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Growth and β-Glucosidase Activity of *Bifidobacterium*

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β-Glucosidase was known to be involved in the mutagenic activation of β-glucosides. The level of β-glucosidase in the feces of adults was 2.7 times higher than that of infants. There was no difference in the percentage of β-glucosidase positive strains among *Bifidobacterium* isolates between adults and infants, corresponding to 90 and 92%, respectively. However, the strains from adults showed 1.9 times higher enzyme activity than those from infants when grown in Brain Heart Infusion medium. β-Glucosidase negative strains could not ferment β-glucosidase substrates, such as cellobiose, salicin, naringin, esculin and arbutin. Presence of β-glucosidase in *Bifidobacterium* did not alter the degree of growth in reconstituted skim milk. The β-glucosidase level was much lower in milk and vegetable medium, although cells grew above 10^8 cfu/ml, than in BHI medium. This study suggests that metabolic activation of the β-glucosides by *Bifidobacterium* β-glucosidase varies significantly depending on types of growth medium.

Many factors influence the composition of normal intestinal microflora. Changes in diet and changes in age can result in changes in the microbial composition and the bacterial enzyme level of the colonic tract. Of particular interest have been the enzymes that are considered responsible for the activation of mutagens; glucuronidases, azoreductases, nitroreductases, and glucosidases (4, 5). β-Glucosidase (β-D-glucoside glucohydrolase, E 3.2.1.21) is classified as a hydrolase which is involved in the hydrolysis of a variety of glycosides as well as cellobiose. Carcinogenic or mutagenic aglycones can be released by the action of fecal glycosidases from various glycosides such as rutin, guercitrin, robinin, cycasin, amygdalin, franguloside, 8-hydroxyquinoline-β-D-glucoside and neocycasin A (6, 14). Maron and Ames (9) used fecalase, an enzyme preparation from human feces containing β-glucosidase for mutagenecity tests of glucosides. In the human large intestine, bacterial cell-bound β-glucosidase is present in high levels (8) and the major bacterial flora responsible for β-glucosidase belong to *Bacteroides, Bifidobacterium, Streptococcus* and *Fusobacterium*. Bifidobacteria are part of the predominant flora and beneficial in the human large intestine. It was suggested that bifidobacteria show higher β-glucosidase activities than other intestinal bacteira (3, 15). The partially purified enzyme from *Bifidobacterium adolescentis* Int-57 was confirmed to convert cycasin to a mutagen in the Ames and SOS chromotests (1). Presently it is suggested that β-glucosidase negative strains should be preferentially used for commercial production of fermented foods utilizing bifidobacteria.

This study was carried out to investigate the level of β-glucosidase of *Bifidobacterium* strains in various growth environments.

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MATERIALS AND METHODS

Strains and Reagents

Various bacteria were isolated from human feces. Feces were obtained from adult volunteers or children with mother's consent. The feces were immediately transported to the lab in an anaerobic dilution medium (10) at 4°C. After 10-fold serial dilutions of 1 g feces, the samples were plated on the appropriate medium and incubated in Gas-Pak jars (BBL) at 37°C for 2.5 days.

The isolation of *Bifidobacterium* was performed on differential BL medium (10) or selective TP (6) medium as described previously. Brown colonies on BL medium or milky white colonies on TP medium belonged to *Bifidobacterium*. Gram staining, F6PPK (fructose-6-phosphate phosphoketolase) test, morphology and anaerobic growth were used to confirm *Bifidobacterium*. Selective isolation of *Bacteroids, Eubacterium, Streptococcus* and

Lactobacillus was performed according to the methods of Mitsuoka (10). Type strains of various bacteria used in this study were purchased from ATCC (American Type Culture Collection). For the fermentation test, 0.5 ml of 10% substrate solution, which was membrane-filtered through a 0.45 μm Acrodisc filter, was added to 9.5 ml of modified MRS medium. After 2.5-days incubation in a Gas-Pak, the tube below pH 5.5 was judged to be fermentation positive. Morphological and physiological characteristics were analysed according to Bergey's Manual of Systematic Bacteriology (13). BHI (brain heart infusion), tryptone, peptone, yeast extract, beef extract and casamino acid were purchased from Difco Co. and all other chemicals were obtained from Sigma Chemical Co.

