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Utilization of fucosylated oligosaccharides by *Bifidobacterium bifidum* BGN4 and expression of cloned BGN4 α-L-fucosidase genes in *E. coli*

Bifidobacterium bifidum BGN4의 퓨코실 올리고당 이용능과 BGN4 유래 α-L-fucosidase 유전자의 클로닝 및 발현 연구

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Department of Food and Nutrition

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Abstract

Utilization of fucosylated oligosaccharides by *Bifidobacterium bifidum* BGN4 and expression of cloned BGN4 α-L-fucosidase genes in *E. coli*

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Bifidobacterium is the most predominant in the intestine of breast-fed infants and beneficial for the maintenance of the balanced microflora in the gut. Human milk oligosaccharides (HMO) is the third most common solid component (15 g/L) in human milk and contains abundant fucosylated oligosaccharides (FO). Therefore, *Bifidobacterium* which can use fucosylated HMO is expected to be selectively enriched in the intestine of breast-fed infants. The utilization of human milk saccharides (HMS) and artificial fucosylated oligosaccharides (2-fucosyllactose, 3-fucosyllactose) by various

Bifidobacterium strains were assessed. *B. bifidum* BGN4 showed specific growth in the presence of 2FL or 3FL. Using the annotated sequences of two theoretical α-L-fucosidase genes (*BBB_0176* and *BBB_1341*) in the genome of *B. bifidum* BGN4, those genes have been cloned in *E. coli* for the expression and characterization of the corresponding enzymes. AFL03772, product of *BBB_0176*, hydrolyzed α-(1→2) and α-(1→3) linkages of 2- and 3-fucosyllactoses; whereas AFL04933, product of *BBB_1341*, hydrolyzed α-(1→3) linkage of 3-fucosyllactose. Based on the results obtained in this study, *B. bifidum* BGN4 has been concluded to have two α-L-fucosidase enzymes to hydrolyze FO. Therefore, *B. bifidum* BGN4 may be used as beneficial bacteria which can preferentially grow in the intestine of the breast-fed infants.

Keywords : *Bifidobacterium bifidum* BGN4, α-L-fucosidase, fucosylated HMO utilization.

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2FL : 2-fucosyllactose

3FL : 3-fucosyllactose

FO : Fucosylated oligosaccharides

HMO : Human milk oligosaccharides

HMS : Human milk saccharides

BMS : Bovine milk saccharides

PCR : Polymerase chain reaction

TLC : Thin layer chromatography

SDS-PAGE : Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PI : Isoelectric point

MW : Molecular weight

IPTG : Isopropyl β-D-1-thiogalactopyranoside

MRS : Mann-Rogosa-Sharpe medium

LB : Luria-Bertani broth

pB buffer : Sodium phosphate buffer

DW : Distilled water

1. Introduction

Human milk is a sole nutrient source and best food for the infants. It is composed of unique proteins, carbohydrates, lipids, minerals, and vitamins which were adapted to satisfy the nutritional and immunological needs of infants. Also it provides wide range of beneficial effects to infants [24, 25, 29].

Human milk contains human milk oligosaccharides (HMO) as the third most common solid component (15 g/L). The breast milk of the other species including bovine milk also has oligosaccharides but human milk has significantly more abundant and diverse oligosaccharides than those of the other milk [22, 23]. HMO is mainly composed of different monosaccharides (D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and sialic acid). Most HMO have lactose at the reducing end, which generally sialylated (3-sialyllactose, 6-sialyllactose) or fucosylated (2-fucosyllactose, 3-fucosyllactose) [5]. HMO contains different oligosaccharide components and various structures among the individuals, Lewis blood type, and during the course of lactation [26, 27]. Among the 200 different identified HMO, 137 HMO were fucosylated and proportion of fucosylated oligosaccharide (FO) is approximately 75% [13]. In contrast, the most

abundant oligosaccharides of bovine milk contain sialylated oiligosaccharides [22, 28]. Therefore, the abundance of FO is a special characteristic of HMO.

FO, the major component of HMO provides some beneficial effects to infants. Various pathogens recognize fucosylated glycans of the epithelial cell as a receptor. FO can act like a cell surface receptor to the various pathogens [5]. By blocking the adhesion of the various pathogens to the epithelial cell glycan layer, FO can protect the intestinal tract of the infants from the attack of the pathogens [5, 12, 14]. Moreover, FO exerts prebiotic effects [21]. Prebiotics is defined as "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" by Gibson *et al* [7]. The fucosylated residues of FO protect themselves from digestive enzymes [8]. The FO reach large intestine, instead of being digested and absorbed as a nutrient, to promote the selective growth of *Bifidobacterium* which is a well-known beneficial bacteria [4, 5]. The selectively promoted beneficial bacteria inhibit the growth of pathogens either by lowering the intestinal pH or through nutrient competition [4, 8].

For the selective growth in the intestine with FO, the bacteria need

ability to hydrolyze fucosylated residue of FO and use the released sugars as energy sources. Accordingly, α-L-fucosidase, an enzyme to hydrolyze fucosyl residue of HMO is essential for the use of FO [3].

Bifidobacterium is known to be the main utilizer of HMO, which contributed to the abundance of bifidobacteria in the intestinal tract of the breast-fed infants compared to adults or formula-fed infants [15]. In the present study, to identify specific *Bifidobacterium* with high ability to use fucosylated HMO, five strains of bifidobacteria were assessed the utilization of HMO and FO. Among them, *B. bifidum* BGN4 which showed the best growth on FO was chosen and its two α-L-fucosidase genes were cloned and expressed in *E. coli* for further characterization.

2. Materials and Methods

Ⅰ. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specifically described. 2-fucosyllactose (2FL) and 3-fucosyllactose (3FL) were purchased from Genechem (Daejeon, Korea).

Ⅱ. Methods

1. Bacterial strains and culture conditions

The bacterial strains used in this work are listed in Table 1. *Bifidobacterium* strains (*B. bifidum* BGN4, *B. longum* RD47, *B. adolescentis* KCTC 3216, *B. infantis* KCTC 3249, *B. breve* KCTC 3419) were obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) and Food Microbiology Laboratory Strain Collection, Seoul National University. They were grown anaerobically in de-Mann-Rogosa-Sharpe (MRS) medium (Becton-Dickinson Company, New Jersey, USA) supplemented with 0.05% (w/v) L-cysteine·HCl at 37°C. To assess the utilization of different carbon sources 2FL (6 mg/ml), 3FL (6 mg/ml), human milk saccharides (HMS,

20 mg/ml), and bovine milk saccharides (BMS, 20 mg/ml) were added into the MRS broth without glucose (KisanBio, Seoul, Korea).

