Synthesis of β -D-Fucosylglucose by β -D-Glucosidase I of *Bifidobacterium breve* clb and Assimilation by Bifidobacteria

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The *p*-nitrophenol-releasing activity, from *p*-nitrophenyl (*p*-NP) β -D-fucoside, of β -D-glucosidase I from *Bifidobacterium breve* clb was enhanced by the addition of many kinds of sugars and alcohols, suggesting the occurrence of a β -D-fucosyl transferring reaction. The enhancement on glucose addition was dependent on the reaction pH, and the concentrations of *p*-NP β -D-fucoside and glucose, and the activity reached 430 % when 100 mM glucose was added to the mixture containing 20 mM *p*-NP β -D-fucoside at pH 4.5. A mixture of transfer products was separated from the other constituents by activated charcoal column chromatography. Further purification of the mixture by paper and thin-layer chromatographies gave four oligosaccharides, which were identified as β -D-fucosylglucoses with β 1 $\rightarrow$ 2, β 1 $\rightarrow$ 3, β 1 $\rightarrow$ 4 and β 1 $\rightarrow$ 6, linkages from the absorption spectra obtained with the phenol-sulfuric acid method, and the results of acid and enzymatic hydrolyses, and methylation analyses. The mixture of oligosaccharides was well assimilated by many bifidobacteria but not by any other intestinal bacteria tested.

Previous investigations on "bifidus factors" revealed that the most important factor for the active proliferation of bifidobacteria in the lower intestine of a host is the carbon source.^{1~3)} Therefore, attempts were made to establish a "bifidus flora" in the intestinal tract by introducing oligosaccharides which are preferentially assimilated by bifidobacteria.^{4,5)} We have studied the glycosidases of bifidobacteria^{6~9)} and demonstrated that β -D-fucoside-hydrolyzing activity was widespread in bifidobacteria, in contrast with in other bacteria, even though the reactions were catalyzed by different enzyme species,⁶⁾ and that the β -D-fucoside-hydrolyzing reaction of β -D-glucosidase I from *Bifidobacterium breve* 203 was enhanced on the addition of some sugars, suggesting a β -D-fucoside transferring reaction.⁷⁾ These results indicated that β -D-fucosyl

derivatives synthesized by *B. breve* β -D-glucosidase I might be specifically assimilated by bifidobacteria.

This paper deals with the characteristics of the β -D-fucosyl transferring reaction of β -D-glucosidase I from *B. breve* clb, which is a strain derived from *B. breve* 203 showing high β -D-glucosidase I productivity,⁹⁾ and the synthesis of β -D-fucosylglucoses by the enzyme as well as the assimilation of the products by bifidobacteria.

Materials and Methods

Enzyme preparation. β -D-Glucosidase I was prepared from glucose-grown cells of *B. breve* clb, which was obtained from *B. breve* 203 through acclimation to cellobiose and which showed 25-fold hydrolyzing activity toward *p*-nitrophenyl (*p*-NP) β -D-fucoside, as compared with the parent strain.⁹⁾ The partially purified preparation

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obtained on DEAE-cellulofine column chromatography and ammonium sulfate fractionation (50~60%) from the cell-free extract showed specific activity of 3.92 units/mg protein and 74% reactivity toward *p*-NP β -D-fucoside compared to that toward *p*-NP β -D-glucoside, and it was free from β -D-glucosidase II and β -D-galactosidase. This preparation showed similar properties to β -D-Glucosidase I from *B. breve* 203, *i.e.*, substrate specificity, optimum pH for the reaction, effects of sugars and so on.⁷⁾ One unit of enzyme activity was defined as the amount which released 1 μ mol of *p*-NP from *p*-NP β -D-glucoside per min at pH 6.0 and 37°C.

Analytical methods. Sugar concentrations were routinely determined by the phenol-sulfuric acid method with glucose as a standard.¹⁰⁾ In the case of disaccharides composed of D-fucose and glucose, the concentrations were calculated as follows by measuring the absorbance at 485 nm (ΔE_{485}) and 420 nm (ΔE_{420}) after colorization by the phenol-sulfuric acid method. The equation used was based on their specific absorption spectra and their molar absorption coefficients at each wavelength.

