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Isolation of Amylolytic *Bifidobacterium* sp. Int-57 and Characterization of Amylase

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The intestinal microflora of humans is an extraordinarily complex mixture of microorganisms, the majority of which are anaerobic microorganisms. The distribution of amylolytic microorganisms in the human large intestinal tract was investigated in various individuals of differing ages using anaerobic culture techniques. A large percentage of the amylolytic microorganisms present belonged to the Genus *Bifidobacteria*. The number of *Bifidobacteria* increased significantly at two years of age. Adults and children above 2 years old carried about 0.8×10^9-2.0×10^{10} colony forming units (CFU/gram) of amylolytic *Bifidobacteria*. Among these amylolytic *Bifidobacteria*, Int-57 was chosen for further studies. Between 65% and 85% of the amylase produced was secreted and the remaining amylase was bound to the cell wall facing the outside . Amylase production could be induced by starch in a stable form. When cells were grown on maltose or glucose, amylase production was much lower than on starch and amylase activity disappeared after 24 hours growth on these media. Partially purified enzymes showed optimum activity at a temperature of 50°C and at an optimum pH of 5.5, respectively. Heat treatment at 70°C for 30 minutes almost completely inactivated amylase. The hydrolysis products of starch were mainly maltose and maltotriose. Soluble starch, amylose, amylopectin, and γ-cyclodextrin(γ-CD) were easily hydrolyzed. The rate of hydrolysis of α-CD and β-CD was slower than that of γ-CD. Carboxymethyl cellulose, β-1,3-glucan and inulin were not hydrolyzed.

The human large bowel is about 150 cm long with a surface area (undissociated) of about 1300 cm^2. It can contain approximately 220g(58~908g) of contents (2, 6). Bacteria are a major component and constitute 40% to 55% of human fecal solids.

Recent dietary studies have shown that a significant (10~40%) portion of starch passes through the human small intestine and reaches the large intestinal tract (2, 4, 5, 12, 19, 20). The starch that enters the large bowel is subject to bacterial degradation and metabolized to produce short chain fatty acids(SCFA), such as acetate, propionate, and butyrate (3, 4, 17, 18, 19). SCFA play an important role as an energy source for colonic epithelial cells, and act as physiological effectors for the large intestine (3, 4, 17, 18, 19). Among amylolytic bacteria in high-producing ruminants, *Streptococcus bovis*, *Ruminobacter amylophilus*, and *Butyrivibrio fibriosolvens* have been characterized (11, 13). *Clostridium* was found to be the main organism responsible for starch degradation in hens (15). Humans have a considerably different microflora. It would be interesting to study which organisms are responsible for starch degradation in humans.

McCarthy *et al.* reported that *Bacteroides vulgatus*, an amylolytic gram negative anaerobe, is one of the most numerous bacterial species in the human colon (12). Macfarlane reported that the predominant amylolytic bacteria belonged to the genera *Bifidobacterium*, *Bacteroides*, *Fusobacterium*, and *Butyrivibrio* (10).

In our study, we found that adults and children older than 2 years of age carried more than 10^8 amylolytic *Bifidobacteria*. In contrast, new-born babies and children under 2 years old carried significantly fewer amylolytic *Bifidobacteria*. While isolated, *Bifidobacteria* secreted extracellular amylase *in vitro*, *Bacteroides vulgatus* pro-

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duced cell-bound amylase. We still do not know whether these differences between our observations and McCarthy's report are due to geographical distribution or experimental condition. In this report we describe the characteristics of a partially purified amylolytic *Bifidobacterium*.

MATERIALS AND METHODS

Materials

Maltooligosaccharides, α-, β-, γ-cyclodextrin, inulin, CMC (carboxy methyl cellulose), mucin, hemin, and trypsin were purchased from Sigma Co..