All broths were filter sterilized through a 0.2 μm PVDF membrane (Pall Corporation, NY, USA) and aliquoted into 96-well plates (Costar 3595, Corning Inc, NY, USA). Milk saccharides were prepared as follows. Human breast milk samples were obtained from healthy women volunteer as approved by the Institutional Bioethics Review Board, Seoul National University (SNUIRB, approval number ; IRB No.1411/001-026). The bovine milk was purchased at a local market (Seoul Milk, Seoul, Korea). The milk saccharides were separated as described by Ward *et al* [17] with a slight modification. Fifty ml of milk was centrifuged at 5,000 × g for 30 min at 4°C, and the fat was removed. Then, 2 volume of ethanol was added, and the mixed samples were incubated overnight at 4°C with stirring. The precipitate was collected by centrifugation at 5,000 × g for 30 min at 4°C and removed. The solvent of supernatant was removed by rotary evaporator and speed vacuum. The solid content of the solution was used as a crude saccharide of human milk and bovine milk. The bacterial growth was measured with a microplate reader (Spectra Max 190, Molecular devices, CA, USA) set at 600 nm. Three replicates were performed for every experimental condition. *E. coli* and BL21 was grown in Luria-Bertani (LB) medium (Becton-Dickinson Company, New Jersey, USA)

at 37°C with vigorous shaking. The LB medium was supplemented with 50 or 100μg/ml ampicillin, 2% agar (Becton-Dickinson Company, New Jersey, USA) and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, TCI, Tokyo, Japan) when necessary.

Table 1. Bacterial strains and plasmids used in this study

Abbreviation	Strains & plasmids	Characteristics
Strains		
B. bifidum BGN4	*Bifidobacterium bifidum* BGN4	Source of two α-L-fucosidases
B. longum RD47	*Bifidobacterium longum* subsp. *longum* RD47	From intestine of adult
B. adolescentis	*Bifidobacterium adolescentis* KCTC 3216	From intestine of adult
B. infantis	*Bifidobacterium longum* subsp. *infantis* KCTC 3249	From intestine of infant
B. breve	*Bifidobacterium breve* KCTC 3419	From intestine of infant
E. coli BL21	*E. coli* BL21(λDE3)	Expression host
E0176	*E. coli* BL21(λDE3) harboring pET21 + *BBB_0176*	Recombinant
E1341	*E. coli* BL21(λDE3) harboring pET21 + *BBB_1341*	Recombinant
Plasmids		
pET21	pET21a(+)	Subcloning vector
p0176	pET21a(+) + *BBB_0176*	Recombinant vector
p1341	pET21a(+) + *BBB_1341*	Recombinant vector

2. Analysis of carbohydrates during culture by thin layer chromatography (TLC)

The carbohydrates in the culture broth were determined by TLC. The broths were loaded on the silica gel plate 60 (Merck, Darmstadt, Germany). The mobile phase was composed of 1-propanol, distilled water (DW), and ethyl acetate (7:2:1, v/v/v). The sulfuric acid–ethanol (1:9, v/v) solution was sprayed and dried. The plate was heated at 120°C, 5 min for visualization.

3. Assessment of the released L-fucose from FO by L-fucose colorimetric assay (Fucose assay)

The separated L-fucose from FO by the hydrolytic action of *Bifidobacterium* or α-L-fucosidases was assessed by combination of L-fucose assay kit (Megazyme, Wicklow, Ireland) and the modified colorimetric method of Cohenford *et al* [6]. The amount of NADPH in the L-fucose assay kit's final reactant was proportional to the sample's L-fucose. The same volume of neocuproine–copper reagent was added to the final reactant; the color turned yellow. This yellow color was measured by the microplate reader at 455 nm. The neocuproine–copper reagent was made by Cohenford *et al*'s instruction [6].

4. Molecular cloning of two α-L-fucosidase genes from *B. bifidum* BGN4

The bacterial strains and plasmids used in this work are listed in Table 1. The complete genome sequence of *B. bifidum* BGN4 (GenBank, http://www.ncbi.nlm.nih.gov/genbank/, accession number : CP001361), a source of two α-L-fucosidase gene sequences (*BBB_0176*, *BBB_1341*), was previously reported [19] at the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/, Entry number : T02135) databases. The expression host *E.coli* BL21(λDE3) (Novagen, WI, USA) and plasmid pET21a(+) (Novagen, WI, USA) were employed to clone and express the two α-L-fucosidase genes.

The chromosomal DNA of *B. bifidum* BGN4 was isolated with the MG^{TM} Cell Genomic DNA Extraction SV kit (MGmed, Seoul, Korea). The lysozyme digestion step (resuspend cell pellet with 30 mg/ml lysozyme and incubate for 30 min at 37°C) was carried out and followed by the manufacturer's instruction for Gram-negative bacteria. The isolated chromosomal DNA was used as template DNA. The primers are designed based on the two α-L-fucosidases sequences in the full genome of *B. bifidum* BGN4. The α-L-fucosidase genes were amplified by Takara LA Taq™ (Takara, Otsu, Japan) with each primer set. The primer sequences used in this work are listed in Table 2. The

amplified *BBB_0176* and *BBB_1341* were purified by PCR/Gel Combo Kit (NucleoGen Inc, Daejeon, Korea) and subcloned into pET21a(+) vectors with T4 ligase (Promega, WI, USA). The cloning vector for *BBB_1341* was treated with CIP (calf intestinal alkaline phosphatase, Promega, WI, USA) after restriction enzyme digestion and purified by PCR/Gel Combo Kit. Each sub-cloned vector was transformed into *E. coli* DH5α and BL21 via heat shock and the $CaCl_2$ method. The positive clones were selected by spreading on LB agar plates supplemented with 50 μg/ml of ampicillin. The plasmids from the selected colonies were purified using a Plasmid Purification Mini Kit (NucleoGen Inc, Daejeon, Korea) and confirmed by Bgl II (Promega, WI, USA) restriction enzyme digestion. As a marker, 1 kb DNA Ladder (Promega, WI, USA) was used.

Table 2. Primer sequences used in this study

Genes and proteins	Primers	Sequences
BBB_0176 (AFL03772)	afuc0176_F (Hind III)	5'-CCT AAG CTT CGA TGA AAC ATA GAG CGA TGT CAT CG -3'
	afuc0176_R (Xho I)	5'-TAT CTC GAG GCA GGC GGA GTG CTT G-3'
BBB_1341 (AFL04933)	afuc1341_F (Xho I)	5'- TTA CTC GAG CGA TGC TAC ACA CAG CAT CAA GAG GAT G -3'
	afuc1341_R (Xho I)	5'- TTA CTC GAG GTA CCG GTT GGA CTT ACG CTT GGC TTC AA -3'

The restriction enzyme recognition sequences are underlined.

5. Assessment of α-L-fucosidase activities in the transformed *E.coli* BL21

BL21 harboring each α-L-fucosidase gene was cultivated in a LB medium (containing 100 μg/ml ampicillin) until an OD 600 was reached 0.5-0.6. Then, IPTG (1 mM) was added to each culture medium and cultivated at 37°C for 6 h with vigorous shaking. After cultivation, the cells were harvested by centrifugation at 8,000 × g for 30 min, 4°C. The 1 g of cell pellet was disrupted by sonication in 5 ml of sodium phosphate buffer (pB buffer, 20 mM, pH 7.8) and centrifuged at 10,000 × g for 30 min, 4°C. The obtained cell free extract was used as a crude enzyme. Cell free extract of BL21 that harbored empty vector pET21a(+) was prepared in the same way as a negative control.