$$\text{Glucose (mm)} = 0.86\Delta E_{485} - 1.20\Delta E_{420}$$

$$\text{Fucose (mm)} = 2.98\Delta E_{420} - 0.81\Delta E_{485}$$

Glucose concentrations were determined with Glucose C-Test Wako (Wako Chemicals). Thin-layer chromatography (TLC) was performed on silica gel plates (Merck, No. 5553) with various solvents. High-performance liquid chromatography (HPLC) was carried out as described previously with slight modification (solvent system: acetonitrile-water-methanol=85:13:2, v/v).⁸⁾ Acid hydrolysis of oligosaccharides was carried out with 3N trifluoroacetic acid (100°C, 4 hr). Enzymatic hydrolyses of oligosaccharides with β -D-glucosidase I, β -D-glucosidase II and α -D-glucosidase of *B. breve* 203, and β -D-galactosidase of *B. longum* 401 were carried out in a mixture (40 μ l) containing 20 μ g of sugar, 8 munits of enzyme and 50 μ mol of potassium phosphate buffer pH 6.0) at 37°C for 2 hr. One unit of enzyme activity was defined as the amount which released 1 μ mol of *p*-NP from each *p*-NP glycoside at 37°C and pH 6.0.

Methylation analysis of oligosaccharides. Hydroxyl groups in the oligosaccharides were analyzed through identification of the partial *O*-methylated alditol acetate derivatives (methylation analysis).¹¹⁾ Gas chromatography (GC) of the derivatives was carried out with a Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector. The temperature of the column (1.5 $\times$ 3 mm) packed with 1% OV-101 on Chromosorb W AW DMCS (80~100 mesh) was maintained at 160°C for 16 min and then raised to 220°C at the rate of 8°C/min. The derivatives were also analyzed with a gas chromatograph-mass spectrometer model M-80 (Hitachi) on a glass column (3 mm $\times$ 1 m) of OV-101. The temperature was programmed from 150°C to 230°C at the rate of 5°C/min.

Sugar assimilation test. Testing of the assimilation of the synthesized sugars by bifidobacteria and other bacteria was carried out with the media described by Yazawa *et al.*¹²⁾ The medium used for lactobacilli was that described by Minami *et al.*¹³⁾ An oligosaccharide solution was filter-sterilized and then added aseptically to the media. Bifidobacteria were cultured under an atmosphere of CO₂, and the other bacteria under air at 37°C for 2 days. Bacterial growth was determined from the turbidity at 610 nm.

Results

β -D-Fucoside-transferring reaction of β -D-glucosidase I with glucose as an acceptor

As described previously, the addition of sugar derivatives containing glucose enhanced *p*-NP liberation from *p*-NP β -D-fucoside by β -D-glucosidase I from *B. breve* clb.⁹⁾ In the reaction mixture with glucose, new products

Fig. 1. Effect of pH on *p*-NP Liberating Activity, from *p*-NP β -D-Fucoside, of β -D-Glucosidase I in the Presence of Glucose.

(A): Mixtures containing 2 mM *p*-NP β -D-fucoside, 40 munits/ml of β -D-glucosidase I and 50 mM various buffers were incubated with (—○—) or without 20 mM glucose (—●—) at 37°C for 5 min. *p*-NP-Liberating activities are expressed relative to that in the case of a mixture containing potassium phosphate buffer (pH 6.0) without glucose. (B): The ratio of the activity with glucose to that without glucose at each pH shown in (A) is presented as a percentage.

Fig. 2. Effect of the Substrate Concentration on β -D-Fucoside-transferring Activity of β -D-Glucosidase I.

The *p*-NP-liberating activity with various concentrations of glucose is expressed relative to that without glucose in mixtures containing 20 m units/ml β -D-glucosidase I, 100 mM citrate-NaOH buffer (pH 4.5) and 2~20 mM *p*-NP β -D-fucoside.

were detected on TLC, which suggests that the transfer of β -D-fucosyl groups to glucose occurred and that the transferring reaction caused an increase in *p*-NP liberation.

As shown in Fig. 1(A), the optimum pH of the *p*-NP liberating reaction, from *p*-NP β -D-fucoside, shifted from 6.0 to 5.0 on the addition of 20 mM glucose. The enhancement on glucose addition was higher in the acidic pH range, and the relative activity with glucose to that without glucose was 180% at pH 4.5 and 210% at pH 4.0 (Fig. 1(B)). No enhancement was observed at above pH 8.0. The enhancement also depended on the concentrations of *p*-NP β -D-fucoside and glucose (Fig. 2), and the relative activity with 100 mM glucose to that without glucose reached 430% in the presence of 20 mM *p*-NP β -D-fucoside (50 mM citrate NaOH buffer, pH 4.5).

Isolation of oligosaccharides

The reaction mixture (338 ml), consisting of 20 mM *p*-NP β -D-fucoside, 100 mM glucose, 100 mM potassium phosphate buffer (pH 6.0) and 13.5 units of β -D-glucosidase I, was in-

Fig. 3. Activated Charcoal Column Chromatography of the Reaction Mixture.

