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Primary structure of ten galactosides formed by transglycosylation during lactose hydrolysis by *Bifidobacterium bifidum**

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ABSTRACT

Oligosaccharides formed by a transgalactosylation reaction during lactose hydrolysis with *Bifidobacterium bifidum* were separated into eight fractions by gel-permeation chromatography and their structures studies determined by trimethylsilylation analysis, methylation analysis, f.a.b.–m.s., g.l.c.–m.s. and enzymic hydrolysis as β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-D-Glc, β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, β-D-Gal*p*-(1→6)[β-D-Gal*p*-(1→4)]-D-Glc, β-D-Gal*p*-(1→2)[β-D-Gal*p*-(1→6)]-D-Glc, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-DGal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, and β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3) β-D-Gal*p*-(1→4)-D-Glc.

INTRODUCTION

β-D-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is known to catalyze not only the hydrolysis of the β-D-galactoside linkage of lactose to give D-glucose and D-galactose but also to carry out transgalactosylation reactions[1–6], the D-galactosyl group of a β-D-galactoside being transferred onto a hydroxylated acceptor. Therefore, when lactose or another carbohydrate is present, it is possible to obtain new glycoside linkages between D-galactose and the acceptor, the number of the formed oligosaccharides varying[7,6] from 2 to 20 depending on the source of the enzyme. Until now, most of the oligosaccharide structures elucidated exhibit preferentially β-D-(1→6)-galactoside linkages[4,7–9]. We describe herein the separation of eight fractions of oligosaccharides formed by transgalactosylation and structure determination of ten compounds which shows a preferential transfer for the β-D-(1→3) position.

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EXPERIMENTAL

Thin-layer chromatography. — T.l.c. was performed on silica gel precoated plates (Kieselgel 60, Merck) with 2:1:1 (v/v) butanol–acetic acid–water as eluent[10]. Sugars were detected by staining with a solution of orcinol (2 g.L) in H_2SO_4–water 1:4 (v/v) and heating for 10 min at 105°.

Structure determination. — Molar carbohydrate composition of each fraction was determined after methanolysis with 0.5M methanolic HCl at 80° for 24 h by g.l.c. after *N*-reacetylation and per(trimethylsilyl)ation according to Kamerling *et al.*[11].

Permethylation was performed on oligosaccharide alditols (reduction with BD_4Na) according to the method of Ciucanu and Kerek[12]. The molecular weight of permethylated oligosaccharide alditols was determined by f.a.b.–m.s. with a Kratos MS 50 RF high-resolution mass spectrometer[13], operated at a 8 keV accelerating potential. An Ion Tech Model B11NF saddle-field, fast-atom source, energized with the B 50 current-regulated power supply, was used with Xe as the bombarding atom (operating conditions, 7.3 kV and 1.2 mA). Partially, *O*-methylated monosaccharides and alditols obtained after methanolysis of the permethylated oligosaccharide alditols were acetylated with 2:1 (v/v) pyridine–acetic anhydride for 15 h at room temperature, and then analyzed by g.l.c.–m.s. using a Girdel Model 30 gas-chromatograph equipped with a fused capillary glass column (0.3mm × 30m) coated with OV-101 (column temperature 120–180°, 2°.min^{-1}; 180–240°, 4°.min^{-1})[14]. Mass spectra were recorded with a Riber Mag 10-10 S mass spectrometer at an ionisation potential of 70 eV.

The anomeric configuration of the D-galactosyl group was determined by use of β-D-galactosidase purified from *Canavalia ensiformis* (Jack bean) and α-D-galactosidase from coffee bean (Boerhinger Mannheim). The enzyme preparations were tested with 4-nitrophenyl α-D-galactopyranoside, 4-nitrophenyl β-D-galactopyranoside, and melibiose as substrates. The enzymic hydrolysis was performed as follows: the oligosaccharide (100 μg) in 0.1M citrate phosphate buffer (pH 3.5 or 6.5) was treated with 0.01 unit of enzyme. After 15 h at room temperature, the reaction was stopped by heating for 3 min at 80°, and the hydrolysis products were analyzed by t.l.c.

Isolation of oligosaccharides. — A 24% lactose solution (84 mL) in 0.1M acetate buffer (pH 4.2) was incubated at 37° with a cell suspension (16 mL) equivalent to 7.5 units of β-D-galactosidase from *Bifidobacterium bifidum* DSM 20456 (1 unit of enzyme activity liberated 1 mmol of 4-nitrophenol .min^{-1}.mL^{-1} from 4-nitrophenyl β-D-galactopyranoside at pH 4.2 and 37°). After 72 h, the reaction was stopped by heating for 5 min in a boiling-water bath, and the mixture was centrifuged for 10 min at 10 000 *g*. The supernatant solution was concentrated in a rotatory evaporator and the resulting syrup was directly applied to a TSK HW 40 S (Fractogel, Merck) double-thermostated column (100 × 1.6 cm). Elution was carried out with water at a flow rate of 40 mL.h^{-1} at 45°.