For the assessment of α-L-fucosidase activities, 2FL (15 mg/ml), 3FL (15 mg/ml), and HMS (300 mg/ml) were added as substrates. Two μl of each substrate and 3 μl of each crude enzyme were mixed and incubated at 37°C for 30 min. The reaction was stopped by heating at 98°C for 5 min. The enzyme reactant was analyzed by using TLC and fucose assay as described above.

6. Purification and characterization of α-L-fucosidases

The 6x his-tag in the pET 21a(+) vector was added to the C terminal of each recombinant protein for purification. The crude enzyme was loaded onto a HisTrap HP column (GE Healthcare, Uppsala, Sweden), and the target protein was eluted with a linear gradient of 0-500 mM imidazole in pB buffer (20 mM, pH 7.8) at a 3 ml/min flow rate. The eluted fraction was applied to a Superdex-200 HR10/30 column (GE Healthcare, Uppsala, Sweden). The column was equilibrated and eluted with pB buffer (20 mM, pH 7.8). All chromatography was conducted by AKTA Explorer 10 protein purification system (GE Healthcare, Uppsala, Sweden). The eluted protein was analyzed by SDS-PAGE to determine the purity of the protein. The eluted fractions were concentrated by 5K Amicon Ultra centrifugal filter (Millipore, MA, USA) when necessary.

7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out with a mini protean 3 cell (Bio-Rad, CA, USA), using 10% SDS gel prepared as described by Sambrook *et al* [1]. The prepared protein samples were mixed with protein 2X sample

buffer (Elpis Biotech, Daejeon, Korea), boiled for 5 min, and electrophoresed at 100 V for 30 min, 200 V for 2-3 h in tris-glycin running buffer (Elpis Biotech, Daejeon, Korea). After electrophoresis, the gels were stained with Power Stain Brilliant Blue R staining solution (Elpis Biotech, Daejeon, Korea) and destained with destaining solution (225 ml of methanol, 50 ml of glacial acetic acid, up to 500 ml with DW). Prestained protein markers (DokDo-MARK™, 7-240kDa, Elpis Biotech, Daejeon, Korea) were used as molecular mass markers.

8. Effects of pH and temperature on α-L-fucosidase activity

AFL03772, the gene product of *BBB_0176*, was purified by aforementioned chromatography in 20 mM pB buffer (pH 7.8). The effect of temperature on AFL03772 was evaluated at 25-55°C using 2FL (15 mg/ml) as substrate. One μl enzyme and 1 μl substrate were reacted in 3 μl DW. To determine the effect of pH, 2 μl of enzyme and 2 μl of substrate were reacted at 37°C in 36 μl of different buffers at pH 4.05-7.48. Citrate buffer (100 mM) was used for pH 4.05-5.71, and pB buffer (20 mM) was used for pH 6.0-7.48. After reaction, the reactants were concentrated to 5 μl with speed vacuum. All reactions were conducted for 4 h and stopped by heating at 98°C for 5 min. The hydrolyzed fucose was determined by the fucose assay.

The effect of temperature on AFL04933, the gene product of *BBB_1341*, was evaluated at 25-65°C. One µl of BL21 (E1341) crude enzyme in pB buffer (20 mM, pH 7.8) and 4 µl substrate (3FL, 8 mg/ml) were reacted. To determine the effect of pH on AFL04933, 2 µl of crude enzyme prepared from E1341 in pB buffer (20 mM, pH 10) and 2 µl substrates (15 mg/ml) were mixed with 36 µl of different pB buffers at pH 7-13.8 and incubated at 37°C for 2h and stopped by heating at 98°C for 5 min. NaOH (5 M) was used for adjusting the pH. After reaction, the reactant was concentrated to 5 µl with speed vacuum.

3. Results and discussion

Profile of partially purified saccharides of human and bovine milk

The TLC analysis in Fig. 1 shows the different profiles of human and bovine milk saccharides. The HMS has oligosaccharide spots below the lactose spot, which was not observed in the BMS. This shows that HMS has higher level of oligosaccharides than bovine milk.

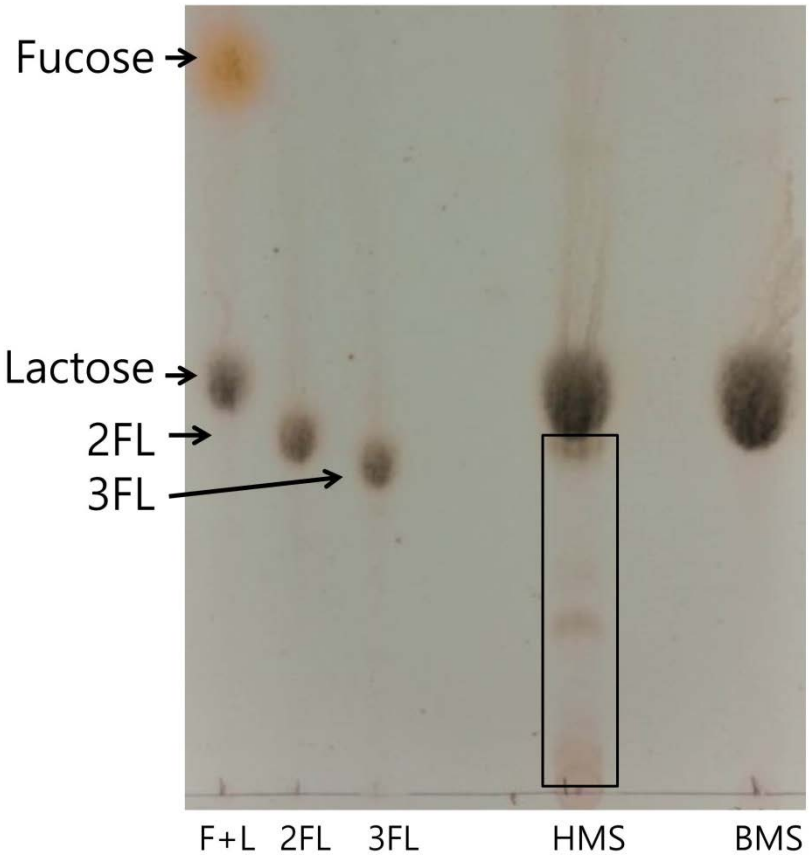

Fig. 1 TLC analysis of partially purified HMS and BMS. F+L : fucose(4%) + lactose(4%), 2FL : 2-fucosyllactose (15 mg/ml), 3FL : 3-fucosyllactose (15 mg/ml), HMS : human milk saccharides (30 mg/ml), BMS : bovine milk saccharides (30 mg/ml)**.** Every spot was loaded with 1μl, except BMS and HMS (5 μl). The oligosaccharide spot in HMS are in the square.

