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Formation of oligosaccharides from lactitol by *Aspergillus oryzae* β-D-galactosidase

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ABSTRACT

Six oligosaccharides were first formed from lactitol by a transgalactosylation reaction catalyzed by *Aspergillus oryzae* β-D-galactosidase. From the results of methylation analysis, MS, and ^{1}H- and ^{13}C-NMR studies, it was concluded that these oligosaccharides are *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucitol, *O*-β-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucitol, *O*-β-D-galactopyranosyl-(1 → 4)-[*O*-β-D-galactopyranosyl-(1 → 6)]-D-glucitol, *O*-β-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucitol, *O*-β-D-galactopyranosyl-(1 → 4)-[*O*-β-D-galactopyranosyl-(1 → 5)]-D-glucitol, and *O*-β-D-galactopyranosyl-(1 → 4)-[*O*-β-D-galactopyranosyl-(1 → 1)]-D-glucitol. The last three are newly observed oligosaccharides.

INTRODUCTION

Many oligosaccharides promoting the growth of *Bifidobacterium* in human intestine have been prepared by a transglycosylation reaction catalyzed by glycosidase [1–6]. They included three trisaccharides containing a lactitol unit, *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucitol [7], *O*-β-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucitol [8], and *O*-β-D-galactopyranosyl-(1 → 4)-[*O*-β-D-galactopyranosyl-(1 → 6)]-D-glucitol [1]. Each was prepared from a corresponding galactosyllactose, formed from lactose by transgalactosylation catalyzed by β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), by reduction with sodium borohydride. However, no study on the oligosaccharides formed from lactitol by transgalactosylation catalyzed by β-D-galactosidase has been reported. The present paper describes the elucidation of the structures of six oligosaccharides formed from lactitol by transgalactosylation catalyzed by β-D-galactosidase from *Aspergillus oryzae*.

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EXPERIMENTAL

Materials.—β-D-Galactosidase (60 units/mg of solid) from *Aspergillus oryzae* was purchased from Shinnihon Kagaku Co. and lactitol was kindly donated by Towa Chemical Industry Co., Ltd. The carbon (Shirasagi) was a product of Takeda Chemical Industries Ltd.

Assay of β-D-galactosidase.—The enzyme solution (1 mL) was added to 0.1 M acetate buffer (pH 5.0, 4.0 mL) containing 10 mM 2-nitrophenyl-β-D-galactopyranoside, and the solution was incubated at 37°. After 10 min, the reaction was stopped by adding 0.5 M Na_2CO_3 (5.0 mL). One unit of enzyme activity is defined as the amount of enzyme needed to liberate 1 μmol of 2-nitrophenol/min under the conditions described.

Preparation and purification of oligosaccharides.—To a solution containing lactitol (6 g) in acetate buffer, pH 5.0 (4 mL) was added the enzyme (300 units). The mixture was kept at 50° for 4 h and then the reaction was stopped by heating at 100° for 5 min. The mixture was applied to an activated charcoal column (5 × 60 cm), the column was washed with water (3 L) to remove monosaccharides, and the adsorbed lactitol was first recovered with aq 5% EtOH (3 L), and the oligosaccharides (0.8 g) were eluted with aq 25% EtOH (3 L).

The oligosaccharide was further purified by chromatography in a column (3 × 55 cm) of charcoal, equilibrated with water, and was eluted with a linear gradient of 0–25% EtOH.

HPLC.—HPLC for analysis and purification of the oligosaccharides was performed on a Waters Model 510, equipped with a refractive index monitor (Erma Optical Works, Model ERC-7510), and a column (6 × 250 mm) of ERC-NH-1181 (Erma CR Inc.) eluted with 7:3 acetonitrile–water at 1.0 mL/min.

TLC.—TLC was performed on a Kiesel gel 60 TLC plate (Merck, Art. 13749) with a solvent system of 3:12:4 butanol–2-propanol–H_2O. The sugar spots on the TLC plate were revealed by heating at 110° after spraying with anilin–diphenylamine.

FABMS.—FABMS of the oligosaccharides was performed in the negative-ion mode, by use of a JEOL JMS-DX-303 mass spectrometer with Xe atoms having a kinetic energy equivalent to 6 kV. Glycerol was used as the matrix solution.

