the circular form is eluted earlier than the open form. The last peaks of partial hydrolyzates of **a, b1, c1, c2, d1, e1,** and **e2** were also found to be the 17th, 18th, 19th, 20th, 21st, 23rd, and 24th peaks, respectively. From these results, it was concluded that the molecular weights of the eight cyclosophoraoses are 2756 (cyclosophoroheptadecaose), 2918 (cyclosophoro-octadecaose), 3080 (cyclosophorononadecaose), 3242 (cyclosophorocicosaose), 3404 (cyclosophorohenicosaose), 3566 (cyclosophorodocosaose), 3728 (cyclosophorotricosaose), and 3890 (cyclosophorotetracosaose). The molecular weight is given by $162.1n$, where n is the degree of polymerization. The ^{13}C-n.m.r. chemical-shifts of these compounds are shown in Table I.

The distributions of these compounds in cyclic (1→2)-β-D-glucan preparations obtained from *Agrobacterium* and *Rhizobium* were examined by 11-MPa l.c.

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS OF CYCLOSOPHORAOSES IN D_2O SOLUTION

Cyclosophoraose	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Cyclosophoroheptadecaose	102.79	82.64	76.28	69.71	77.18	61.50
Cyclosophoro-octadecaose	102.50	82.76	76.31	69.74	77.06	61.56
Cyclosophorononadecaose	103.02	83.34	76.28	69.56	77.18	61.53
Cyclosophoroeicosaose	102.59	82.61	76.39	69.79	77.15	61.59
Cyclosophoroheneicosaose	102.88	83.28	76.31	69.62	77.15	61.56
Cyclosophorodocosaose	102.88	83.08	76.36	69.68	77.18	61.59
Cyclosophorotricosaose	102.67	83.05	76.33	69.68	77.12	61.59
Cyclosophorotetracosaose	102.99	83.40	76.33	69.62	77.18	61.59

on a column of μBondapak carbohydrate. The four elution-patterns distinguished are shown in Fig. 5. Cyclic (1→2)-β-D-glucans of *R. trifolii* AHU 1134, *R. phaseoli* AHU 1133, and *R. lupini* KLU were found to be composed almost entirely of cyclosophoroheptadecaose [Type I cyclic (1→2)-β-D-glucan]; those of *R. trifolii* 4S, *R. trifolii* IFO 13337, *R. japonicum* IFO 13338, and *R. leguminosarum* 303 are composed mainly of cyclosophorononadecaose and cyclosophoroeicosaose [Type II cyclic (1→2)-β-D-glucan]; those of *R. meliloti* J7017 and *R. meliloti* IFO 13336 are composed mainly of cyclosophoroeicosaose, cyclosophoroheneicosaose, and cyclosophorodocosaose [Type III cyclic (1→2)-β-D-glucan]; and those of nine strains of *Agrobacterium* and *R. trifolii* J60 are composed mainly of cyclosophorononadecaose, cyclosophoroeicosaose, cyclosophoroheneicosaose, and cyclosophorodocosaose [Type IV cyclic (1→2)-β-D-glucan]. This grouping is consistent with that based on the patterns on the paper chromatogram (see Fig. 1).

We had reported[7] that, judging by the results of field-desorption, mass spectrometry and paper chromatography of the hydrolyzate of the glucan, the cyclic (1→2)-β-D-glucan of *Agrobacterium* is composed of a heptadecaose and an octadecaose, but we could not detect the components of higher molecular weight, because of the weak resolutions achieved with the techniques then employed.

The structures of extracellular, acidic polysaccharides produced by *Agrobacterium* and *Rhizobium* used in this work have all been elucidated, except those formed by three strains[15,19–21]. Judging from the results of methylation and component analysis (unpublished data), the structures of the acidic polysaccharides of the three strains *R. trifolii* IFO 13337, *R. japonicum* IFO 13338, and *R. leguminosarum* 303 are probably the same as those of the acidic polysaccharides of *R. leguminosarum* strains 128c53 and 128c63 and *R. trifolii* strains NA30 and 0403, elucidated by Robertsen *et al.*[22]. Interestingly, we noticed that the structure of the acidic polysaccharide is closely connected with the type of cyclic (1→2)-β-D-glucan: three strains of *Rhizobium* elaborating Type I cyclic (1→2)-β-D-glucan produce the same acidic polysaccharide composed of D-glucose, D-galactose, and pyruvic acid in the molar ratios of 12:4:3 (ref. 20); four strains of *Rhizobium* forming Type II