Growth of *Bifidobacterium* strains in the presence of fucosyl carbohydrates

Fig. 2 shows the growth of each *Bifidobacterium* in MRS medium with 2FL, 3FL, HMS, and BMS as carbon sources. All of the experimental *Bifidobacterium* strains showed similar growth patterns in the presence of HMS and BMS probably because both HMS and BMS contained abundant amount of lactose. Interestingly, *B. bifidum* BGN4 showed the best growth in the presence of 2FL and 3FL as a sole carbon source.

When each culture broth was analyzed for the sugars, *B. bifidum* BGN4 shows the hydrolyzed monomers of fucosyl lactoses (fucose, galactose, and glucose) at the 3FL and 2FL groups (Fig. 3 : lane 4 in 3FL and 2FL groups). This result suggests that *B. bifidum* BGN4 has the best hydrolyzing ability for fucosyl residues and lactose among the experimental bifidobacteria. At the HMS group, *B. breve* (Fig. 3 : lane 2 in HMS group) revealed a light oligosaccharide spot after consuming all of the lactose which had initially covered the undigested oligosaccharides spots. These spots for oligosaccharide and lactose disappeared when *B. bifidum* BGN4 was cultured and produced a light orange spot for fucose (Fig. 3 : lane 4 in HMS group). It means that

fucosyl oligosaccharides contained in HMS was hydrolyzed and consumed by *B. bifidum* but not by *B. breve.*

Fig. 4 shows the amount of released L-fucose in 5 μl of each culture broth. The 2FL and 3FL groups showed considerable level of released fucose in the culture broth of *B. bifidum* BGN4. The culture broth of the other strains did not show released fucose.

The comparison of the released fucose by experimental *Bifidobacterium* between HMS and BMS revealed that only *B. bifidum* BGN4 released higher level of fucose in HMS than BMS. It confirms that HMS has higher level of FO than BMS which were efficiently hydrolyzed by *B. bifidum* BGN4. These results are consistent with the data obtained from Fig. 2 showing that *B. bifidum* BGN4 grew the best in the presence of 2, 3-FL and from Fig. 3 suggesting that *B. bifidum* BGN4 can hydrolyze FO.

In the previous research, various strains of *Bifidobacterum* tested for its growth in HMO and FO. *B. infantis* showed noticeable growth in HMO and FO, while *B. bifidum* also showed growth but to a lower degree than *B. infantis* [2, 9, 11, 16, 17, 18, 21] which are different from this study. Several *B. longum* strains showed different pattern in the utilization of the HMO and FO [20] whereas *B. longum* strains which can use HMO contained common HMO utilization genes in their genomes [10]. These results suggest that the utilization ability of FO

and HMO is rather strain specific than species specific characteristics.

The present study also showed that the degree of utilization of FO differs among different strains of *Bifidobacterium*. As shown in Fig. 2-4. *B. bifidum* BGN4 showed the noticeable utilization and hydrolysis of 2FL, 3FL, and FO components contained in HMS. Other tested bifidobacteria did not show noticeable growth in FO. Accordingly, *B. bifidum* BGN4 was chosen for further studies.

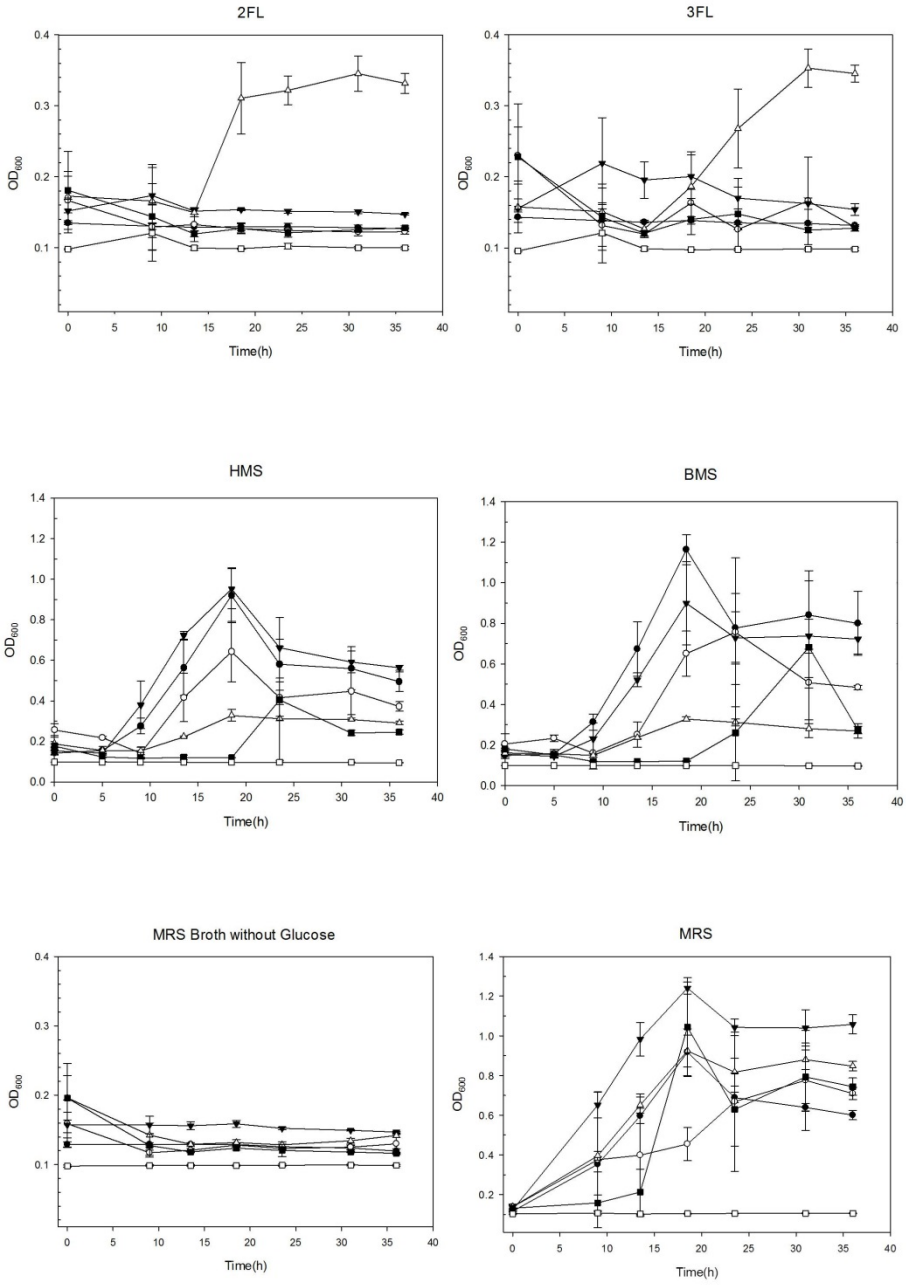
2FL
3FL
HMS
BMS
MRS Broth without Glucose
MRS
OD600
Time(h)

Fig. 2 Growth curves of experimental bifidobacteria grown in MRS broth with different carbon sources. Each medium was added with 2FL (2-fucosyllactose, 6 mg/ml), 3FL (3-fucosyllactose, 6 mg/ml), HMS (human milk saccharides, 20 mg/ml), BMS (bovine milk saccharides, 20 mg/ml). Each medium was incubated with *B. adolescentis* KCTC 3216 (●), *B. breve* KCTC 3419 (○), *B. longum* subsp. *infantis* KCTC 3249 (▼), *B. bifidum* BGN4 (△), *B. longum* subsp. *longum* RD47 (■). Control (□) was not inoculated. The data are expressed as the average of triplicate determinations. Bars of standard deviations are also presented.

