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Note

A new, extracellular oligosaccharide, kojihexaose, from *Rhizobium japonicum* strain 561

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D-Glucans and D-gluco-oligosaccharides having α-(1→2) linkage(s) have rarely been found as natural products. Among them, kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) has been isolated from koji extract[1], beer[2], honey[3], and starch hydrol[4], as well as from a partial acetolyzate of *Leuconostoc mesenteroides* NRRL B-1299 dextran[5]. This disaccharide is also known as the carbohydrate component of the membrane teichoic acid[6] from *Streptococcus faecalis* strain 39.

Kojitriose is the carbohydrate component of the membrane teichoic acid[6] isolated from *Streptococcus faecalis* strain 8191. It has also been isolated from a partial acetolyzate of *Leuconostoc mesenteroides* NRRL B-1299 dextran[7].

As regards (1→2)-α-D-glucan, only one report has been published, by Kent[8]. He isolated an antigenic lipopolysaccharide from the culture filtrate of human-type *Mycobacterium tuberculosis* strain H37 or A33, and characterized the carbohydrate moiety of this lipopolysaccharide as α-D-(1→2)-linked D-glucan by determination of its optical rotation and by methylation analysis.

It is known that the strains belonging to such slow-growing rhizobia as *Rhizobium japonicum* produce various kinds of extracellular polysaccharides[9,10]. Recently, Dudman and Jones[11] isolated two D-glucans from the culture filtrate of *Rhizobium japonicum* strain 3I 1b 71a, and characterized them as branched D-glucans composed of β-D-(1→3)- and β-D-(1→6)-linked residues. Dudman[12] also suggested the presence of an α-D-(1→2)-linked D-glucan in the culture filtrate of *Rhizobium japonicum* 3I 1b 135. Using another strain of *Rhizobium japonicum* (561), we have now obtained, as an extracellular product, a D-glucose polymer having α-D-(1→2)-linkages. Unexpectedly, this product is not a polysaccharide, but a hexasaccharide.

We now describe the isolation and characterization of a new oligosaccharide, kojihexaose.

RESULTS AND DISCUSSION

On complete hydrolysis with acid, the purified, extracellular oligosaccharide from *Rhizobium japonicum* strain 561 yielded only glucose, which was characterized by paper chromatography and gas–liquid chromatography (g.l.c.). Gel filtration of this oligosaccharide showed that its elution volume was exactly the same as that of maltohexaose (see Fig. 1). Thus, this extracellular oligosaccharide was shown to be a glucohexaose.

In both paper chromatography and paper electrophoresis, it showed a single spot (R_G 0.11, or M_G 0.29). It was not stained on a paper chromatogram by spraying with triphenyltetrazolium chloride (TTC)[13]. The slow electrophoretic migration, as well as the negative staining with TTC, suggested the presence of a (1→2) linkage at the reducing end.

G.l.c. analyses of both the methanolyzate and the alditol acetate derivatives obtained from the permethylated, extracellular oligosaccharide showed peaks corresponding in relative retention-times to those of the methyl glycosides and the alditol acetates of 2,3,4,6-tetra- and 3,4,6-tri-*O*-methyl-D-glucose. These results confirmed that the extracellular oligosaccharide has only (1→2) linkages.

The purified oligosaccharide was readily soluble in water, and had $[\alpha]_D$ +166.3° (*c* 1.6, water), suggesting that the glycosidic linkages are α. The presence of α-linkages was confirmed by its i.r. spectrum. The extracellular oligosaccharide showed infrared absorption peaks at 916 and 838 cm^{-1}, characteristic of an α-D-glucosidic linkage, but no absorption peak at 890 cm^{-1}.

On partial hydrolysis with acid, the extracellular oligosaccharide gave glucose, kojibiose (R_G 0.68), and oligosaccharides, not stained by TTC, having R_G values of

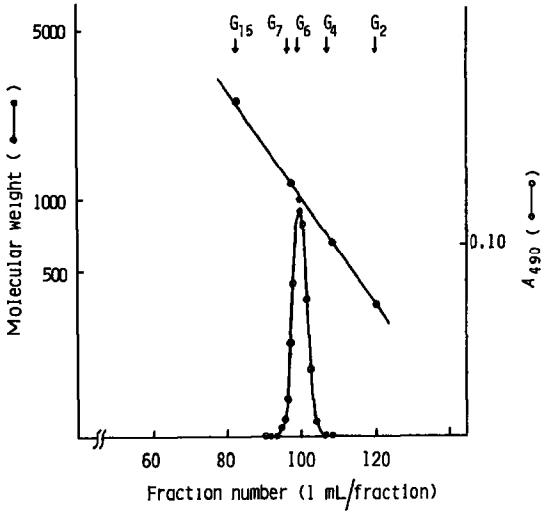

Fig. 1. Determination of the molecular size of the extracellular oligosaccharide by gel filtration on Toyopearl HW-40 F.