Culture Media

Anaerobic bacteria were isolated from human feces. Feces were obtained from adult volunteers and from children and were immediately transported to the lab in an anaerobic dilution medium at 4℃ (16). After 10-fold serial dilutions of 1g of feces, the samples were plated on a BHI-starch medium (BHI,0.05% cysteine, 0.001%(0.01g) hemin, 0.001g (0.0001%) vitamin K, and 0.5% starch). After 2 to 3 days of incubation at 37℃ in a Gas-Pak, Gram's Lugol solution was poured onto the plates. Colonies with clear zones were counted and used for further experiments.

Isolation media were used for liquid culture. Sugars were added after filtration through a 0.45 μm Acrodisc membrane filter.

Strain Characterization and Identification

For the fermentation test, 0.5 m*l* of 10% substrate solution, which was membrane-filtered through a 0.45 μm Acrodisc filter, was added to 9.5 m*l* of PYF basal medium (16). After 2.5 days incubation in a Gas-Pak, the pH of the growth media was measured. The tube below pH 5.5 was judged to be fermentation positive. Morphological and physiological characteristics were studied based on "Bergey's Manual of Determinative Bacteriology" (21).

Amylase Activity Assay

The hydrolytic activity of amylase was assayed by measuring the reducing sugar, as described by Miller (14) with modification. The mixture, containing 0.5 m*l* of 1% soluble starch and 0.25 m*l* of 0.1 M citrate buffer (pH 5.5), was prewarmed for 5 minutes at 50℃. The enzyme solution was added to the prewarmed solution and incubated at 50℃ for 2 hours. The reaction was terminated by adding 3 m*l* of DNS(dinitrosalicilic acid) solution. After boiling for 5 minutes in a water bath, the absorbance of the colored solution was measured with a spectrophotometer at 550 nm.

One unit of amylase activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 μmole of maltose from starch per 2 hours at 50℃.

For iodine staining value determination, 0.2 m*l* samples were withdrawn from the reaction tube and placed into tubes containing 0.2 m*l* of 0.1 M HCl. Then, 4 m*l* of iodine solution (0.3g of iodine and 0.7g of KI per liter) was added. The absorbance was then measured at 620nm against a water-iodine blank.

Preparation of Enzyme, Protein Determination and SDS-PAGE

Int-57 bacteria anaerobically cultured in a medium containing starch at 37℃ for 2 days. The culture medium was then centrifuged at 10,000×*g*. The culture medium was precipitated with 50% saturated ammonium sulfate and centrifuged. The supernatant was precipitated again with 75% saturated ammonium sulfate. The pellet was dissolved in a 20 mM Tris/HCl, pH 7.5 phosphate buffer solution and dialyzed 4 times against the same buffer. Then, the concentrated sample was applied to a Sephacryl S-1000(Pharmacia Co.). The fractions with the highest activities were pooled and subjected to 10% SDS-PAGE for molecular mass determination (8). The proteins were denatured by mixing with a 25 μl loading buffer solution, and boiling at 100℃ for 3 minutes. All samples were run in duplicate. Activity staining of the SDS-PAGE gel was performed as described by Bahri *et al.* (1), using half of the gel. The gel was overlaid on starch-agar slides with weight at 37℃ overnight. After removal of gels, the starch-agar was stained with 0.03% I_2 in a 0.07% KI solution. The other half of the protein gel was stained with Coomassie brilliant blue R.

Thin Layer Chromatography (TLC) of the Hydrolyzates

TLC was done with a Kiesel gel 60 plate (Merck Co. Ltd) with a solvent system of isopropanol/ethyl acetate/water (3:1:1, v/v/v). After 5 hours of development the TLC plate was dried and visualized by spraying with 50% sulfuric acid in a methanol solution, and heating at 110℃.