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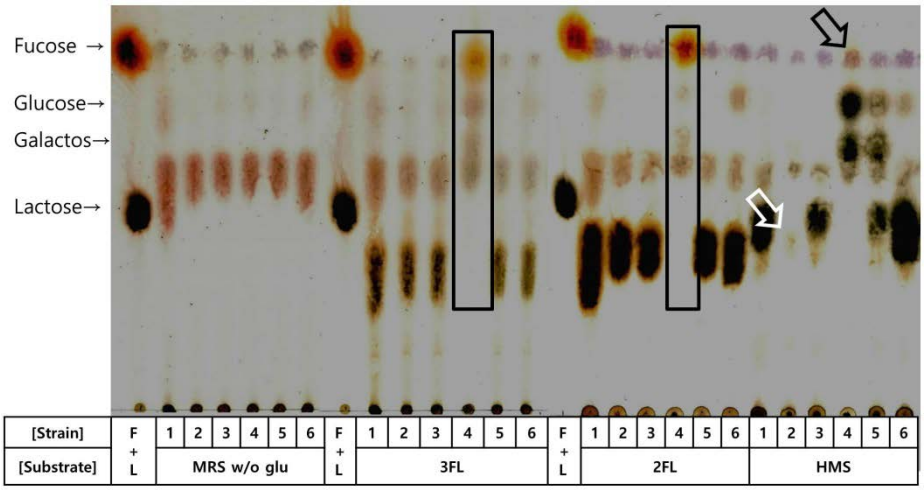

Fig. 3 TLC analysis of saccharides in the culture broth after cultivation of several bifidobacteria strains in the medium with 3FL, 2FL, and HMS. F+L : fucose(4%) + lactose(4%). 1 : *B. adolescentis* KCTC 3216, 2 : *B. breve* KCTC 3419, 3 : *B. longum* subsp. *infantis* KCTC 3249, 4 : *B. bifidum* BGN4, 5 : *B. longum* subsp. *longum* RD47, 6 : Control (non-fermented). MRS w/o glu : MRS without glucose, 3FL : MRS with 3-fucosyllactose (6 mg/ml), 2FL : MRS with 2-fucosyllactose (6 mg/ml), HMS : MRS with human milk saccharides (20 mg/ml). Each spot was loaded with 5 μl of culture broth except for F+L (1 μl). Hydrolyzed product of 2FL and 3FL by *B. bifidum* BGN4 are boxed and released fucose from HMS by *B. bifidum* BGN4 is indicated by black arrow. HMO in HMS which is not hydrolyzed by *B. breve* KCTC 3418 is indicated by white arrow.

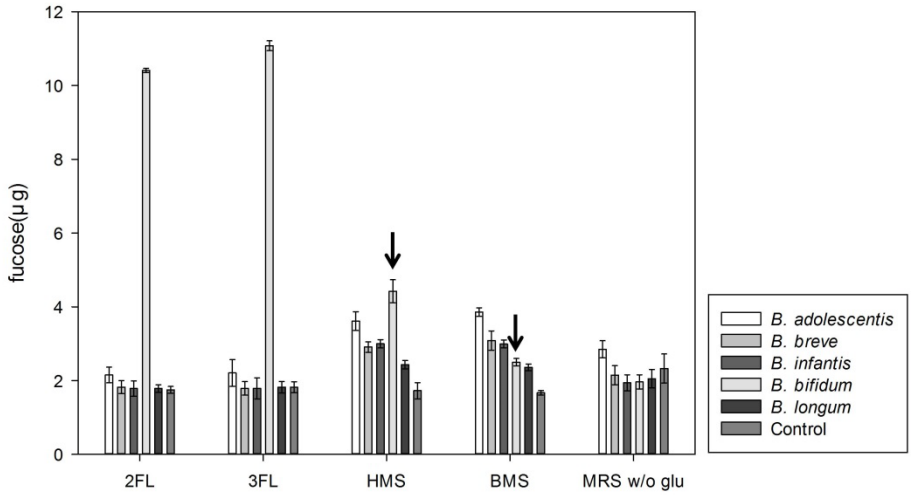

Fig. 4 Released fucose in the 5μl of media after culture of several *Bifidobacterium* strains. 2FL : 2-fucosyllactose (6 mg/ml), 3FL : 3-fucosyllactose (6 mg/ml), HMS : human milk saccharides (20 mg/ml), BMS : bovine milk saccharides (20 mg/ml), MRS w/o glu : MRS without glucose. Each fucose assay was performed using 5μl of culture broth. The data are expressed as the average of triplicate determinations. Bars of standard deviations are also presented. Released fucose from HMS and BMS by *B. bifidum* BGN4 are indicated by down arrows.

Cloning and activity assessments of α-L-fucosidase genes

Two open reading frames of putative α-L-fucosidase genes, *BBB_0176* (5,880bp) and *BBB_1341* (4,482bp), were identified in the full genome data of *B. bifidum* BGN4. The annotated sequences were used to design PCR primers, and to deduce the theoretical pI/MW (isoelectric point / molecular weight) using the ExPASy (The Expert Protein Analysis System, http://www.expasy.org).

BBB_0176 was PCR amplified (Fig. 6, a) and digested with Hind Ⅲ and Xho Ⅰ. It was ligated with Hind Ⅲ and Xho Ⅰ digested pET21 to construct p0176 and transformed into *E. coli* BL21 (Fig. 5). It is expected to produce recombinant protein 'AFL03772 + 6x His tag' with MW and pI of 206 kDa and 5.45, respectively. *BBB_1341* was PCR amplified (Fig. 6, b) and digested with Xho Ⅰ. It was ligated with Xho Ⅰ digested pET21 to construct p1341 and transformed into *E. coli* BL21 (Fig. 5). It is also expected to produce recombinant protein 'AFL04933 + 6x His tag' with MW and pI of 159 kDa and 5.15, respectively. Each recombinant *E.coli* BL21 strain harboring p0176 and p1341 was designated as E0176 and E131, respectively.

The plasmid DNA purified from the two clones of α-L-fucosidase genes (p0176 and p1341) were digested by Bgl II for confirmation by band pattern and size estimation. The expected 11,316 bp band from the

p0176 (Fig. 6 : c) and 6,952 and 2,983bp bands from p1341 (Fig. 6 : d) were shown.