NMR studies.—The ^1H- and ^{13}C-NMR spectra of oligosaccharides were obtained for a 2–3% solution in D_2O at 20° with a Varian XL-400 spectrometer. Chemical shifts (δ) are expressed downfield relative to the signal of sodium 4,4-dimethyl-4-silapentanoate (TSP). The assignments were based on ^{13}C–^1H correlation, DEPT, COSY, and COSY RL methods [10–12].

Methylation analysis.—Each dried oligosaccharide was methylated by the Hakomori method [13], and the reaction was monitored with triphenylmethane [14]. The methylated products were purified [15] in a Sep-pak C_{18} cartridge (Waters Assoc.), and were hydrolyzed with M trifluoroacetic acid at 100° for 3 h. The hydrolyzates were reduced with M $NaBH_4$ in aq 95% EtOH containing NH_4OH at room

temperature for 3 h, and then converted into the partially methylated alditol acetates. These were analyzed by GLC (Hewlett–Packard 5890) in a DB-1 capillary column (30 m × 0.53 mm i.d.) at 150–220° (2°/min) with a split ratio of 60:1. GLC–MS of the partially methylated alditol acetates was performed on a Hitachi M-80 mass spectrometer, equipped with the same column and under the same conditions as for GLC analysis, and operated at an ionization voltage of 20 eV and an ion source at 180°.

RESULTS AND DISCUSSION

Preparation and purification of oligosaccharides.—The effects of the reaction temperature (40 and 50°), lactitol concentration (40, 50, and 60%), and enzyme concentration (7.5, 15, and 30 units/g of reaction mixture) on the oligosaccharide formation were investigated. The maximum yield of oligosaccharide (~ 20% of the total sugars) was obtained when a 60% (w/w) lactitol solution containing 30 units/g of reaction mixture at pH 5.0 was incubated at 50° for 4 h (see Fig. 1). After termination of the reaction by heating, the oligosaccharides were partially purified by charcoal column chromatography (Fig. 2) showing the presence of at least six different oligosaccharides which was confirmed by HPLC analysis (Fig. 3A). The HPLC elution pattern of each oligosaccharide from an NH_2-column was different from that from charcoal column chromatography. The oligosaccharides separated by charcoal column chromatography, F1–F6, correspond to Peaks 3, 6, 4, 5, 1, and 2 of the HPLC chromatogram. Therefore, the oligosaccharides F1–F6

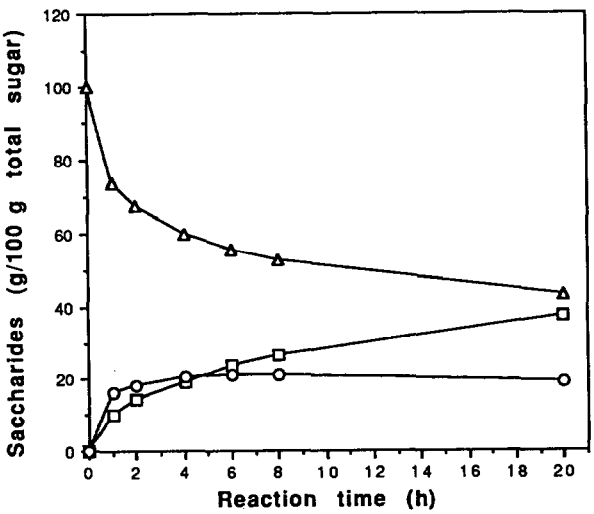

Fig. 1. Lactitol hydrolysis and oligosaccharide formation by β-D-galactosidase (300 units) acting on a solution of lactitol (6 g) in 100 mM acetate buffer (pH 5.0, 4 mL) at 50°: (○) oligosaccharides, (△) lactitol, and (□) monosaccharides (galactose and glucitol).

