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FORMATION OF A CYCLOINULO-OLIGOSACCHARIDE FROM INULIN BY AN EXTRACELLULAR ENZYME OF *Bacillus circulans* OKUMZ 31B

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ABSTRACT

A strain of *Bacillus circulans* OKUMZ 31B, isolated from soil, has been shown to produce an extracellular enzyme that converts inulin into cycloinulo-oligosaccharides. The main product was identified as cycloinulo-hexaose. The enzyme is arbitrarily designated as cycloinulo-oligosaccharide fructanotransferase.

INTRODUCTION

Inulin fructotransferase [EC 2.4.1.93] catalyzes the formation of cyclic di-D-fructose (di-D-fructose anhydride III), from inulin through an intramolecular trans-fructosylation reaction[1,2]. The enzyme has been found in some bacterial strains, such as *Arthrobacter ureafaciens, A. grobiformis*[3], and *A. ilicis*[4]. It has also been shown that the levan fructotransferase of *A. ureafaciens* produces a homologous compound, di-D-fructose anhydride IV, from levan by the same type of reaction[5]. These results led us to consider that other types of fructotransferase that would catalyze the formation of a cycloinulo-oligosaccharides from inulin might exist. We attempted the isolation of bacterial strains that would produce such an enzyme.

One isolated bacterial strain (*Bacillus circulans* OKUMZ 31B) produced an enzyme that converts inulin into novel cycloinulo-oligosaccharides. We describe here the characterization and identification of the cycloinulo-oligosaccharide that is the main product of the enzyme reaction.

MATERIALS AND METHODS

Materials. — Inulin (dahlia) and β-fructofuranosidase [EC 2.4.1.26] (*Candida utilis*, 620 units/mg) were purchased from Sigma. Endo-inulinase[6] of *Aspergillus niger*-12 was a generous gift from Dr. T. Nakamura (Faculty of Agriculture, Miyazaki University, Miyazaki Japan). A mixture of inulo-oligosaccharides for d.p.

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standards was prepared from inulin by partial acid hydrolysis, as described previously[2].

General methods. — Melting points were determined with a Yanagimoto micro melting-point apparatus and are uncorrected. ^{13}C-N.m.r. spectra were recorded at 25 MHz with a JEOL FX-100 spectrometer for solutions in D_2O (internal 1,4-dioxane, 67.4 p.p.m. relative to the signal for Me_4Si). For f.d.–m.s., a JEOL D-300 mass spectrometer was used. G.l.c.–m.s. was performed on a Shimadzu GCMS-7000S apparatus; glass column 2.6 mm × 2 m packed with 3% OV-255 on Gas Chrom Q, injection temp. 230°, column temp. 200°, carrier gas He at 17 mL/min, separator temp. 240°, ionization voltage 70 eV, accelerating voltage 3 kV. H.p.l.c. was performed out on a Waters M600 instrument, employing a column (3.9 × 150 mm) of μBondasphere 5 μm NH_2, with elution by 1:4 (v/v) MeCN–water at 1 mL/min, and differential refractomeric detection (Waters model R-401 detector). Optical rotations were measured with a Jasco DIP 180 type polarimeter having a 1.0-dm light path. T.l.c. plates were developed twice on Silica Gel G-254 (Merck) with 1:4:1 (v/v) 1-butanol–2-propanol–water. Sugars were detected with naphthoresorcinol–H_2SO_4 reagent[7].

Cultivation. — The strain used for the test was isolated from soil and identified as a strain of *Bacillus circulans*. The solid agar culture-medium used for the isolation and storage of bacteria was composed of inulin (15 g), $NaNO_3$ (2 g), $MgSO_4 \cdot 7\ H_2O$ (0.5 g), KCl (0.5 g), a trace of $FeCl_3$, and KH_2PO_4 (0.5 g) per 1 L of distilled water, adjusted to pH 7 with 2M NaOH. The liquid culture medium for the enzyme preparation had approximately the same composition as just mentioned except that 10 g of yeast extract was added. A loop of cultured bacteria was inoculated into a 150-mL aliquot of the medium in a 500 mL Sakaguchi flask that was shaken (110 strokes/min) for 4 days at 30°.