The TLC assay in Fig. 7 and the fucose assay in Fig. 8 showed that the crude enzyme of BL21 harboring empty vector (Fig. 7 : 2, Fig. 8 : pET21) did not show fucose releasing activity for all substrates. On the other hand, the crude enzyme of E0176 (Fig.7 : 3, Fig. 8 : E0176) released fucose by hydrolyzing the α-(1→2) linkage of 2FL and α-(1→3) linkage of 3FL. The crude enzyme of E1341 (Fig. 7 : 4, Fig. 8 : E1341) showed more than doubled hydrolyzing activity for the α-(1→3) linkages of 3FL compared to E0176, but not for 2FL.

In the case of HMS, E0176 showed stronger hydrolysis activity compared to E1341 (Fig. 8), probably because E0176 can hydrolyze both α-(1→2) and α-(1→3) linkages of fucosylated residue of FO in HMO, whereas E1341 can only hydrolyze α-(1→3) linkages.

As a result, the two recombinant α-L-fucosidases were found to hydrolyze two major FO, 2FL and 3FL, present in HMO. This observation was similar to that of the two α-L-fucosidases of *B. bifidum* JCM1254, which were previously shown to successfully utilize fucosylated HMO [3].

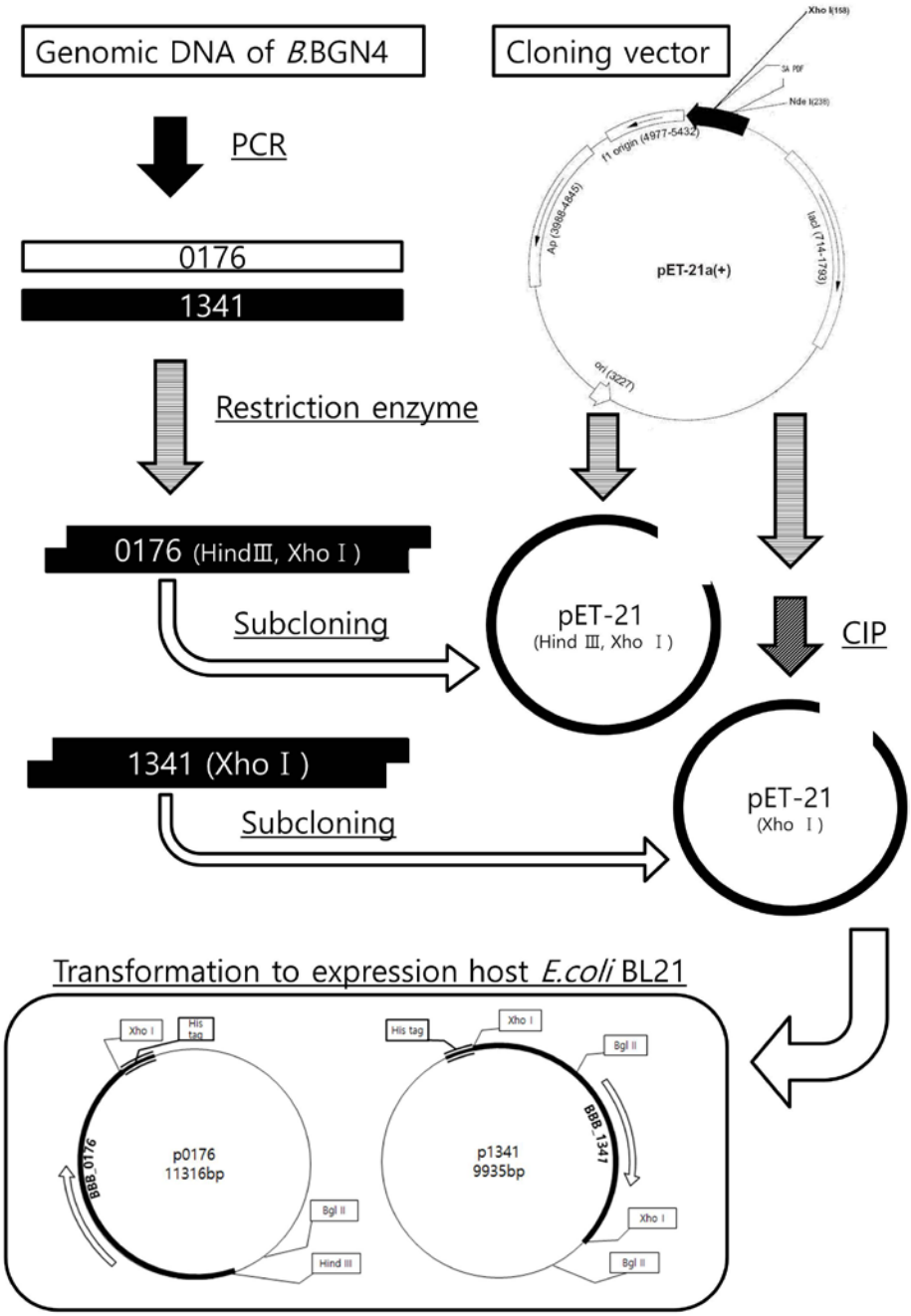

Fig. 5 Cloning strategy of each α-L-fucosidase genes (*BBB_0176, BBB_1341*) from *B. bifidum* BGN4.

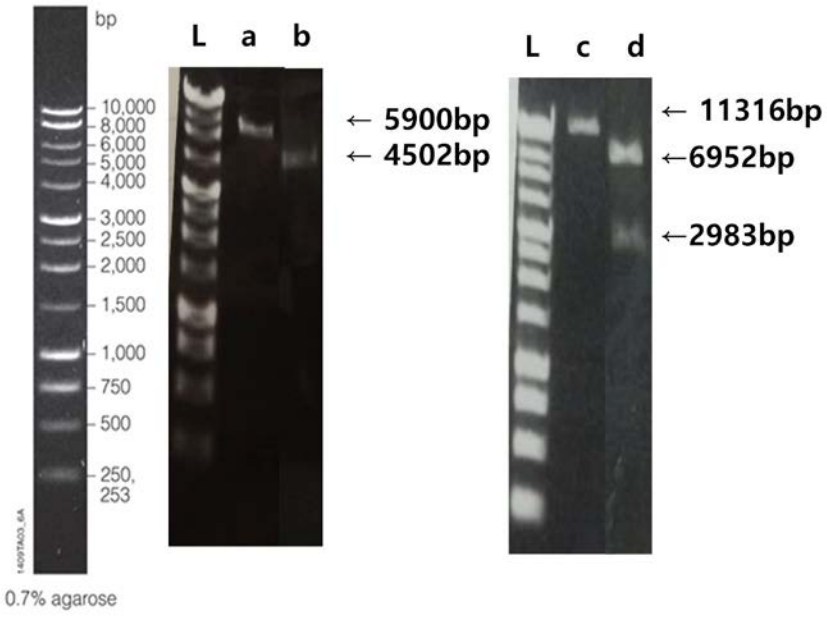

Fig. 6 PCR amplification of two a-L-fucosidase genes from *B. bifidum* BGN4 and Bgl II digestion of p0176 and p1341. L : 1 kb DNA ladder, a : PCR amplified *BBB_0176*, b : PCR amplified *BBB_1341*, c : p0176 digested by Bgl II, d : p1341 digested by Bgl II