Cell Growth

Cell growth of *Bifidobacterium* and various bacterial strains were carried out in the media of BHI, skim milk, fruits and vegetables. Incubations were carried out statically at 37°C for 36 h. For the growth of cells in reconstituted skim milk medium, commercial skim milk powder was dissolved at a concentration of 10% in water and treated at 110°C for 10 min. Fruit and vegetable media were also used for cultivation of *Bifidobacterium*. Mashed and ground fruits or vegetable were centrifuged. The supernatant fraction was pH-adjusted at pH 7.0 and flushed with N_2 gas. This was heated for 30 min at 90°C, inoculated with $1\text{-}5\times10^6$ cfu/ml *Bifidobacterium* cells and incubated at 37°C.

β-Glucosidase Assay

Fresh feces (1 g) obtained from 14 adults and 11 infants were suspended in 0.1 M sodium phosphate buffer (pH 6.0) to produce 10% (w/v) slurries. This suspension was centrifuged at 5,000×g for 10 min at 4°C to yield a crude particulate fraction and a supernatant fraction. Cell culture broth grown in various media was also centrifuged in the same manner. The pellet from feces or culture broth contained most of the β-glucosidase activity. The pellet was resuspended in 10 ml 0.1 M sodium phosphate buffer and washed twice. The resultant pellet fraction was then sonicated (XL2020 sonicator, New York). β-Glucosidase activity was assayed at 45°C in a mixture containing 0.5 mM p-nitrophenol-β-glucoside (PNPG), 10 mM sodium phosphate buffer (pH 6.0) and the enzyme solution. This reaction was terminated by adding 0.5 M sodium carbonate solution. p-Nitrophenol (PNP) released was quantified at 400 nm using PNP as a standard. One unit of enzyme activity was defined as the amount which released 1 μmol of PNP per min.

RESULTS AND DISCUSSION

β-Glucosidase Activity of the Human Fecal Bacteria

Various intestinal bacteria were isolated in this lab or obtained from ATCC or other commercial suppliers. These strains were tested for β-glucosidase production (Table 1). When examined on BHI-medium, the levels of β-glucosidase activity of *Bifidobacterium* strains were generally higher as compared to *Bacteroides, Eubacterium, E. coli, Lactobacillus, Streptococcus* which are also resident flora of the intetinal tract. Tochikura *et al.* (15) also reported that Bifidobacteria showed higher β-glucosidase activity than *Staphylococcus, Escherichia coli, Proteus,*

Table 1. β-Glucosidase activities of the various bacteria obtained from the ATCC, feces and commercial yoghurts.

Microorganisms	Sources	Number of strains tested	Number of β-glucosidase producing strains	β-Glucosidase activity (mU/ml culture)
Bifidobacterium adolescentis	ATCC 15703	1	1	11.4
Bifidobacterium animalis	ATCC 25527	1	1	2.48
Bifidobacterium bifidum	ATCC 29521	1	1	3.57
Bifidobacterium infantis	ATCC 15697	1	1	9.74
Bifidobacterium longum	ATCC 15707	1	1	3.91
Bifidobacterium thermophilum	ATCC 25525	1	1	10.24
Bifidobacterium adolescentis	Int 57	1	1	12.73
Bifidobacterium strains	commercial yoghurts	11	11	1.88±1.11*
Bifidobacterium strains	adult feces	49	44	10.98±4.85
Bifidobacterium strains	infant feces	63	58	5.77±2.70
Bacteroids strains	adult feces	8	8	1.83±1.26
Lactobacillus strains	commercial yoghurts	18	2	3.52
Lactobacillus strains	adult feces	6	1	8.74
Lactobacillus acidophilus	ATCC 32820	1	0	
Streptococcus strains	adult feces	3	2	0.63
Clostridium butyricum	ATCC 19398	1	0	
Eubacterium limosum	ATCC 8486	1	0	

Bacterial cultures were grown in the BHI medium for 12 to 18 h. Bacterial cells were harvested and resuspended in the assay buffer. The cells were disrupted with a sonicator and assayed for β-glucosidase activity. *When the number of strains tested were greater than three, the S.D. values were shown.

Streptococcus and *Lactobacillus* tested in their study.

All of the commercial *Bifidobacterium* strains tested exhibited β-glucosidase activity. Among the 49 *Bifidobacterium* isolates from adults, 44 strains showed β-glucosidase while 58 out of 63 strains from infants showed activity. Thus the frequency of β-glucosidase positive strains was not significantly different between adult and infant isolates and the majority of *Bifidobacterium* in human intestine were able to produce β-glucosidase.