RESULTS

Synthesis, fractionation, and structure of D-*galactosyl oligosaccharides.* — Transgalactosylation reactions gave a maximal yield when a high lactose concentration was used at the optimum pH of 4.2, which is different from the optimum pH for lactose hydrolysis, pH 6.5. During 72 h, *Bifidobacterium bifidum* cells produced four major transgalactosylation reaction products (Fig. 1, lane B); tri- and higher oligosaccharides represented more than 15% of the total sugar composition.

After removal of the enzyme by heating and centrifugation, five fractions were obtained by gel chromatography on TSK HW 40 S (Fig. 2). Two oligosaccharides (F.5, F.6) were isolated in one run. The other fractions shown in Fig. 1 were separated by repeated gel filtrations of the different oligosaccharide mixtures under the same conditions. These conditions and the small amounts of fractions F.2, F.4 and F.8 did not allow the preparation of pure oligosaccharides from these three fractions.

The structures of ten oligosaccharides could be determined. All presented the following characteristics: (a) they possess a D-galactopyranosyl group in a nonreducing terminal position, as demonstrated by the presence of methyl 2,3,4,6-tetra-*O*-methyl galactoside by methylation analysis (Table I). (b) The *β*-D configuration of the galactose residues shown by the total hydrolysis of each oligosaccharide fraction by jack bean *β*-D-galactosidase. *α*-D-Galactosidase did not cleave the neosynthesized oligosaccharides.

Fraction F.1. — This fraction was composed of a pure disaccharide, as indicated by molar carbohydrate composition and f.a.b.–m.s. of the reduced and permethylated oligosaccharide showing an ion at m/z 494 $(M + Na)^+$ (Table II). Methylation analysis

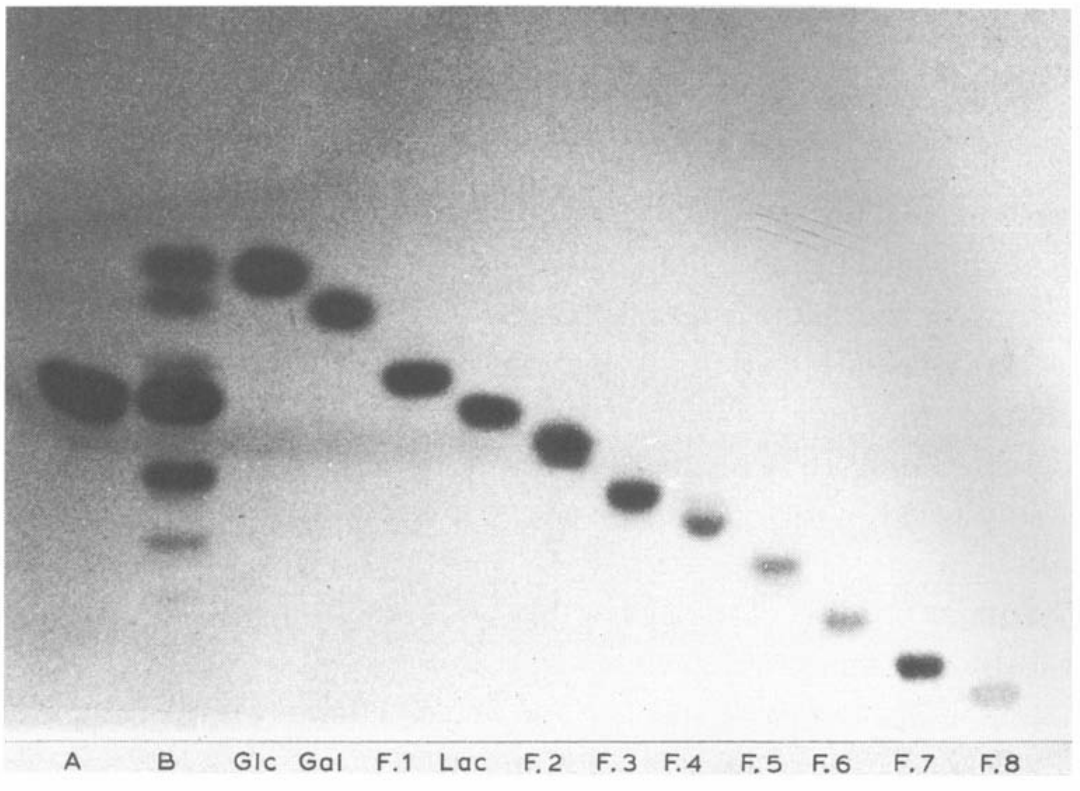

Fig. 1. T.l.c. of incubation mixture and oligosaccharide fractions. Incubation mixture: (A) Time 0; (B) time 72 h. Standarts: Glc, glucose; Gal, galactose; and Lac, lactose. F.1–F.8: oligosaccharide fractions.