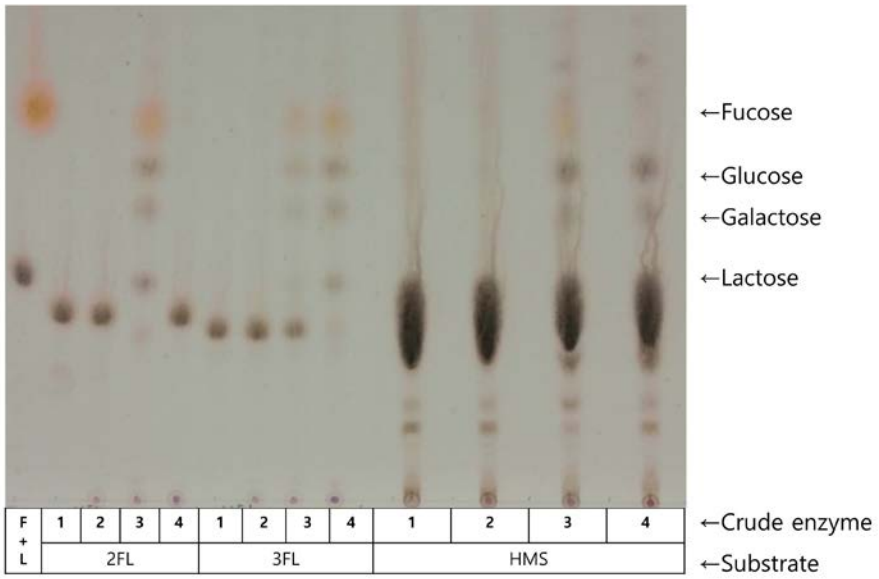

Fig. 7 TLC of hydrolysis products of 2FL, 3FL, and HMS using crude enzyme of α-L-fucosidases obtained from recombinant *E. coli* BL21. F+L : fucose (4%) + lactose (4%), 1 : Distilled water, 2 : Cell free extract from BL21 harboring pET21a(+), 3 : Cell free extract from E0176, Cell free extract obtained from E1341, 2FL (2-fucosyllactose, 15 mg/ml), 3FL (3-fucosyllactose, 15 mg/ml), HMS (human milk saccharides, 300 mg/ml). Two μl of substrates and 3μl of each crude enzyme were incubated at 37°C, 30min. Every spot was loaded with 2μl of reactant, except for F+L (1ul).

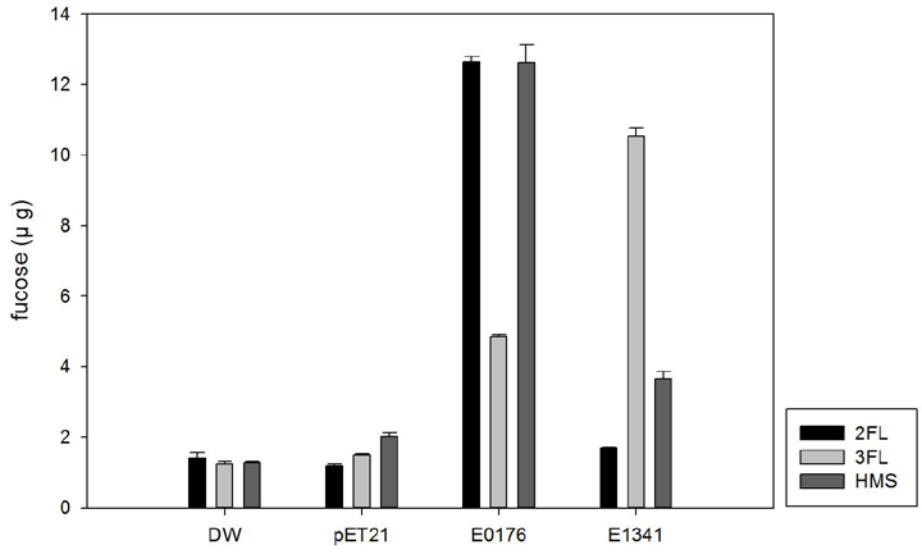

Fig. 8 Hydrolyzed fucose from 2FL, 3FL, and HMS by crude enzymes of each recombinant *E. coli* BL21.

DW : Distilled water (control), pET21 : Cell free extract from BL21 harboring pET21a(+), E0176 : Cell free extract from BL21 harboring p0176, E1341 : Cell free extract obtained from BL21 harboring p1341, 2FL (2-fucosyllactose, 15 mg/ml), 3FL (3-fucosyllactose, 15 mg/ml), HMS (human milk saccharides, 300 mg/ml). Each fucose assay was performed using 5μl of reactant (2μl of substrates and 3μl of each crude enzyme, 37°C, 30min). The data are expressed as the average of triplicate determinations. Bars of standard deviations are also presented.

Purification of α-L-fucosidases

His-tag purification and gel filtration were conducted to purify the recombinant α-L-fucosidase. After the purification by his-tag column, AFL03772 (recombinant protein of α-L-fucosidase gene *BBB_0176*) showed a protein band with a molecular mass of around 200 kDa, which corresponds with the molecular mass of 206 kDa deduced from gene sequence. However, the his-tag purified protein yielded two major bands on SDS-PAGE (Fig. 9 : lane C5). This eluted fraction was further purified to obtain one major band via gel filtration with Superdex 200 column (Fig. 9 : lane 6).

In the case of AFL04933 (recombinant protein of α-L-fucosidase gene *BBB_1341*), crude enzyme was used for the characterization because of the low purification efficiency of his-tag column purification.

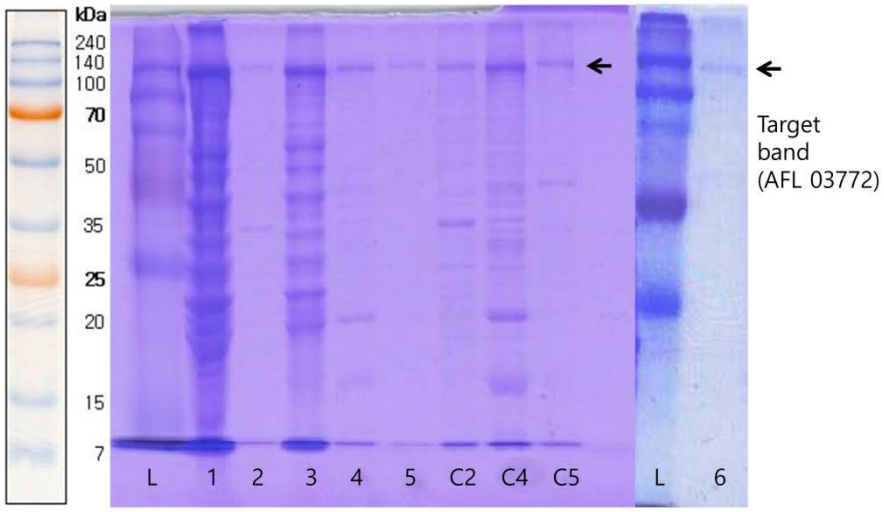

Fig. 9 SDS-PAGE of AFL03772 (recombinant protein of α-L-fucosidases gene *BBB_0176*) purified by His-tag purification and gel filtration. L : ladder, 1 : Crude enzyme of BL21 harboring p0176, 2 : Elution fraction 1, 3 : Flow through, 4 : Elution fraction 2, 5 : Elution fraction 3, C2 : Concentrated 2, C4 : Concentrated 4, C5 : Concentrated 5, 6 : Concentrated gel filtration purified C5.