Enzyme preparation. — The cultured medium (390 mL) was centrifuged at 10 000*g* at 0° for 15 min to remove the bacteria. To the supernatant solution, chilled in ice, 1 vol. of cold acetone (−10°) was slowly added at 0–5° under stirring. The precipitate formed was collected by centrifugation at 10 000*g* for 15 min at 0°, and washed with ice-cold acetone, ice-cold Et_2O, and dried *in vacuo* over $CaCl_2$. The yield was 840 mg.

Isolation of the cycloinulo-oligosaccharide. — A mixture containing 3% inulin and 200 mg of enzyme in 100 mL of 50mM phosphate buffer (pH 7.0), was incubated under toluene for 4 days at 30°. The reaction was stopped by heating for 5 min at 100°. To remove insoluble material, the mixture was centrifuged at 5 000*g* for 15 min, and the supernatant solution was evaporated to a syrup at 40° under diminished pressure; yield 4.2 g. The syrup was suspended in 40 mL of 80% (v/v) aq. EtOH and centrifuged. The precipitate was resuspended in 40 mL of 80% EtOH and centrifuged. Two supernatant solutions were combined and evaporated to a syrup at 40° under diminished pressure; yield 0.49 g. The dried material was dissolved in 5 mL of distilled water. Isolation of the cycloinulo-oligosaccharide was performed by charcoal-column chromatography [4.5 × 20 cm, with a mixture of

Celite No. 535 (60 g) and Tokusei-Shirasagi charcoal (30 g); Takeda Pharm. Co. Ltd.]. Prior to chromatography, the column was prewashed successively with 1 L of 50% EtOH and 1.5 L distilled water. After the sample solution had been applied, the column was washed with 1 L of distilled water and 1 L of 15% EtOH, and then eluted with 20% EtOH. The flowrate was 10 mL/h, and 10-mL fractions were collected. The ketohexose content of the fraction was determined by the resorcinol–HCl method[8]. Fractions containing the cycloinulo-oligosaccharide were combined and the solution was evaporated to a syrup at 40° under diminished pressure; yield 0.18 g. The syrup was dissolved in 2 mL of 70% (v/v) EtOH and subjected to partition chromatography with a column of QAE-Toyopearl 550C (Tosoh Corp., SO_4^{2-} form). The sample was applied to the column [2.5 × 39 cm; prior to packing, the ion-exchanger was washed with 3 vol. of M $(NH_4)_2SO_4$ and 3 vol. of distilled water, and equilibrated with 70% EtOH]. The column was eluted with 70% EtOH and 10-mL fractions were collected at a flowrate of 60 mL/h. The fractions containing the cycloinulo-oligosaccharide were combined and evaporated at 40° under diminished pressure to syrup, yield 0.12 g. The syrup was dissolved in 2 mL of MeOH at 40° and refrigerated for a few days at 4°. The dendritic crystals that formed were collected, washed with cold MeOH, and recrystallized in the same manner; yield (after drying *in vacuo* over $CaCl_2$) 76 mg.

Methylation analysis. — The purified oligosaccharide was methylated, using dimethylsulfinyl carbanion in dry dimethyl sulfoxide and methyl iodide, according to the Hakomori procedure[9]. The methylated sample (5 mg) was treated with aqueous 90% formic acid (1 mL) for 5 h at 100°. The formic acid was then evaporated under diminished pressure, and the residue treated with 0.15M H_2SO_4 (1 mL) for 16 h at 100°. The hydrolyzate was made neutral with $BaCO_3$, evaporated, and the residue treated with aq. $NaBD_4$ (1 mL, ~18 mg) for 5 h at room temperature. The mixture was made neutral with Amberlite MB-3 resin, evaporated, and the resulting methylated alditol acetylated with 1:1 acetic anhydride–pyridine (1 mL) for 1 h at 100°. The solution was evaporated, and the residue was suspended in H_2O and extracted with hexane. The extract was subjected to g.l.c.–m.s.[10].

RESULTS AND DISCUSSION

The formation of several oligosaccharides in the cultured medium of the bacterium was observed by t.l.c. (Fig. 1, lane 2). When the medium was treated with β-fructofuranosidase, one oligosaccharide, which migrated almost to the position of d.p. 6, was not hydrolyzed (Fig. 1, lane 3). The same result was obtained in an experiment using the products formed from inulin with the acetone-powder enzyme, and so the oligosaccharide was isolated from the mixture. The elution profile obtained by partition chromatography on a column of QAE-Toyopearl is shown in Fig. 2. The oligosaccharide eluted as the main peak from the column was further purified by crystallization, when it gave a single spot on t.l.c. (Fig. 1, lane 4) and a single h.p.l.c. peak.