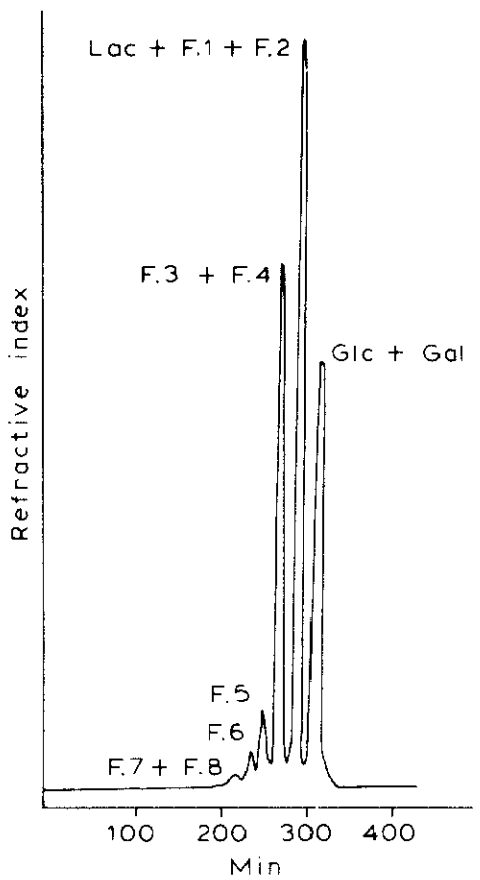

Fig. 2. Elution pattern of a gel-chromatograph on a double TSK HW 40 S column (1000 × 16 mm), thermostated at 45°, of a concentrated incubation-mixture at a flow rate of 40 mL.h^{-1}. Fractions (4.5 mL) were collected and the detection was carried out with a differential refractometer R.401 (Waters Associates): Glc, glucose; Gal, galactose; Lac, lactose; and F.1–F.8, oligosaccharide fractions.

(Table I) showing 1.0 residue of methyl 2,3,4,6-tetra-*O*-methylgalactoside and 1.07 residue of 1,2,4,5,6-penta-*O*-methylglucitol, and enzymic hydrolysis, established the structure as **1**.

Fraction F.2. — The two oligosaccharides forming this fraction were identified as disaccharides by f.a.b.–m.s. analysis which showed an ion at *m/z* 494 $(M + Na)^+$. The sugar composition gave a galactose-to-glucose ratio of 1.2:1 (Table II). This slight excess of galactose was confirmed by methylation analysis; the two methylated hexitols separated by g.l.c. showed the same mass spectrum corresponding to 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylhexitol in addition to methyl 2,3,4,6-tetra-*O*-methylgalactoside (Table I). The g.l.c.–m.s. analysis showed that 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylgalactitol represented only 10% of the total hexitol composition. This and the result of f.a.b.–m.s. analysis led to the conclusion that F.2 contains about 90% **2** and 10% of **3**.

Fraction F.3. The major product of transgalactosylation reaction was identified as a trisaccharide by an f.a.b.–m.s. analysis showing an ion at *m/z* 698 $(M + Na)^+$ and by molar ratio determination indicating two galactose and one glucose residues (Table II). The methylation analysis showed the presence, in addition to methyl 2,3,4,6-tetra-*O*-methylgalactoside, of methyl 3-*O*-acetyl-2,4,6-tri-*O*-methylgalactoside and 4-O-acetyl-1,2,3,5,6 penta-*O*-methylglucitol (Table I). On the basis of these results, enzymic hydrolysis, and f.a.b–m.s. analysis, the structure of F.3 oligosaccharide is **4.**

TABLE I

Relative retention times and carbohydrate molar composition of oligosaccharides obtained from lactose by transglycosylation with *Bifidobacterium bifidum*