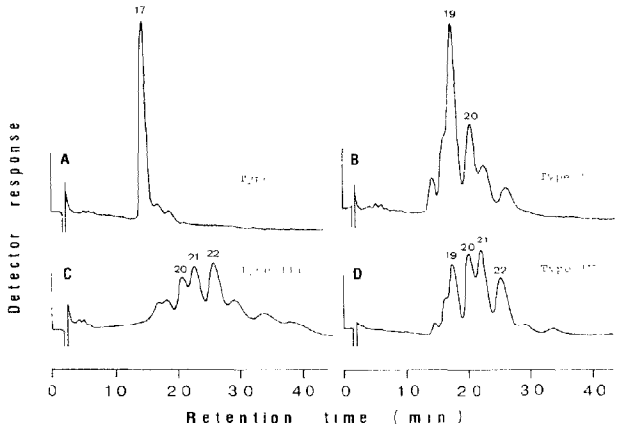

Fig. 5 Liquid chromatograms of the cyclic (1→2)-β-D-glucans of *R. trifolii* AHU 1134 (A), *R. trifolii* IFO 13337 (B), *R. meliloti* IFO 13336 (C), and *A. radiobacter* IFO 12665 (D) on a column of μBondapak carbohydrate. [The elution patterns of the cyclic (1→2)-β-D-glucans of *R. phaseoli* AHU 1133 and *R. lupini* KLU, *R. trifolii* 4S, *R. japonicum* IFO 13338 and *R. leguminosarum* 303, *R. meliloti* J7017, and other strains of *Agrobacterium* and *R. trifolii* J60 were similar to A, B, C, and D, respectively. Numerals 17, 18, 19, 20, 21, and 22 indicate cyclosophoroheptadecaose, cyclosophoro-octadecaose, cyclosophorononadecaose, cyclosophoroeicosaose, cyclosophorohenecosaose, and cyclosophorodocosaose, respectively.]

cyclic (1→2)-β-D-glucan produce the same, or quite similar, polysaccharides composed of D-glucose, D-galactose, D-glucuronic acid, and pyruvic acid in the molar ratios of 5:1:2:2 (refs. 21 and 22; unpublished data), although the polysaccharide of one (*R. trifolii* 4S) of the four strains does not have a terminal D-galactosyl residue in the side chain[21]; nine strains of *Agrobacterium* and one strain of *Rhizobium* producing Type IV cyclic (1→2)-β-D-glucan produce[15] the same acidic polysaccharide (succinoglycan-type polysaccharide) composed of D-glucose, D-galactose, and pyruvic acid in the molar ratios of 7:1:1; two strains of *Rhizobium* producing Type III cyclic (1→2)-β-D-glucan produce succinoglycan-type polysaccharide or similar polysaccharide containing D-glucuronic acid and D-riburonic acid at the terminal of the side chain, although the structure of the other part is the same as that of succinoglycan[19].

As the ^{13}C-n.m.r. chemical-shifts of C-1 and C-2 of the eight cyclosophoraoses differ slightly, depending on the molecular size, the main components in a (1→2)-β-D-glucan preparation can be inferred from the chemical shifts. The (1→2)-β-D-glucans of *A. tumefaciens* IIBV7 and *A. radiobacter* 1000 reported by

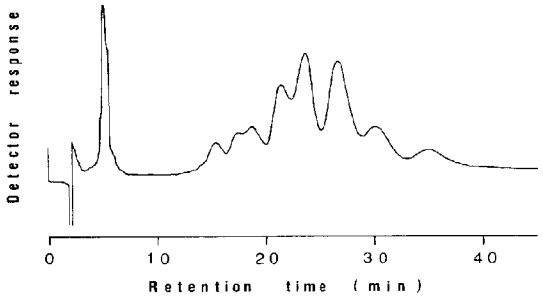

Fig. 6. Liquid chromatogram of the cyclic (1→2)-β-D-glucan fraction of *R. meliloti* J7017 on a column of μBondapak carbohydrate.

Barreto-Bergter *et al.*[4], showing 2 signals for C-1 and 3 signals for C-2, may be Type IV cyclic (1→2)-β-D-glucan, peculiar to *Agrobacterium* strains. The cyclic (1→2)-β-D-glucan of *R. meliloti* K-24 reported by Zevenhuizen[10], showing one signal for C-1 (δ_c 102.8) and 2 signals for C-2 (δ_c 83.2 and 82.9), may be composed mainly of cyclosophoroheneicosaose and cyclosophorodocosaose, being similar to Type III cyclic (1→2)-β-D-glucan.

In the preparation of cyclic (1→2)-β-D-glucan from *R. meliloti* J7017, materials of low molecular weight, eluted at 4–6 min, were detected by l.c. on a column of μBondapak carbohydrate, as shown in Fig. 6. The materials were collected from the effluent. The $[\alpha]_D^{20}$ value was +166.8° (*c* 0.25, water), and methylation analysis gave the alditol acetates of 2,3,4,6-tetra- and 3,4,6-tri-*O*-methylglucose, suggesting that these materials are D-gluco-oligosaccharides having an α-(1→2) linkage. The D-gluco-oligosaccharides may be related to the kojihexaose recently reported by Watanabe *et al.*[23] as an extracellular oligosaccharide of *R. japonicum* strain 561.

Cyclic (1→2)-β-D-glucan was also found in cells of *Rhizobium*[9–11], as already described. Consequently, the cyclic (1→2)-β-D-glucans isolated from the cells of *R. trifolii* 4S and *R. trifolii* AHU 1134 by 75% ethanol extraction by the method of Zevenhuizen[10] were, by l.c. on a column of μBondapak carbohydrate, compared with those from the culture filtrates of the same strains. The elution patterns were the same, indicating that the extracellular (from the culture filtrates) and the intracellular (from the cells) cyclic (1→2)-β-D-glucans were composed of the same components.

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