The fractions eluted with 15% ethanol (Nos. 210~280) were combined and then concentrated by evaporation (Fraction III).

cubated at 37°C for 4 hr. After boiling for 5 min, the mixture was applied to an activated charcoal column (40×6 cm), which was then developed with an increasing ethanol concentration gradient. As shown in Fig. 3, glucose and D-fucose were eluted with water and 5% ethanol (Fractions I and II), respectively. Four components were eluted with 15% ethanol (Fraction III), which were colored grey with diphenylamine reagent¹⁴⁾ on TLC, in contrast with glucose (blue) and D-fucose (green). They were broadly eluted on HPLC at 10.6~18.6 min, while standard D-fucose and glucose at 6.7 and 9.0, respectively. Analysis with glucose oxidase showed that Fraction III was contaminated by a small amount of glucose (less than 3.9%). *p*-NP and *p*-NP β -D-fucoside were not eluted, even with 50% ethanol.

Then a portion of Fraction III was further purified by paper chromatography with an upper layer of acetic acid-buthanol-water (4:1:5, v/v) and TLC with acetone-chloroform-methanol-water (75:10:10:5, v/v). The isolated compounds, A, B, C and D, gave single spots on TLC with *R_f* values of 1.19, 0.93, 0.67 and 0.45, respectively, the ratio being

about 4:2:3:1.

Identification of the products

After treatment with trifluoroacetic acid, all the isolated compounds gave two spots on TLC, which corresponded to those of glucose and D-fucose. They were also hydrolyzed by β -D-glucosidase I of *B. breve* 203 and β -D-galactosidase of *B. longum* 401, but not by β -D-glucosidase II or α -D-glucosidase of *B. breve* 203. When colored by the phenol-sulfuric acid method, they showed identical spectra to that of a mixture of glucose and D-fucose (1:1):

they showed maximum absorption at 485 nm and a small peak at 420 nm. From the absorbance at the two wavelengths, the molar ratio of glucose and D-fucose in all products was calculated to be 1:1. On methylation analysis (Table I), the 1-hydroxyl groups of all the fucoses, and the 3-, 4-, 2- and 6-hydroxyl groups of the glucose moiety of products A, B, C and D were found to be involved in the linkages, respectively. These results indicated that the products are D-fucopyranosyl (β 1 $\rightarrow$ 3) glucopyranoside, D-fucopyranosyl (β 1 $\rightarrow$ 4) glucopyranoside, D-fucopyranosyl

Table I. RETENTION TIMES OF PARTIAL O-METHYLATED ALDITOL ACETATES ON A 1% OV-101 COLUMN

Alditol derivatives	Rt ^a	RRt ^a	Products
2,3,4-Tri-O-methyl-(1,5-di-O-acetyl) fucitol	3.11	0.65	A,B,C,D
2,3,4,6-Tetra-O-methyl-(1,5-di-O-acetyl) glucitol	4.77	1.00	—
3,4,6-Tri-O-methyl-(1,2,5-tri-O-acetyl) glucitol	6.79	1.42	C
2,4,6-Tri-O-methyl-(1,3,5-tri-O-acetyl) glucitol	6.89	1.44	A
2,3,6-Tri-O-methyl-(1,4,5-tri-O-acetyl) glucitol	7.26	1.52	B
2,3,4-Tri-O-methyl-(1,5,6-tri-O-acetyl) glucitol	7.85	1.65	D

^a The relative retention times (RRt) were calculated on the basis of the retention time (Rt) of 2,3,4,6-tetra-O-methyl-(1,5-di-O-acetyl) glucitol.

Table II. ASSIMILATION OF β -D-FUCOSYLGLUCOSES BY INTESTINAL INHIBITANTS

Strain	Growth (O.D. 610 nm) on			Assimilation
	Glucose (0.5%)	Glucose (0.02%)	Fraction III (0.5%)	
<i>B. adolescentis</i> 304	1.11	0.09	0.85	+
<i>B. bifidum</i> 101	0.41	0.05	0.31	+
<i>B. bifidum</i> 201	0.45	0.05	0.28	+
<i>B. bifidum</i> 301	0.47	0.05	0.49	+
<i>B. breve</i> 108	0.29	0.05	0.19	+
<i>B. breve</i> 203	0.89	0.10	0.86	+
<i>B. breve</i> 204	0.26	0.05	0.26	+
<i>B. infantis</i> 105	0.93	0.05	0.08	—
<i>B. longum</i> 305	0.83	0.07	0.07	—
<i>B. longum</i> 401	0.24	0.06	0.05	—
<i>E. coli</i> IFO 3301	0.23	0.01	0.01	—
<i>E. coli</i> IFO 3806	0.42	0.09	0.09	—
<i>Pr. mirabilis</i> IFO 3045	0.51	0.27	0.28	—
<i>L. acidophilus</i> IFO 3205	1.41	0.76	0.76	—
<i>L. casei</i> IFO 3425	2.70	0.30	0.30	—
<i>L. fermenti</i> IFO 3071	2.70	0.30	0.30	—
<i>L. pluntarum</i> IFO 3070	2.78	0.10	0.10	—
<i>Str. lactis</i> IFO 12546	2.23	0.27	0.24	—
<i>Str. faecalis</i> IFO 3181	0.43	0.14	0.14	—
<i>Staph. aureus</i> IFO 3183	0.43	0.09	0.08	—