Fraction	R_{lac}[a]	*Gal- to Glc-Molar ratio*	*Sugar in reducing position*	*Nature of oligosaccharides*[b]	*Ion at* m/z *(M + Na)*$^+$
F.1	1.10	1 : 1	Glc	Disaccharide	494
F.2	0.91	1.2 : 1	Glc + Gal (trace)	Disaccharide	494
F.3	0.75	1.9 : 1	Glc	Trisaccharide	698
F.4	0.67	2.2 : 1	Glc + Gal (trace)	Trisaccharide	698
F.5	0.55	2.9 : 1	Glc	Tetrasaccharide	902
F.6	0.41	3.9 : 1	Glc	Pentasaccharide	1106
F.7	0.28	4.8 : 1	Glc	Hexasaccharide	1310
F.8	0.20	5.35 : 1	Glc	Heptasaccharide	1514

[a] Mobility on t.l.c. in 2:1:1 (v/v) butanol–acetic acid–water (2:1:1 by vol.) relative to lactose. [b] Based on f.a.b.–m.s. analysis of reduced and permethylated oligosaccharides.

Fraction F.4. — This minor fraction of the transgalactosylation reaction was composed of different trisaccharides which were characterized by f.a.b.–m.s. analysis showing an ion at *m/z* 698 $(M + Na)^+$ (Table II) and by methylation analysis indicating that this fraction is a mixture of linear and branched trisaccharides, as shown by the presence of two tri-*O*-methylgalactosides, three penta-*O*-methylhexitols, and two tetra-*O*-methylhexitols (Table I). The structure of the branched trisaccharides were determined by mass spectrometry which showed the presence of 4,6-di-*O*-acetyl-1,2,3,5-

TABLE II

Molar ratios[a] of methyl *O*-acetyl-*O*-methylglycosides and *O*-acetyl-*O*-methylhexitols derived from the methanolyzate of permethylated, reduced oligosaccharides

Methyl ethers	*Fractions*							
	F.1	*F.2*	*F.3*	*F.4*	*F.5*	*F.6*	*F.7*	*F.8*
Methyl galactoside								
2,3,4,6-tetra	1	1	1	1	1	1	1	++
2,4,6-tri			0.92	0.44	1.7	2.65	3.99	++++
2,3,6-tri				0.16				
Galactitol								
1,2,3,4,5-penta		0.15		0.1				
Glucitol								
1,2,4,5,6-penta	1.07							
1,2,3,5,6-penta			0.83	0.12	0.74	0.87	0.72	+
1,2,3,4,5-penta		1.04		0.42				
1,3,4,5-tetra				0.15				
1,2,3,5-tetra				0.21				

[a] Relative to methyl 2,3,4,6-tetra-*O*-methylgalactoside.

tetra-*O*-methyl- and 2,6-di-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-glucitol. On the basis of these results and enzymic hydrolysis, structures **5** and **6** are proposed.

The low amount of linear trisaccharides of F.4 did not allow an exact determination of the structures.

Fraction F.5. — The molar ratio of galactose to glucose in this fraction was 2.9:1 and the partial f.a.b. mass spectrum of the permethylated, reduced oligosaccharide showed an ion at *m/z* 902 $(M + Na)^+$ (Table II), indicating that this fraction is constituted by a tetrasaccharide. G.l.c.–m.s. analysis showed exactly the same methylated methyl galactosides and hexitols as did Fraction F.3, but with an increase of the ratio of methyl 2,4,6-tri-*O*-methyl- to 2,3,4,6-tetra-*O*-methyl-galactoside (0.92:1 for F.3 and 1.7:1 for F.5) (Table I). These results and that of enzymic hydrolysis indicated structure **7**.

Fraction F.6. — This fraction consisted of a pentasaccharide as indicated by the ratio of galactose to glucose of 3.9:1 and the f.a.b.–m.s. analysis showing an ion at *m/z* 1106 $(M + Na)^+$ (Table I). The g.l.c.–m.s. analysis showed the same methyl *O*-methylglycosides and hexitol as observed for Fractions F.3 and F.5. The molar ratio between the internal hexosyl residue (methyl 2,4,6-tri-*O*-methylgalactoside) and the terminal group (methyl 2,3,4,6-tetra-*O*-methylgalactoside) was 2.65:1 (Table I), indicating the formation of β-D-(1→3) linkages. These results and that of enzymic hydrolysis suggested structure **8**.

Fraction F.7. — F.a.b.–m.s. analysis of the reduced and permethylated oligosaccharide of Fraction F.7 indicating an ion at *m/z* 1310 $(M + Na)^+$, a galactose-to-glucose molar ratio of 4.8:1 (Table II), and the methylation analysis ratio of methyl 2,3,4,6-tetra-*O*-methylgalactoside to methyl 2,3,6-tri-*O*-methylgalactoside to 1,2,3,5,6-penta-*O*-methylhexitol of 1:4:1 (Table I) indicated the presence of a hexasaccharide. These results and that of enzymic hydrolysis established the structure of the linear hexasaccharide as **9**.