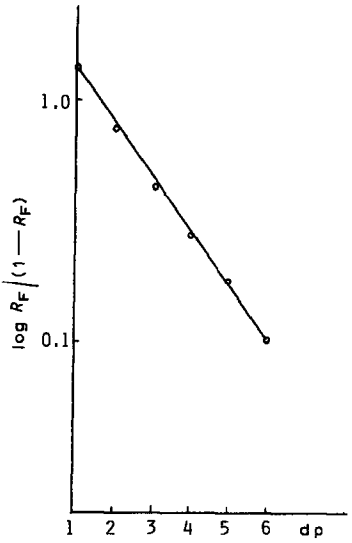

Fig. 2. Paper-chromatographic mobilities of the components of the partial hydrolyzate of the extracellular oligosaccharide with acid. [Developed three times by the ascending method with 6:4:3 (v/v) 1-butanol–pyridine–water.]

0.44, 0.29, and 0.18, together with the original oligosaccharide (R_G 0.11). A linear relationship was observed when log $R_F/(1 - R_F)$ for each of the products of partial hydrolysis of the extracellular oligosaccharide was plotted against the degree of polymerization[14], as shown in Fig. 2. These results again indicated that the oligosaccharide has only α-(1→2) linkages.

In the ^{13}C-n.m.r. spectrum, a chemical shift at 77.1 p.p.m. was identical with the C-2 resonance involved in the glucosidic linkage of kojibiose[15]. Some additional peaks were observed in a region slightly higher (76.0, 75.5, and 75.2 p.p.m.) than that of the C-2 resonance of kojibiose (77.1 p.p.m.). These peaks were different from the chemical shifts of linkage carbon atoms involved in oligosaccharides having other linkages, and could, presumably, be assigned to the resonances of the C-2 atoms in the middle units. These results lend support to the foregoing view that the extracellular oligosaccharide consists of α-(1→2)-linked D-glucosyl residues.

D-Gluco-oligosaccharides of the α-D-(1→2) series that are larger than kojitriose have never been reported, and, hence, the present oligosaccharide is a newly isolated and characterized one. It remains to be established, however, whether this oligosaccharide is a general product of the strains of *Rhizobium japonicum*.

EXPERIMENTAL

Organism, and its culture. — Slow-growing rhizobia, *Rhizobium japonicum* strain 561, were obtained from the Agricultural Experimental Station, Tsukuba, Ibaraki Prefecture, Japan. The organism, sustained on an agar slant, was precultured

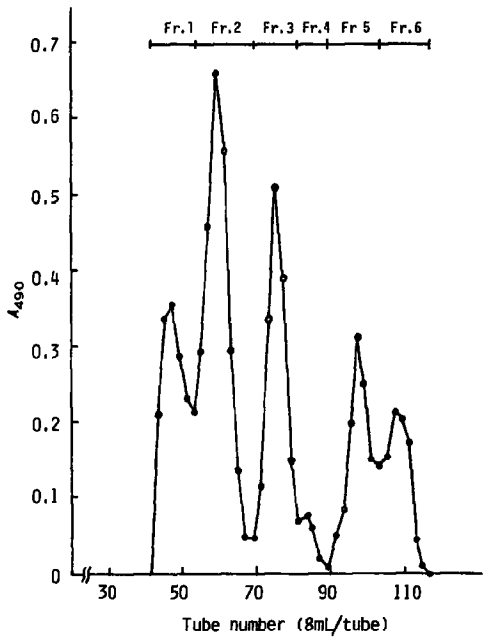

Fig. 3. Elution profile of the dialyzate from the culture filtrate of *Rhizobium japonicum* strain 561 on Bio-Gel P-2.

for 5 days at 28°, with shaking, in the following medium (100 mL): D-mannitol (10 g), L-glutamic acid (1 g), K_2HPO_4 (0.2 g), KH_2PO_4 (0.3 g), $MgSO_4 \cdot 7\ H_2O$ (0.15 g), $CaCl_2 \cdot 2\ H_2O$ (50 mg), $FeCl_3$ (6 mg), NaCl (50 mg), and bouillon (1 g) per L (pH 6.8). A portion (100 mL) of the precultured, mycelial suspension was inoculated into 1 L of the same medium in 3-L flasks, and cultivation was continued for 9 days at 28°.