Effect of Trypsin Treatment and Sonication of the Cell-bound Amylase

For trypsin treatment 10ml of culture medium was centrifuged. The pellet was washed and resuspended in 0.5ml of reaction buffer solution (20 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM Tris, pH 7.2) containing 0.5 mg or 1 mg trypsin. After 4hr of incubation at 37℃ with gentle shaking, cell pellets were harvested and assayed for remaining amylase activity. For sonication, cell pellets were resuspended in a 0.5 m*l* buffer solution. Amylase activity was measured before and after sonication.

RESULTS

Distribution of Amylase Positive Strains among

Individuals

The number of colonies showing clear zones on a BHI+starch medium, after addition of Lugol's iodine solution, was counted. Results are shown in Table 1. The number of amylase positive colonies was much higher in individuals older than 2 years of age. New-born babies and young children less than 2 years old harbored less than 10^8 amylolytic bacteria/gram of wet feces. This suggests that the bacterial flora can adjust to the food pattern of the host during growth. When we examined the anaerobic flora in rats, according to the feed composition, protein-rich feeding promoted a fast-growing facultative anaerobic flora whereas the control group, carbohydrate-rich and fat-rich foods promoted an increase in the number of amylolytic *Bifidobacteria* (unpublished data). Amylolytic bacteria were isolated from several individuals and the colonies were whitish, opaque, and round. When 25% skim milk was added they grew faster while other colonies were inhibited. Also, the molecular weights of the amylase isolated from most of the strains were identical on SDS-PAGE and activity staining gel. Therefore, we concluded that most of the amylase positive strains isolated in this study belonged to the same group. All the amylolytic isolates showed positive fructose-6-phosphoketolase (Bifidoshunt enzyme) and acetate/lactate production which is characteristic of *Bifidobacterium* (9). Among the *Bifidobacterium*, Int-57 was selected and characterized, as shown in Table 2. Int-57 grown in BHI medium is illustrated in Fig. 1. An identification of *Bifidobacterium* Int-57 to the species level was not pursued.

Effect of Adding Various Substrates to the Growth Medium

Table 1. Distribution of amylase producing microorganism in the feces of various individuals.

Age	Sex	Number of positive colonies/gram wet feces
3 days	M	4.2×10^8
21 days	F	$<10^8$
1 month	F	$<10^8$
7 months	M	$<10^8$
9 months	F	1.2×10^9
11 months	F	$<10^8$
15 months	M	1.0×10^8
3 years	M	3.3×10^9
5 years	F	2.7×10^9
7 years	M	4.5×10^9
24 years	F	0.8×10^9
26 years	M	9.3×10^9
32 years	F	2.0×10^{10}
36 years	M	4.5×10^9

The effect on amylase production of adding various substrates to the growth medium is shown in Fig. 2.

When 0.5% starch was added the amylase activity of the culture supernatant was 4 times higher than in the control (no sugar added). When 0.5% glucose was added a low level of amylase activity resulted in the early growth phase, but disappeared after 24 hours

Table 2. Description of the *Bifidobacterium* sp. Int-57 strain isolated from a sample of human feces.

Morphology	: straight or curved cells		
Size	: 0.5×1.0-4.0 μm		
Gram staining	: +		
Mobility	: −		
Catalase	: −		
F6PPK	: +		
α-glucosidase	: +	β-glucosidase	: +
α-galactosidase	: +		
β-galactosidase	: +	phospho-β galactosidase	: −
α-fucosidase	: −		
β-xylosidase	: +	α-arabinofuranosidase	: +
β-glucuronidase	: −		
Spore formation	: −		
Fermentation			
glucose	: +	galactose	: +
mannose	: −	xylose	: +
D-arabinose	: −	L-arabinose	: −
fructose	: +	gluconate	: +
sorbitol	: −	mannitol	: +
maltose	: +	sucrose	: +
lactose	: +	cellobiose	: +
raffinose	: +	trehalose	: −
starch	: +	inulin	: +
salicin	: +		

Fig. 1. A photomicrograph of *Bifidobacterium* sp. Int-57 grown in BHI medium.