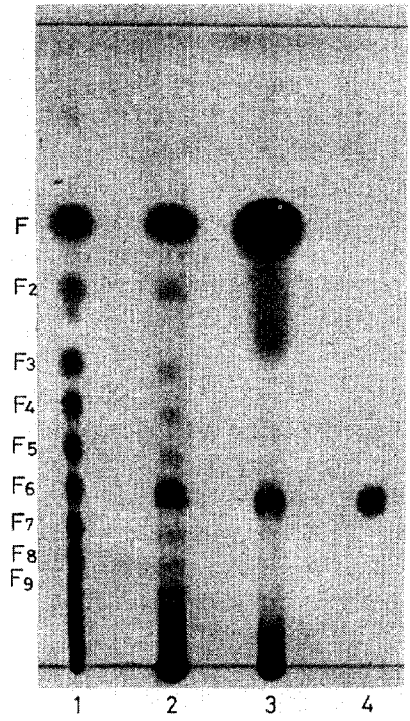

Fig. 1. T.l.c. of the products from inulin in the cultured medium of *Bacillus circulans* OKUMZ 31B, and the purified oligosaccharide. [Samples loaded on t.l.c. plate were: standard inulo-oligosaccharides, lane 1; the medium incubated for 2 days, lane 2; its digest by β-fructofuranosidase, lane 3; and the isolated oligosaccharide, lane 4. F and F_2–F_9 are abbreviations for fructose and inulo-oligosaccharides, d.p. 2–9.]

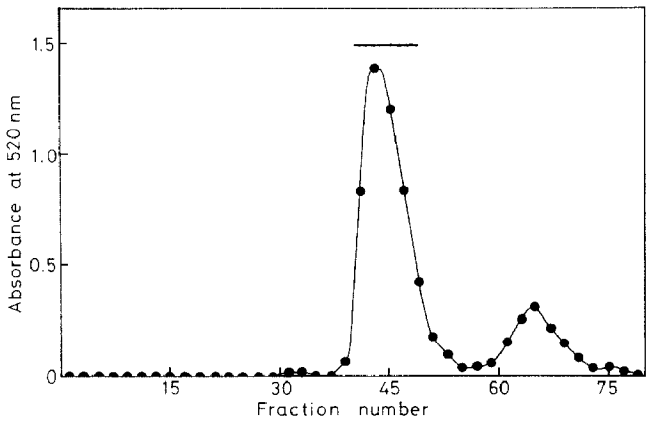

Fig. 2. Partition chromatography on a column of QAE-Toyopearl. [The fractions combined for further purification are indicated by the horizontal bar.]

The isolated oligosaccharide was nonreducing and consisted solely of D-fructose. It had m.p. 231–233° (dec.) and $[\alpha]_D^{20}$ −64.6° (*c* 1.08, H_2O). F.d.–m.s. analysis of (Fig. 3) suggested a mol. wt. of 972 *m/z*: 995 $(M + Na)^+$, in agreement with the mol. wt. calculated for D-fructohexaose anhydride.

Anal. Calc. for $C_{36}H_{60}O_{30} \cdot 3\ H_2O$: C, 42.11; H, 6.48. Found: C, 42.04; H, 6.42.

From these results, the oligosaccharide was presumed to be a cyclized D-fructohexaose.

To provide further evidence for a cyclic structure of the oligosaccharide, it was subjected to digestion by *Aspergillus niger* endo-inulinase (Fig. 4). The oligosaccharide was hydrolyzed by the endo-inulinase, and an oligosaccharide eluted on h.p.l.c. at the same retention time as inulo-hexaose was the principal initial reaction-product (Fig. 4B); this was considered to be a linear D-fructohexaose because it was completely hydrolyzed to D-fructose by the action of β-fructofuranosidase (Fig. 4C).

The data here described strongly suggested that the oligosaccharide is a cyclofructohexaose.