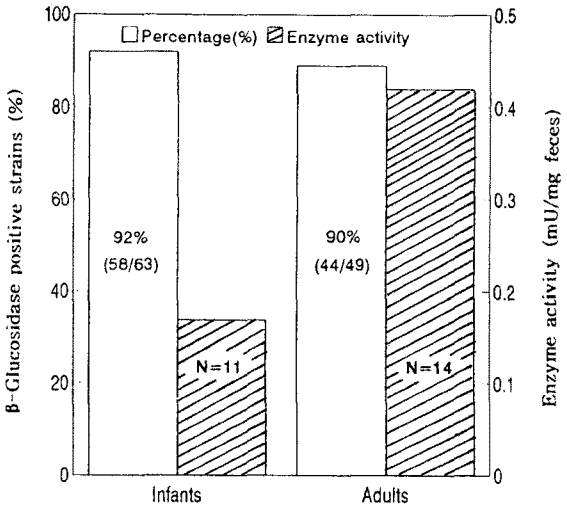

Fig. 1. Percentage of β-glucosidase positive strains among isolated *Bifidobacterium* and enzyme activity of the feces from 11 infants and 14 adults.

When the average value of the β-glucosidase activity between the 44 strains from adults and 58 strains from infants grown in BHI medium were compared, adult-originated strains showed 1.9-fold higher activity (Table 1). However it is not yet known whether relatively higher production of β-glucosidase of the adult *Bifidobacterium* strains observed in vitro can be also observed in human intestine.

β-Glucosidase levels in the 14 adult feces tested were 2.7-fold higher than that from the 11 infants (Fig. 1). Since β-glucosidase is produced by several kinds of bacteria in the large intestine, the higher level of β-glucosidase in adults cannot yet be attributed solely to the presence of strong β-glucosidase *Bifidobacterium* strains in the adult large intestine. Also the kinetics of enzyme production in the large intestine may be different from that in the *in vitro* culture media due to differences in the growth rate and environmental conditions. Since many glycosides produced by human intestinal bacteria are inducible (8, 12), their activities probably reflect the relative availabilities of β-glucosidase inducer substrates in infants and adults.

Fermentation Pattern of β-Glucosidase Negative *Bifidobacterium*

β-Glucosidase negative strains are favored for the production of fermented foods because β-glucosidase is involved in the production of carcinogenic compounds (1). β-Glucosidase negative strains isolated and tested in this study belonged to *B. longum, B. bifidum* and *B. infantis* according to their fermentation patterns. These strains were not able to ferment β-glucosidase substrates: salicin, cellobiose, naringin, esculin and arbutin (Table 2). A β-

Table 2. Carbohydrate utilization of β-glucosidase positive and negative *Bifidobacterium* strains.

Substrates	β-Glucosidase negative strains				Mutant	β-Glucosidase positive strains				
	BGN1	BGN2	BGN3	BGN4	M6	INT57	A5	A6	A7	A8
Arabinose	+	+	+	−	−	+	+	+	+	
Arbutin	−	−	−	−	−	+	−	+	+	−
Cellobiose	−	−	−	−	−	+		+	+	+
Esculin	−	−	−	−	−	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	−	+	+	+	+		+
Maltose	+	+	+	−	−	+	+	+	+	+
Melezitose		+	+	−	−	+	+		−	+
Melebiose	+	+	+	+	−	+	+	+	+	+
Naringin	−	−	−	−	−	+	+	−	+	+
Raffinose	+	+	+	+	−	+	+	+	+	+
Ribose	+	+	+	−		+	+	+		−
Salicin	−	−	−	−	−	+	−	+	+	+
Sucrose	+	+	+	−	+	+		+	+	+
Starch	−	−	−	−		+	−	−	+	+
Trehalose	−	−	−	−		−	−	−		+
Xylose	+	+	+	−		+	+		−	−

−, No growth; +, Moderate growth ($O.D._{600}$ above 0.6 and pH below 4.5).

glucosidase negative mutant strain of *B. adolescentis* Int57 also lost the ability to ferment various β-glucosidase substrates tested. This strain lost the ability to ferment several other sugars, the reason for which is not yet understood. Referring to Bergey's Manual (13) the fermentation ability of cellobiose of *B. bifidum* and *B. longum* varies between strains. This might be due to the varying level of β-glucosidase activity of each strain.