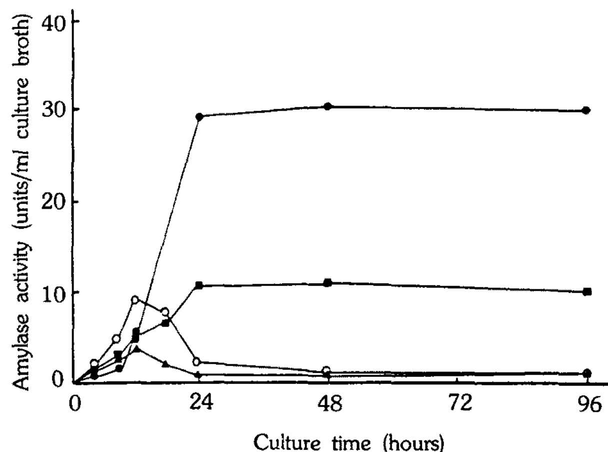

Fig. 2. Effects of adding glucose, maltose and starch on the production of Int-57 amylase during growth.

growth. When grown on maltose the level of amylase activity was higher than when grown on glucose, but amylase activity disappeared after 24 hours growth. For this test, all the culture media were dialyzed to remove unused sugars. Amylase produced on starch-supplemented media and control media was stable for several days during culture. It is unknown why amylase activity on glucose-supplemented and maltose-supplemented media disappeared. We do not know yet if it is from the production of some kind of protease or amylase inhibitor on glucose- or maltose-supplemented media. *Bifdobacterium* studied in this investigation followed the general regulation pattern of amylase studied in other organisms; low amylase activity on glucose-media and high amylase activity on starch- media. Addition of 0.5% of NaCl and mucin had no effect on amylase production.

Location of Int-57 Amylase

Most of the amylase was secreted during growth. The ratio of secreted amylase to cell-bound amylase was nearly constant throughout the incubation period (data not shown). Of the amylase produced, 87%, 70% and 75% was secreted when grown on starch, control and inulin respectively while the remaining 13%, 30% and 35% was cell-associated (Table 3). When cloned in *E. coli* the amylase was still secreted, but in a lower molecular weight form(unpublished data). It would be interesting to study the signal sequence of the *Bifidobacterium* amylase and the interaction of the signal sequence with the cell membrane in *Bifidobacterium* and *E. coli*. We tested whether the cell-associated amylase was present in the cytoplasmic space or on the cell wall.

Trypsin treatment of the cell-associated amylase abolished most of the amylase activity, as shown in Table 4, suggesting that cell-associated amylase is present on the cell wall facing outside of the cell. Sonication of the cell before or after trypsin treatment didn't increase the amylase activity. Washing with 1 or 2 M NaCl didn't release amylase from the cell. Additionally, sonication of the isolated and commercial amylases as controls did not decrease the amylase activity. These results suggest that the amylase level inside the cell is minimal. In the future we hope to study whether the *Bifidobacterium* amylase is also secreted at a detectable level in a natural host environment.

Table 3. Amylase activity of cell-bound and secreted forms after 24 hrs growth.

	BHI	BHI+Starch	BHI+Inulin
Secreted amylase (%)	75	87	70
Cell bound amylase (%)	25	13	30

The results are shown in relative activity (%)

Table 4. Effect of trypsin treatment on the cell-bound amylase.

	control	+0.5 mg trypsin	+5 mg trypsin
Cell-bound amylase activity after trypsin treatment (%)	100	34.8	13.6
After sonication of the above prepartion (%)	104.5	41.4	19.6

10 *ml* culture of Int-57 was washed and resuspended in 0.5 *ml* of reaction buffer (20 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM Tris, pH 7.2). After 4 hr incubation at 37℃ with gentle shaking, cell pellets were harvested and assayed. The results are shown in relative activity (%).