The oligosaccharide was subjected to methylation analysis to establish the fructofuranosidic linkages. After acid hydrolysis, the partially methylated alditol acetate was subjected to g.l.c.–m.s. The product showed only one peak, having the retention time of 35.0 min in g.l.c. The peak was identified by m.s. as a 3,4,6-tri-*O*-methylalditol; it showed peaks at *m/z* 234, 205, 190, and 161 (Fig. 5). These data indicated that the methylation analysis of the oligosaccharide led to acetylated 2-deuterio-3,4,6-tri-*O*-methylglucitol, consistent with a (2→1)-linked cyclofructo-oligosaccharide.

The ^{13}C-n.m.r. spectrum of the oligosaccharide is shown in Fig. 6. Six signals were observed, and it is evident that all of the D-fructofuranosyl residues in this oligosaccharide have the same conformations. The signal for the anomeric carbon atom at 103.7 p.p.m., is indicative of polymeric β-D-fructofuranosyl residues. The resonances at 82.3, 79.0, and 75.2 p.p.m. are attributable to the C-5, C-3, and C-4, respectively. The resonance at 63.3 p.p.m. pertains to the primary carbon atom

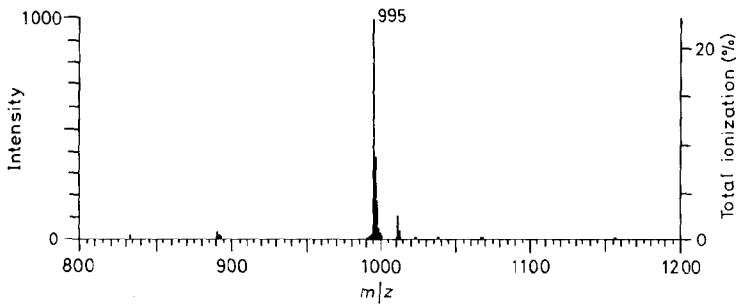

Fig. 3. F.d.–m.s. of the oligosaccharide.

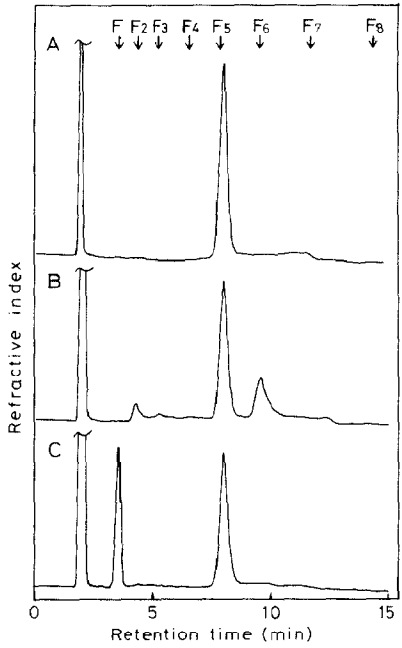

Fig. 4. Digestion test of the oligosaccharide by *Aspergillus niger* endo-inulinase. [The mixture, containing 1 mg of the isolated oligosaccharide and 1 unit of the endo-inulinase in 0.25 mL of 20mM acetate buffer (pH 5.0), was incubated for 4 h at 40°. At zero time (A), and after termination of the reaction by heating at 100° (B), 10-μL aliquots were analyzed by h.p.l.c. Subsequently, 31 units of β-fructofuranosidase in 50 μL of water was added to a 50-μL aliquot of the mixture, which was incubated for 2 h at 40°, with subsequent injection of a 20-μL aliquot into the h.p.l.c. apparatus (C). F and F_2–F_8 are abbreviations for fructose and inulo-oligosaccharides d.p. 2–8, and the arrows indicate the corresponding retention times.]

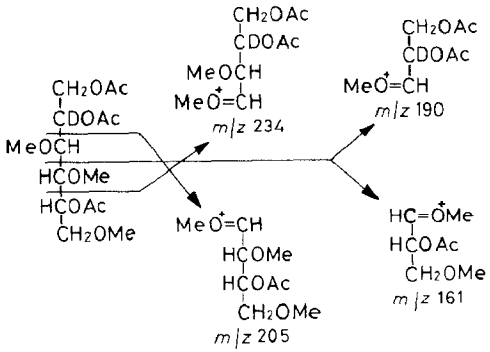

Fig. 5. Fragmentation pattern by g.l.c.–m.s. of the methylated alditol acetate derived from the oligosaccharide.