(β 1 $\rightarrow$ 2) glucopyranoside and D-fucopyranosyl (β 1 $\rightarrow$ 6) glucopyranoside, respectively.

Assimilation of β -D-fucosylglucoses by bifidobacteria

As shown in Table II, 7 strains of *B. breve*, *B. bifidum* and *B. adolescentis* could grow on Fraction III as a carbon source, but *B. longum*, *B. infantis* and other intestinal inhabitants tested could not. All strains scarcely grew on 0.02% glucose, which corresponded to the amount of glucose contaminating Fraction III.

Discussion

β -D-Glucosidase I showed the unique ability of the transfer the β -D-fucosyl moiety to many sugar and alcohol derivatives.⁷⁾ The reaction rate might be higher than that of hydrolysis because the amount of *p*-NP released increased on the addition of glucose. The increase in *p*-NP release depended on the pH (Fig. 1), and the concentrations of the substrate and glucose (Fig. 2).

Four oligosaccharides were isolated from the reaction mixture with glucose. They were identified as β -D-fucosylglucoses with β 1 $\rightarrow$ 2, β 1 $\rightarrow$ 3, β 1 $\rightarrow$ 4 and β 1 $\rightarrow$ 6, linkages, from the absorption spectra obtained with the phenol-sulfuric acid method, and the results of TLC analyses of the acid and enzymatical hydrolyzates as well as methylation analyses. The amounts of the isomers accumulated varied (β 1 $\rightarrow$ 3: β 1 $\rightarrow$ 2: β 1 $\rightarrow$ 4: β 1 $\rightarrow$ 6=4:3:2:1), the ratio being similar to that of the activities of β -D-glucosidase I toward various β -D-glucosyl disaccharides⁷⁾: it reacted with laminaribiose (β 1 $\rightarrow$ 3), cellobiose (β 1 $\rightarrow$ 4) and gentiobiose (β 1 $\rightarrow$ 6), in the ratio of 5:3:1. The yield of the β -D-fucosyl group in the product mixture was about 50%, based on the substrate *p*-NP β -D-fucoside, at pH 6.0, after 4 hr reaction with 20 mM *p*-NP β -D-fucoside and 100 mM glucose. Further work to optimize the reaction time, temperature, enzyme concentration and solvent system might lead to a more efficient transferring-reaction.¹⁵⁾

Though some β -D-glucosidases have been

reported to catalyze the β -D-glucosyl-transferring reaction to alcohols or glucose from arylglucose,^{16~18)} their β -D-fucosyl-transferring reaction has not been elucidated. The oligosaccharide formation from lactose by β -D-galactosidase has been well documented: the proportion of the transfer to the hydrolysis varies with the reaction time,¹⁹⁾ lactose concentration,²⁰⁾ and enzyme source²¹⁾ and some β -D-galactosidases accumulated a disaccharide,²²⁾ some several disaccharides²³⁾ and others many oligomers.²⁴⁾

Some oligosaccharides were enzymatically synthesized which are assimilable by bifidobacteria exhibiting comparably high selectivity: galsucrose and lactosucrose were prepared with levan-sucrase,¹³⁾ transgalactosylated oligosaccharides with β -D-galactosidase²⁵⁾ and fructooligosaccharides with invertase.⁵⁾ The mixture of β -D-fucosylglucoses in this study showed excellent selectivity: β -D-fucosylglucoses could be assimilated by many bifidobacteria but not other intestinal inhabitants, including *Escherichia coli*, *Streptococcus faecalis* and some lactobacilli. Though further investigations are necessary on the metabolism of individual isomers by bifidobacteria, and on the assimilation by other intestinal inhabitants such as *Bacteroides*, *Clostridia* and so on, the results suggest the possibility of synthesizing new "bifidus factors" by using glycosidase specifically found in bifidobacteria.

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