Molecular Weight of Secreted Amylase

No amylase band was observed on activity-staining gel prepared from amylase negative isolates or from *E. coli*. When the concentrate of *Bifidobaterium* culture supernatants was loaded on SDS-PAGE and blotted on starch-agarose after renaturation, the major 66 KD band was observed, and also a very faint higher molecular weight form was noticed (Fig. 3). We didn't examine the faint upper band further but the upper band might be an aggregated, non-processed or different form of amylase. When partially purified on an ammonium sulfate fraction and filtered through Sephacryl S-1000, only the 66 KD band was obtained. The faint upper band was eliminated. This partially purified preparation was used for further analysis.

Examination of the Amylase Type

Confirmation that the enzyme was an endo-type amylase was obtained by comparison of plots of reduction in iodine-staining value versus release of reducing power,

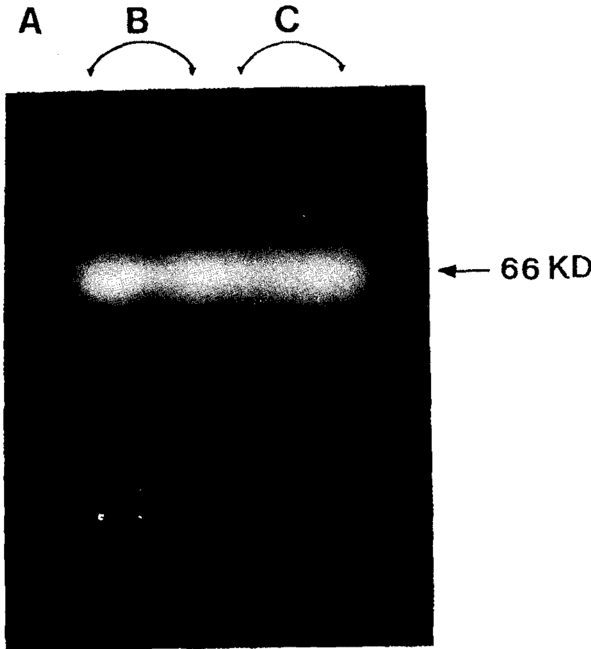

Fig. 3. Iodine staining of Int-57 amylase on an agarose gel containing soluble starch.
The agarose gel was overlaid on the reactivated SDS-PAGE gel overnight at 37℃ before staining: A. Culture supernatant of an amylase negative isolate, B. Partially purified Int-57 amylase, C. Int-57 culture supernatant

with commercial barley β-amylase and *Aspergillus oryzae* α-amylase. The reducing value of *Bifidobacterium* amylase fell sharply during the reaction period, while the iodine staining value decreased at a slower rate (Fig. 4). This pattern is the same as *Asperillus* α-amylase and different from the pattern of barley β-amylase. Consequently, *Bifidobacterium* amylase is an endo-type enzyme. However, it does not necessarily mean that the cleavage sites on starch are chosen randomly by *Bifidobacterium* amylase. The major products, G5, G4 and G3, were distinct from the beginning of cleavage and a significant level of series of cleavage products at higher molecular weight forms was not observed (Fig. 5). Several other endo-type amylases have also been reported to have a preferential cleavage recognition pattern (7). The final cleavage products were mostly G2 and G3, with glucose formed minimally. *Streptococcus precox* amylase also primarily produced G2 (22). These two amylases may be closely related.

Optimum Temperature and pH

The optimum temperature for amylase activity was 50℃ and the optimum pH was 5.5 (Fig. 6, Fig. 7). After incubation of the enzyme at 70℃ for 1 hour before assay, the activity decreased sharply, suggesting that *Bifidobacterium* amylase is unstable at 70℃. A BHI medium of pH adjusted to between 7.0 and 7.5 was best for maximal production of amylase. Several amylases showed optimum activity at a temperature of 50℃. Ca^{2+} ion concentration did not modulate activity. The temperature stability of the enzyme was examined in the presence and absence of 5 mM $CaCl_2$ by preincubation