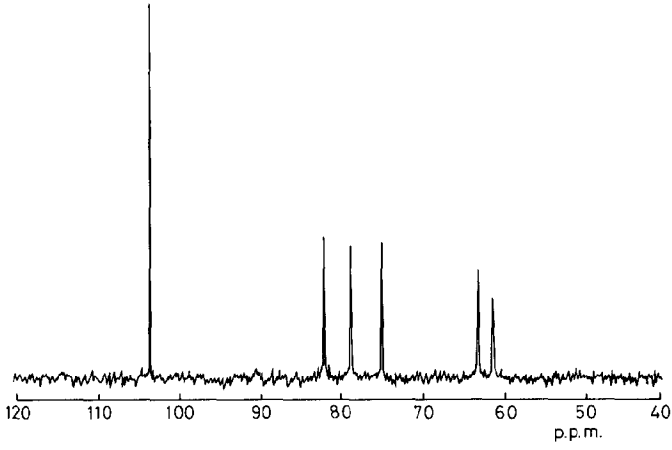

Fig. 6. ^{13}C-N.m.r. spectrum of the oligosaccharide.

(C-6). The substituted primary carbon atom (C-1) resonates at 61.4 p.p.m.; no significant downfield shift was observed.

The data described here indicate that the extracellular enzyme of this bacterial strain produces cycloinulo-hexaose (Fig. 7) as the main product from inulin

We also isolated small amounts of cycloinulo-heptaose and -octaose from the enzyme digest of inulin as byproducts of the enzyme reaction. Structural determinations of these products are under way. We presume that the enzyme acts on inulin to form cycloinulo-oligosaccharides through an intramolecular transfructosylation reaction. For the present, we arbitrarily designate the enzyme as cycloinulo-oligosaccharide fructanotransferase.

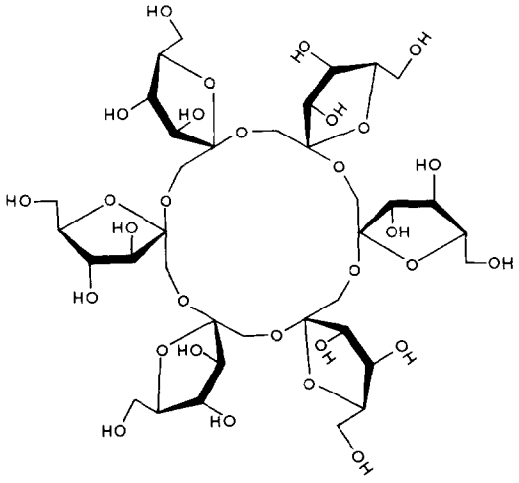

Fig. 7. Schematic structure of the oligosaccharide.

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REFERENCES

1 T. UCHIYAMA, S. NIWA, AND K. TANAKA, *Biochim. Biophys. Acta,* 315 (1973) 412–420.
2 T. UCHIYAMA, *Biochim. Biophys. Acta,* 397 (1975) 153–163.
3 K. HARAGUCHI, M. KISHIMOTO, K. HAYASHI, S. KOBAYASHI, AND K. KAINUMA, *Agric. Biol. Chem.,* 52 (1988) 291–292.
4 M. KAWAMURA, S. TAKAHASHI, AND T. UCHIYAMA, *Agric. Biol. Chem.,* 52 (1988) 3209–3210.
5 K. TANAKA, T. UCHIYAMA, K. YAMAUCHI, Y. SUZUKI, AND S. HASHIGUCHI, *Carbohydr. Res.,* 99 (1982) 197–204.
6 T. NAKAMURA, T. KUROKAWA, S. NAKATSU, AND S. UEDA, *Nippon Nogei Kagaku Kaishi,* 52 (1978) 159–166.
7 M. GHEBREGZABHER, S. RUFINI, B. MONALDI, AND M. LATO, *J. Chromatogr.,* 127 (1976) 133–162.
8 J. H. ROE, J. H. EPSTEIN, AND N. P. GOLDSTEIN, *J. Biol. Chem.,* 178 (1949) 839–845.
9 S. HAKOMORI, *J. Biochem. (Tokyo),* 55 (1964) 205–208.
10 S. EBISU, K. KATO, S. KOTANI, AND A. MISAKI, *J. Biochem. (Tokyo),* 18 (1975) 879–887.