Yaeshima *et al.* (16) reported that the difference between *B. longum* and *B. animalis* is the lack of β-glucosidase and fermentation of salicin by *B. longum* and the presence of these factors in *B. animalis*. However, in our tests β-glucosidase level varied in *B. longum* and most strains posessed β-glucosidase activity albeit at a lower level than in other *Bifidobacterium* species.

Growth and β-Glucosidase Activity in Milk and Plants

Four β-glucosidase positive strains and eight β-glucosidase negative strains were grown in reconstituted skim milk media (Table 3). Presence of β-glucosidase did not alter the degree of growth in the milk media since no correlation was observed between the degree of cell growth and the posession of β-glucosidase. β-Glucosidase negative strains grew well as β-glucosidase positve strains. However, *B. adolescentis* Int57 and *B. adolescentis* ATCC 15703 grew poorly. The poor growth of *B. adolescentis* in milk was thought to be due to poor production of vitamins (2) while high-accumulating strains of vitamin such as *B. bifidum* and *B. infantis* grew well.

The β-glucosidase level was much lower in milk medium than in BHI medium (Table 4). *B. adolescentis* Int57 is strong in β-glucosidase production in BHI medium. The very low production of β-glucosidase in *B. adolescentis* Int57 in milk might be due to the poor growth of the cells and repression of β-glucosidase by lactose in milk medium. *B. longum* ATCC 15707 which was grown well in milk medium showed still low β-glucosidase production. Park *et al.* previously showed that the production of β-glucosidase in *B. adolescentis* Int57 was considerably increased by cellobiose and decreased by lactose and glucose though the degree of cell growth

Table 3. Growth of various β-glucosidase positive and negative strains in reconstituted skim milk (10%).

Strains	Log of viable cell counts (cfu/ml)					pH
	0*	12	15	22	30	30
β-glucodsidase negative strains						
B. longum BGN2	6.4	8.6	8.9	8.7	8.1	4.4
B. longum BGN3	6.6	8.5	8.7	8.3	7.7	4.5
B. bifidum BGN4	6.2	8.5	8.6	8.1	7.9	4.5
B. bifidum CH3	6.5	7.8	7.3	7.2	7.0	5.5
B. bifidum CH4	6.0	7.8	7.5	7.3	7.2	5.4
B. bifidum CH1.6	6.2	7.8	8.5	8.2	8.2	4.6
B. infantis K2	6.6	8.8	8.7	7.7	7.7	4.3
Bifidobacterium sp. M-6	6.2	7.9	7.7	7.6	7.8	5.3
β-glucosidase positive strains						
B. longum ATCC 15707	6.7	8.4	8.6	8.5	8.2	4.8
B. adolescentis ATCC 15703	6.3	6.9	7.2	7.4	7.0	5.4
B. adolescentis Int-57	6.3	6.9	7.3	7.4	7.1	5.4
B. bifidum KU1	6.2	7.7	8.6	8.3	7.8	4.5

* Incubation hours.

Table 4. Growth and β-glucosidase activity in various growth media.

	B. adolescentis Int57		*B. longum* ATCC 15707	
	Viable cell counts (cfu/ml)	β-Glucosidase activity (mU/ml culture)	Viable cell counts (cfu/ml)	β-Glucosidase activity (mU/ml culture)
Grape	3.0×10^6	– *	4.2×10^6	–
Apple	2.2×10^7	below < 0.03	2.5×10^7	below < 0.03
Orange	8.0×10^8	below < 0.03	8.2×10^8	below < 0.03
Carrot	8.1×10^8	below < 0.03	7.8×10^8	below < 0.03
Reconstituted skim milk	1.1×10^8	0.14	8.9×10^8	0.16
BHI	7.9×10^8	10.5	6.4×10^8	2.80

Cells were grown in each medium at 37°C for 20 hours and β-glucosidase activity was measured as described in Materials and Methods. * Negative in enzyme activity.

was similar (11).

The growth of *Bifidobacterium* on vegetables and fruits varied (Table 4). Grapes did not allow growth, while oranges and carrots were good media for growth of *B. adolescentis* Int57 and *B. longum* ATCC 15707. The activity of β-glucosidase of the *Bifidobacterium* strains grown on oranges and carrots was also very weak although cell numbers were above 10^8 cfu/ml. In conclusion, the production of β-glucosidase varied considerably depending on the growth conditions. Further studies are needed to determine the contribution of *Bifidobacterium* β-glucosidase to the metabolism of various glucosides in the large intestine and its role in *Bifidobacterium*-fermented foods.

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