The effects of temperature and pH on α-L-fucosidase activities

The optimal temperature of α-L-fucosidases were determined to be 35°C (Fig. 10 : a) for AFL03772 and 45°C (Fig. 10 : c) for AFL04933, respectively, when investigated in the pB buffer (20 mM, pH 7.8).

The effect of pH on AFL03772 was investigated from 4.05 to 7.48, and the optimal pH was observed at 6 - 6.54 (Fig. 10 : b). In the case of AFL04933, the optimal pH was 10 when examined in the range of 7 - 12 (Fig. 10 : d).

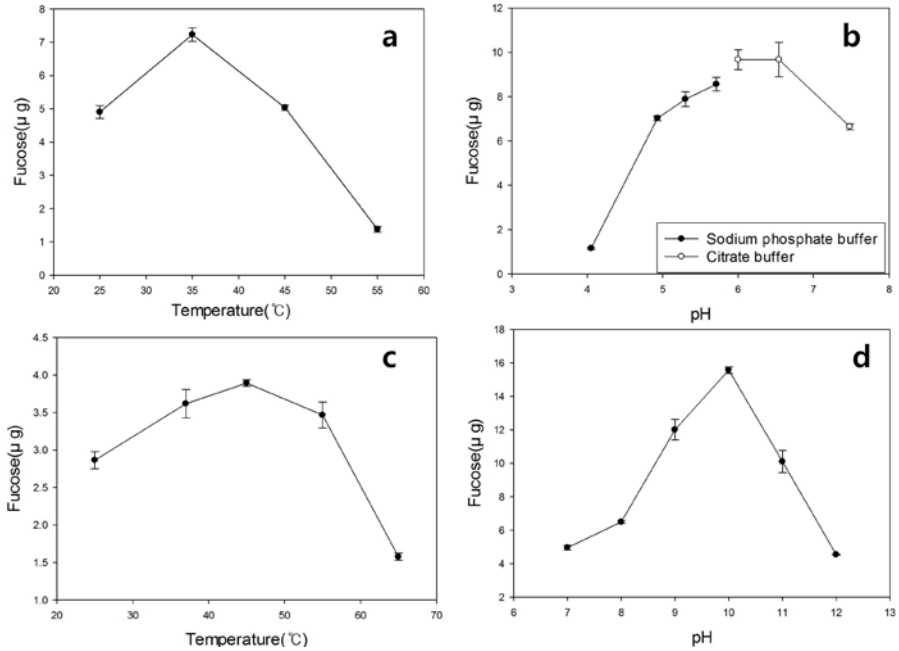

Fig. 10 Effects of temperature and pH on the activities of AFL03772 (recombinant protein of *BBB_0176*) and crude AFL 04933 (recombinant protein of *BBB_1341*). a : Effect of temperature on AFL03772, b : Effect of pH on AFL03772, c : Effect of temperature on AFL04933, d : Effect of pH on AFL04933. The data are expressed as the average of triplicate determinations. Bars of standard deviations are also represented.

4. Conclusion

In this research, the selective utilization of fucosylated oligosaccharides - which are a major component of HMO - has been confirmed for *B. bifidum* BGN4. In addition, the corresponding genes of two α-L-fucosidases which are essential for the hydrolysis and utilization of fucosylated HMO were expressed in *E. coli* BL21 and purified for characterization. This is the first report to confirm the activity and characteristics of the annotated hypothetical fucosidase genes in the genome of *B. bifidum* BGN4. Also, the crucial role of α-L-fucosidases for the preferential utilization of FO and successful growth of *B. bifidum* BGN4 in FO was confirmed. *B. bifidum* BGN4 may successfully colonize the intestinal tracts of the breast-fed infants by using fucosylated HMO and may confer beneficial effects on the breast-fed infants.

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국문초록

*Bifidobacterium*은 모유 영양아의 장내에서 우세한 성장을 보이는 유익균이며, 아기 장내 균총의 균형 유지에 유익한 영향을 미친다. 모유 올리고당 (Human milk oligosaccharide, HMO)은 모유에서 세번째로 많은 고형 성분(15 g/L)이며 퓨코실 올리고당이 (fucosylated oligosaccharides, FO) 약 75%의 높은 비율로 존재한다. 따라서 모유에서 소화되지 않고 대장에 도달한 퓨코실 올리고당을 이용할 수 있는 *Bifidobacterium*은 모유 영양아의 장내에서 선택적인 성장이 가능할 것으로 생각된다.

몇 종류의 *Bifidobacterium* 에 대하여 모유의 당과, 인공적으로 합성된 모유 퓨코실 올리고당의 주요 구조 2가지 (2-fucosyllactose, 3-fucosyllactose) 의 이용능이 측정되었다. 실험한 *Bifidobacterium* 중 *B. bifidum* BGN4 가 2FL과 3FL에 의해 선택적으로 성장이 촉진되었으며 모유 당에서 fucose를 가수분해하였다.

이전에 보고된 *B. bifidum* BGN4의 유전체 서열에서 퓨코실 올리고 이용에 필수적인 효소인 α-L-fucosidase로 예상되는 유전자 2개 (*BBB_0176* and *BBB_1341*) 의 서열이 확인되었다. 이들을 클로닝하여 *E. coli* BL21에서 발현시킴으로서 각각의 유전자

가 발현된 효소 단백질의 활성과 특성을 확인하였다. *BBB_0176* 유전자가 발현된 AFL03772 단백질은 2FL의 α-(1→2)과 3FL의 α-(1→3) 결합에 대해 가수분해능을 보였으며, *BBB_1341* 유전자가 발현된 AFL04933 단백질은 3FL의 α-(1→3) 결합에 대한 가수분해능이 확인되었다. 본 연구에서는 *B. bifidum* BGN4 가 모유 속의 퓨코실 올리고 이용에 필요한 α-L-fucosidase 효소를 지니고 있다는 것을 확인함에 따라 *B. bifidum* BGN4를 모유 영양아의 장내에서 선택적인 성장이 가능한 유익균으로서 이용할 수 있는 가능성을 제시하였다.

주요어 : *Bifidobacterium bifidum* BGN4, α-L-fucosidase
퓨코실 모유 올리고당 이용능

학 번 : 2013-21513