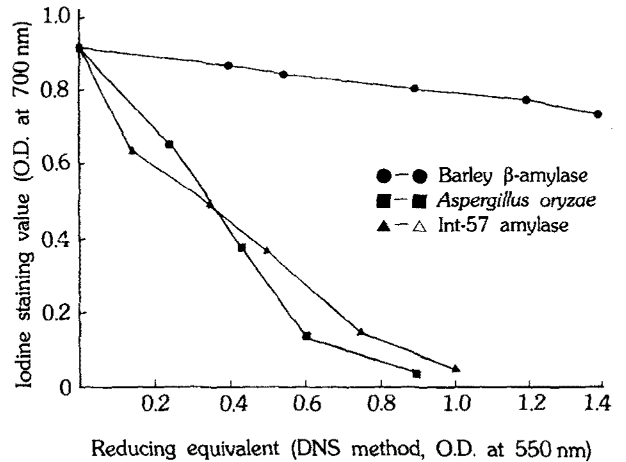

Fig. 4. A comparison of decrease in iodine staining value and increase in reducing equivalent for starch with Int-57 amylase, Barley β-amylase and *Aspergillus oryzae* α-amylase.

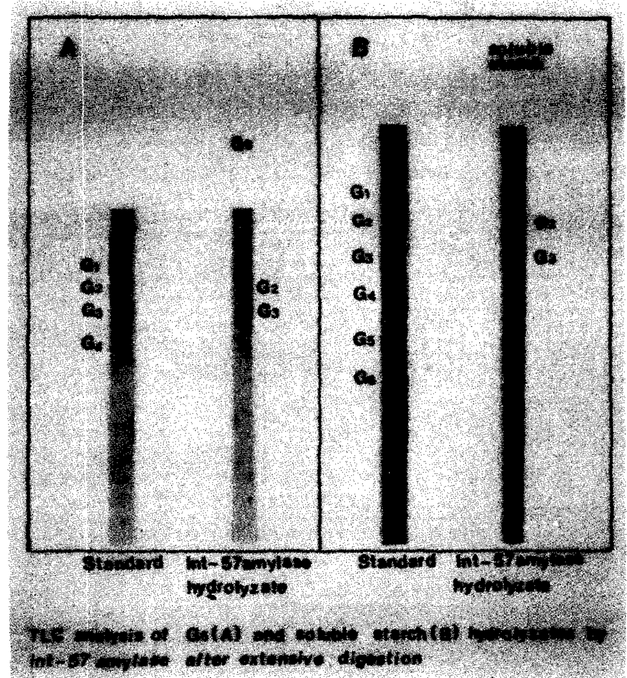

Fig. 5. TLC analysis of G_6 (A) and soluble starch (B) hydrolyzates by Int-57 amylase after extensive digestion.

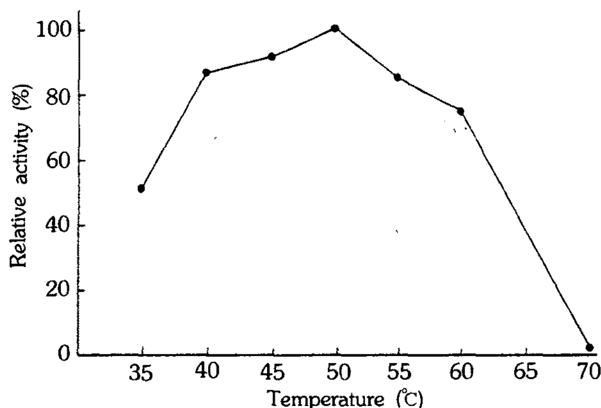

Fig. 6. Effect of temperature on the activity of amylase obtained from Int-57 strain.