Fraction F.8. — The small amount of this fraction did not allow further purification. T.l.c. (Fig. 1) and the molar ratio of galactose-to-glucose of 5.35:1 (Table I) showed that Fraction F.8 did not contain a pure oligosaccharide, but that it was contaminated by Fraction F.7. F.a.b.–m.s. analysis showed that the major component of this fraction was a heptasaccharide giving an ion at *m/z* 1514 $(M + Na)^+$ (Table II).

The determination of the molar ratio of methyl *O*-methylglycosides and *O*-methylhexitols also indicated F.7 contamination, but g.l.c.–m.s. analysis of the methylated sugars showed the same three methyl *O*-methylglycosides and *O*-methylhexitol as shown by Fractions F.3, F.5, F.6, and F.7, *i.e.*, methyl 2,3,4,6-tetra-*O*-methyl- and 2,4,6-tri-*O*-methyl-galactoside, and 1,2,3,5,6-penta-*O*-methylglucitol (Table I). On the basis of these results and that of enzymic hydrolysis, the structure of the heptasaccharide was established as **10**.

DISCUSSION

Bifidobacterium bifidum exhibits transgalactosylation properties during incubation with lactose. Eight oligosaccharide fractions were separated by gel-filtration on TSK HW 40 S and ten structures established.

Until now, the β-D-(1→6) linkage has been reported preferentially formed during transgalactosylation reactions[9], the β-D-(1→3) linkage being formed only in lactose hydrolysis by *Aspergillus oryzae*[6] in addition to the major β-D-(1→6) linkage. Recently, the minor formation of β-D-galactopyranosyl-(1→3)-D-glucose was also observed as a result of the reversed hydrolysis activity of β-D-galactosidase[15]. In the present study, we show that the β-D-galactosyl-(1→3) linkage is the most important oligosaccharide

β-D-Gal*p*-(1→3)-D-Glc
1

β-D-Gal*p*-(1→6)-D-Glc
2

β-D-Gal*p*-(1→6)-D-Gal
3

β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
4

β-D-Gal*p*-(1→6)-D-Glc
4
↑
1
β-D-Gal*p*
5

β-D-Gal*p*-(1→6)-D-Glc
2
↑
1
β-D-Gal*p*
6

β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
7

β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
8

β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-Glc
9

β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
10

β-D-Gal*p*-(1→3)-[β-D-Galp(1→3)]$_{n=0-4}$-β-D-Gal*p*(1→4)-D-Glc
11

linkage formed during the transgalactosylation reaction catalyzed by *Bifidobacterium bifidum*. Except for two branched trisaccharides detected in minor amounts in Fraction F.4, all of the trisaccharides and higher oligosaccharides obtained are linear and contain a β-D-galactosyl-(1→3)-group.

The action of *Bifidobacterium bifidum* on lactose gave a series of linear oligosaccharides having the general structure **11**. Compound **4** (3′-galactosyllactose) was isolated previously from human urine[16], bovine colostrum[17], and human milk[18].

A series of galactosides identical with **11** has already been found in marsupial milk[19,20]. The eventual function of these compounds would be to decrease the osmotic pressure in the intestinal lumen in relation to the absence of external lactase on the enterocyte membranes and thus to prevent diarrhea[21]. *Bifidobacterium bifidum* is an enterobacterium, described for the first time by Tissier[22], that can only grow in the intestine of infant fed with breast milk. In general, the establishment of this bacterium in the intestinal mucosa is a mark of good health (decrease of infant mortality and morbidity). It is possible that the compounds produced by *Bifidobacterium bifidum* are involved in the adherence of bacteria in the large intestine, and studies are presently being undertaken to correlate the production of neosynthesized oligosaccharides with possible biological effects.

In addition to the formation of β-(1→3), β-(1→6), and β-(1→4)-D-galactosyl linkages (the last two being the usual transgalactosylation reaction products), the formation of branched oligosaccharides appears to be an artefact of the transgalactosylation reaction. Recent results (not shown) indicate that *Bifidobacterium bifidum* could produce preferentially β-D-(1→3) linkages under acidic condition (pH 4.2–4.5) and β-D-(1→6) linkages at the optimum pH of lactose hydrolysis (pH 6). Only the purification of the enzymic system will ascertain whether β-D-(1→3), -(1→4), and -(1→6) linkages are formed by the same enzyme.

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