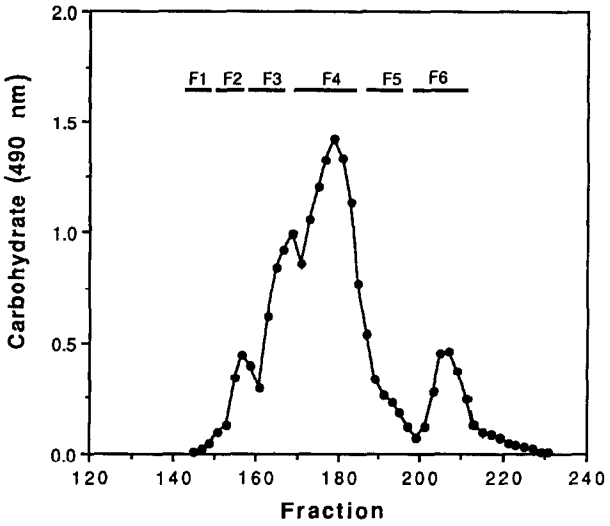

Fig. 2. Charcoal column chromatography of oligosaccharide fraction (see Fig. 1). After removal of monosaccharides and lactitol, the oligosaccharide (0.8 g) fraction was eluted with 25% EtOH and further purified with a linear gradient of 0–25% EtOH from a charcoal column (3×55 cm): (•) carbohydrate (phenol–H_2SO_4 method).

were further purified by HPLC, and each oligosaccharide (P1–P6) was eluted as a single peak (Fig. 3B–G). The purity of each oligosaccharide P1–P6 was confirmed by a single spot on TLC (data not shown). The yield of P1–P6 was 12, 14, 11, 27,

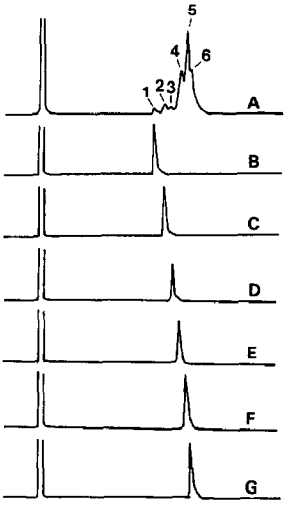

Fig. 3. HPLC of oligosaccharides formed from lactitol by β-D-galactosidase from *Aspergillus oryzae*. A solution (20 μL) containing 20 mg/mL of each oligosaccharide was injected: (A) oligosaccharides eluted from charcoal column with 25% EtOH; (B) P1; (C) P2; (D) P3; (E) P4; (F) P5; and (G) P6 (see Fig. 2).

TABLE I

Characterization of purified oligosaccharides P1–P6

HPLC peak	Sugar (mol ratio) [a]		FABMS	^{1}H-NMR (δ)	H-1 (δ)	$[\alpha]_D^{21}$ [b] (degrees)
	Gal	Glcol	$(M-1)^+$			
P1	2.0	0.9	505	4.55	4.61	[c]
P2	2.0	0.9	505	4.57	4.62	[c]
P3	2.0	1.0	505	4.56	4.61	[c]
P4	2.0	0.9	505	4.46	4.54	+2.3
P5	2.0	1.0	505	4.46	4.59	+9.7
P6	2.0	1.0	505	4.42	4.53	+2.3

[a] The sugar constituents were estimated after hydrolysis with M trifluoroacetic acid for 3 h at 100°. [b] For a solution in water. [c] Not determined.

35, and 30 mg, respectively. The percentage of area corresponding to P1–P6 on the chromatograms was 3.5, 5.6, 3.0, 23.0, 40.9, and 24.0, respectively.

Characterization of oligosaccharides P1–P6.—The six oligosaccharides consisted of galactose and glucitol in the molar ratio of 2:1, and their mol wt was estimated at 506 by FABMS. The ^{1}H-NMR spectra showed two H-1 protons at δ 4.4–4.6 as a doublet (J 7.4–7.6 Hz) characteristic for a β-D-glycoside [16] indicating that the six oligosaccharides were β-D-linked trisaccharides (see Table I).

Methylation analysis and ^{13}C-NMR spectroscopy of oligosaccharides P1–P6.—Oligosaccharides P1–P6 were methylated and the partially methylated alditol acetates were identified by GLC–MS. In the ^{13}C-NMR spectra of P1–P6, every signal was assigned on the basis of comparison with the spectra of glucitol [17] and lactitol by the COSY, COSY RL, DEPT, and ^{13}C–^{1}H correlation methods.

Oligosaccharide P1.—Methylation analysis gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, and 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol in the molar ratio of 1:1:1 (Table II). The ^{13}C-NMR spectrum (Table III) showed that the terminal, nonreducing galactosyl group and the glucitol residue gave chemical shifts similar to those of lactitol. The C′-4 signal of the internal galactose unit was shifted to a lower field (δ 80.1) by the glycosylation. From these results, the structure of oligosaccharide P1 was determined as β-D-Gal$p$-(1 → 4)-β-D-Gal$p$-(1 → 4)-D-Glcol (**1**).