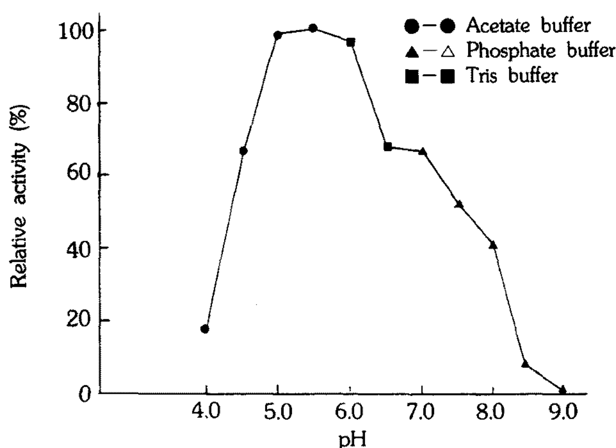

Fig. 7. Effect of pH on the activity of Int-57 amylase.

in the absence of a substrate. $CaCl_2$ did not affect the heat stability. When the same concentration of other substrates was added to the assay buffer, amylopectin and soluble starch were degradaded at a fastest rate (Table 5). Among α-CD, β-CD and γ-CD, γ-CD was hydrolyzed most rapidly, followed by β-CD and α-CD. γ-CD is a ring form of an 8 glucose oligomer, indicating that *Bifidobacterium* amylase can easily cleave a relaxed ring.

β-1,3-glucan, inulin and CMC were not hydrolyzed at all. When mashed and boiled rice was incubated with Int-57 amylase, clearing of the rice stew progressed every day.

DISCUSSION

The result that the number of amylolytic *Bifidobacterium* is considerably increased as one grows during childhood is interesting, and it suggests that the large intestinal microflora has a close relationship to the change

Table 5. Substrate specificity of Int-57 amylase.

Substrate	Relative activity (%)
soluble starch	72
amylopectin	100
pullulan	6.2
glycogen	17.5
α-CD	2.0
β-CD	3.0
γ-CD	22.5
β-glucan	0
inulin	0
CMC	0

in dietary patterns; i.e. from breast or bottle- feeding to the weaning diet. To our knowledge this phenomenon has never been reported. It would be valuable to know if the flora producing other kinds of enzymes is also altered according to age or diet. Our result is in contrast with McCarthy *et al.* (20) who reported that *Bacteroides vulgatus*, an amylolytic gram negative anaerobe, is one of the most numerous bacterial species in the colon. *Bacteroides vulgatus* produced cell bound amylase whereas *Bifidobacteria* yielded extracellular amylase.

It is possible that amylolytic *Bifidobacteria* adapted better to Korean food. Further studies should be carried out to clarify this situation. Not all the *Bifidobacteria* isolated in this study produced amylase. Only about half of the *Bifidobacterium* isolates were positive on amylase production. The role of starch hydrolysis by these amylolytic *Bifidobacteria* in the large intestinal tract needs to be examined *in vivo* for the physiological aspect. The benefit of starch degradation by these amylolytic bacteria might belong, not just to *Bifidobacteria,* but also to other microorganisms.

When *Bifidobacterium* Int-57 amylase was cloned in *E. coli*, the major portion of the amylase produced was secreted (unpublished data). In most cases, cloned gene products were known to be located in periplasmic or intracellular space in *E. coli*. It is unknown how the secretion signal of *Bifidobacterium* is recognized in *E. coli*. Int-57 amylase which was not secreted, yet which was cell wall-bound facing outside, lost activity after trypsin treatment. When examined *in vitro*, starch stimulated amylase production at higher levels than glucose or maltose, and the amylase produced on starch was stable. This suggests that the level of amylase in the large intestine is constantly changing according to the level of starch that reaches the large intestine. A partially purified amylase preparation was examined for starch hydrolysis products. Maltose and maltotriose were the major final products identified.

Thus, maltose and maltotriose utilizers would benefit from starch hydrolysis by amylolytic *Bifidobacterium*. Amylase showed optimum activity at a pH of pH 5.5, which is close to the normal pH of the healthy large intestine. At 37℃ amylase activity would be 1/3 of the activity at 50℃.

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