Isolation of the extracellular oligosaccharide. — After completion of the cultivation, the culture filtrate was evaporated, and dialyzed against distilled water in a cellulose tube overnight. Evaporation of the dialyzate gave extracellular products of low molecular weight (yield, 1.5 g from 2 L of the culture medium). A portion of the dialyzate (300 mg) was dissolved in distilled water (6 mL), and applied to a column (3.5 × 150 cm) of Bio-Gel P-2. The column was eluted with distilled water. Fractions (8 mL each) of eluate were collected, and the carbohydrate content of each was determined by the phenol–sulfuric acid method. The dialyzate was separated into six fractions (see Fig. 3). Four such runs were made, and the combined fraction 3 (80 mg) was purified by repeated filtration on the same column. The purified fraction 3 was used as the purified, extracellular oligosaccharide.

Estimation of the molecular size of the extracellular oligosaccharide. — The purified, extracellular oligosaccharide (300 μg) was dissolved in distilled water (1 mL), and applied to a column (1.75 × 80 cm) of Toyopearl HW-40 F. The column was eluted with distilled water, and the eluate was separated, by means of a fraction

collector, into tubes containing 1 mL each. The carbohydrate content of each tube was determined by the phenol–sulfuric acid method. The column was calibrated with the standard malto-oligosaccharides (G_2, G_4, G_6, G_7, and G_{15}).

General methods. — All evaporations were conducted under diminished pressure below 40°. Optical rotations were measured with a Jasco DIP-4 polarimeter. Paper chromatography was performed on Toyo No. 50 filter paper by the triple ascending method with 6:4:3 (v/v) 1-butanol–pyridine–water[16]. Paper electrophoresis was performed on Toyo No. 50 filter paper at 15 V/cm with 0.1M sodium tetraborate. The silver nitrate dip-method[17] was used for the detection of sugars. Gas–liquid chromatography was performed with a Yanagimoto Model G-80 gas chromatograph fitted with a flame-ionization detector.

Complete hydrolysis of the extracellular oligosaccharide with acid. — Complete hydrolysis of the oligosaccharide was achieved by heating a sample (1 mg) with 2M trifluoroacetic acid (1 mL) for 4 h at 100°, and the hydrolyzate was evaporated.

Partial hydrolysis of the extracellular oligosaccharide with acid. — Partial hydrolysis of the oligosaccharide was conducted by heating a sample (1 mg) with 0.2M trifluoroacetic acid (1 mL) for 2 h at 100°, and the hydrolyzate was evaporated.

Sugar analysis of the extracellular oligosaccharide. — After complete acid hydrolysis of the oligosaccharide, the hydrolyzate was converted into the corresponding alditol trifluoroacetyl derivative[18], which was analyzed by gas–liquid chromatography with a glass column (0.4 × 200 cm) packed[19] with 1.5% of QF-1 on Chromosorb W. Detection of the component sugar was also performed by paper chromatography.

Methylation analysis. — The oligosaccharide was methylated by the method of Hakomori[20]. A portion of the methylated oligosaccharide was methanolyzed by an established procedure[21]. The methanolyzate was analyzed by gas–liquid chromatography, under conditions reported previously[22]. Another portion of the methylated oligosaccharide was hydrolyzed, and the sugars were converted into the corresponding alditol acetates[23]. The partially methylated alditol acetates were analyzed by gas–liquid chromatography with a glass column (0.4 × 100 cm) packed with 3% of OV-210 on Supelcoport[24].

^{13}C-N.m.r. spectrum. — The extracellular oligosaccharide (20 mg) was dissolved in deuterium oxide (0.5 mL). The ^{13}C-n.m.r. spectrum was recorded with a JEOL FX-100 spectrometer.

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