Oligosaccharide P2.—Methylation analysis gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, and 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol in the molar ratio of 1:1:1 (Table II). The ^{13}C-NMR spectrum (Table III) showed that the terminal, nonreducing galactosyl group and the glucitol residue gave chemical shifts similar to those of lactitol. The C′-3 signal of the internal galactose unit was shifted to a lower field (δ 84.8) by the glycosylation. From these results, the structure of oligosaccharide P2 was determined as β-D-Gal$p$-(1 → 3)-β-D-Gal$p$-(1 → 4)-D-Glcol (**2**).

Oligosaccharide P3.—Methylation analysis gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol and 4,5-di-*O*-acetyl-1,2,3,6-tetra-*O*-methylglucitol in the molar ra-

TABLE II

The alditol acetates obtained from the hydrolyzate of permethylated oligosaccharides P1–P6

Methylated alditol acetate derivatives	Mol ratio						MS fragments (m/z)	Linkage
	P1	P2	P3	P4	P5	P6		
2,3,4,6-Me_4Gal	1.0	1.0	2.0	1.0	2.0	2.0	101, 117, 129, 145, 161, 205	Gal-(1 →
2,3,6-Me_3Gal	1.0						99.101, 113, 117, 129, 131 161, 173, 233	→ 4)-Gal
2,4,6-Me_3Gal		0.8					101, 117, 129, 161, 173, 233 277	→ 3)-Gal
2,3,4-Me_3Gal				0.9			99, 101, 117, 129, 159, 161 173, 189, 233	→ 6)-Gal
1,2,3,5,6-Me_5Glcol	0.9	0.8		0.8			89.101, 113, 133, 157, 173 185, 205, 249	→ 4)-Glcol
1,2,3,6-Me_4Glcol			0.9				87, 89, 99, 101, 113, 129, 131 143, 157, 173, 185, 203, 233, 277	→ 4) → 5)-Glcol
1,2,3,5-Me_4Glcol					0.9		89, 101, 117, 127, 133, 143 157, 159, 201, 233, 277	→ 4) → 6)-Glcol
2,3,5,6-Me_4Glcol						0.9	89, 101, 113, 117, 129, 131, 161, 173, 205, 277	→ 4) → 1)-Glcol

tio of 2:1 (Table II). In the ^{13}C-NMR spectrum (Table IV), the chemical shifts of the terminal, nonreducing galactosyl groups, were similar to those of the terminal galactosyl group of lactitol. The C-4 and C-5 signals of the glucitol residues were

TABLE III

^{13}C-NMR data of oligosaccharides P1, P2 and P4 purified from lactitol hydrolyzate

Atom	Chemical Shifts (δ)				
	Glcol	Lactol	P1	P2	P4
C-1	65.3	65.5	65.4	65.5	65.4
C-2	75.8	75.1	74.9	75.0	75.0
C-3	72.5	72.3	72.4	72.4	72.3
C-4	73.9	82.1	82.4	82.2	82.6
C-5	73.8	74.0	74.1	74.0	74.1
C-6	65.6	64.9	64.9	64.9	64.9
C-1′		105.9	106.0	105.6	105.9
C-2′		73.9	74.3	73.1	73.6
C-3′		75.4	75.7	84.8	75.5
C-4′		71.5	80.1	71.3	71.5
C-5′		78.0	77.2	77.6	76.5
C-6′		63.9	63.4	63.8	71.9
C-1″			107.2	107.2	106.2
C-2″			74.3	73.9	73.8
C-3″			75.8	75.4	75.3
C-4″			71.5	71.4	71.5
C-5″			78.0	77.9	78.0
C-6″			63.9	63.8	63.9

TABLE IV

^{13}C-NMR data of oligosaccharides P3, P5, and P6 purified from lactitol hydrolyzate

Atom	Chemical Shifts (δ)				
	Glcol	Lactol	P3	P5	P6
C-1	65.3	65.5	65.5	64.9	73.5
C-2	75.8	75.1	75.1	75.5	74.2
C-3	72.5	72.3	71.9	72.0	72.2
C-4	73.9	82.1	82.0	81.3	81.9
C-5	73.8	74.0	81.0	72.4	73.9
C-6	65.6	64.9	62.5	72.8	65.0
C′,C″-1		105.9	105.5, 105.7	105.8, 105.9	105.9, 106.0
C′,C″-2		73.9	73.9, 73.9	73.7, 73.9	73.5, 73.7
C′,C″-3		75.4	75.4, 75.5	75.3, 75.4	75.4, 75.5
C′,C″-4		71.5	71.5, 71.6	71.5, 71.5	71.4, 71.5
C′,C″-5		78.0	77.9, 78.0	77.9, 78.0	77.9, 78.0
C′,C″-6		63.9	63.9, 64.0	63.9, 63.9	63.7, 63.8

shifted to a lower field (δ 82.0 and 81.0, respectively) by the glycosylation. From these results, the structure of oligosaccharide P3 was determined as β-D-Gal*p*-(1 → 4)-[β-D-Gal*p*-(1 → 5)]-D-Glcol (**3**).

Oligosaccharide P4.—Methylation analysis gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol, and 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol in the molar ratio of 1:1:1 (Table II). The ^{13}C-NMR spectrum (Table III) showed that the terminal, nonreducing galactosyl group and the glucitol unit of P4 gave chemical shifts similar to those of lactitol. The C-6′ signal of the internal galactose unit was shifted to lower field (δ 71.9) by the glycosylation. The assignment of this signal was confirmed by the DEPT method. From these results, the structure of oligosaccharide P4 was determined as β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glcol (**4**).

Oligosaccharide P5.—Methylation analysis gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol and 4,6-di-*O*-acetyl-1,2,3,5-tetra-*O*-methylglucitol in the molar ratio of 2:1 (Table II). In the ^{13}C-NMR spectrum (Table IV), the chemical shifts of the terminal, nonreducing galactosyl groups were similar to those of the terminal galactosyl group of lactitol. The C-4 and C-6 signals of the glucitol unit were shifted to the lower field (δ 81.3 and 72.8, respectively) by the glycosylation. The assignment of the C-6 signal was confirmed by the DEPT method. From these results, the structure of oligosaccharide P5 was determined as β-D-Gal*p*-(1 → 4)-[β-D-Galp-(1 → 6)]-D-Glcol (**5**).

Oligosaccharide P6.—Methylation analysis gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol and 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylglucitol in the molar ratio of 2:1 (Table II). In the ^{13}C-NMR spectrum (Table IV), the chemical shifts of the terminal, nonreducing galactosyl groups were similar to those of the terminal galactosyl group of lactitol. The C-1 and C-4 signals of the glucitol unit were

Chart 1.

Structures of oligosaccharides purified from lactitol hydrolyzate with *Aspergillus oryzae* β-D-galactosidase.

1 β-D-Galp-(1 → 4)-β-D-Galp-(1 → 4)-D-Glcol

2 β-D-Galp-(1 → 3)-β-D-Galp-(1 → 4)-D-Glcol

β-D-Gal
1
↓
5

3 β-D-Galp-(1 → 4)-D-Glcol

4 β-D-Galp-(1 → 6)-β-D-Galp-(1 → 4)-D-Glcol

β-D-Gal
1
↓
6

5 β-D-Galp-(1 → 4)-D-Glcol

β-D-Gal
1
↓
4

5 β-D-Galp-(1 → 1)-D-Glcol

shifted to the lower field (δ 73.5 and 81.9, respectively) by the glycosylation. The assignment of the C-1 signal was confirmed by the DEPT method. From these results, the structure of oligosaccharide P6 was determined as β-D-Gal*p*-(1 → 4)-[β-D-Gal*p*-(1 → 1)]-D-Glcol (**6**).

In this paper, we report the formation of six different oligosaccharides during lactitol hydrolysis with *A. oryzae* β-galactosidase. The structures are shown in Chart 1. All oligosaccharides were trisaccharides containing a lactitol unit; oligosaccharides P3, P4, and P6 have not been previously reported. Although oligosaccharides P1, P2, and P5 were previously obtained by reduction of galactosyllactose with sodium borohydride [1,7,8], their formation during lactitol hydrolysis with β-D-galactosidase had not been reported. Oligosaccharides containing β-(1 → 3)-, β-(1 → 4)-, or β-(1 → 6)-linked D-galactopyranosyl groups, formed by the transgalactosylation with β-D-galactosidase, have been obtained earlier [1,4,8], but oligosaccharides containing β-(1 → 5)- or β-(1 → 1)-linked groups, such as P3 and P6, have not been previously obtained. These oligosaccharides have a high potential for food and pharmaceutical applications.

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