Analyses of Bifidobacterial Glycosidases Involved in the Metabolism of Oligosaccharides

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Received for Publication, October 9, 2009

Many bifidobacteria produce an endo-α-N-acetylgalactosaminidase that liberates the O-linked galactosyl β-1,3N-acetylgalactosamine (GNB) from intestinal mucin glycoproteins. The molecular cloning of the Bifidobacterium longum enzyme was completed using information in public databases. The enzyme constitutes a novel glycoside hydrolase (GH) family 101 member. The gene encoding a specific 1,2-α-L-fucosidase was cloned from B. bifidum. The recombinant enzyme specifically hydrolyzes the terminal α-1,2-fucosidic linkages of various oligosaccharides, including human milk oligosaccharides and blood group substances. Analysis of its primary structure revealed that this enzyme constitutes a novel GH family 95 member. We also solved the crystal structure of its catalytic domain. We assumed that these bifidobacterial enzymes are involved in the metabolism of oligosaccharides in mucin glycoproteins that are abundant in the intestine. Some bifidobacteria strains produce a lacto-N-biosidase that releases galactosyl β-1,3N-acetylgalactosamine (LNB) from human milk oligosaccharides, but the other enteric bacteria do not. This disaccharide is one of the building blocks in human milk oligosaccharides and is rarely found in other mammalian milks. The lacto-N-biosidase gene was cloned from B. bifidum and we hypothesized that this enzyme is crucially involved in the degradation of human milk oligosaccharides. The genes encoding sialidase and α-1,3/4-L-fucosidase were also cloned from B. bifidum. These enzymes release modified sialic acid and L-fucose from human milk oligosaccharides, respectively. A solute-binding protein of a putative ABC transporter specific for GNB and LNB was also discovered, and its gene was cloned from B. longum. We named it GNB/LNB-binding protein and crystallized it. Isothermal titration calorimetry measurements revealed that this protein specifically binds GNB and LNB. We speculate that bifidobacteria have a novel GNB/LNB metabolic pathway.

Key words: human milk oligosaccharides; lacto-N-biose I; mucin glycoprotein; galacto-N-biose; glycosidases

INTRODUCTION

Bifidobacteria colonize the lower intestinal tract, an environment poor in mono- and di-saccharides, because sugars are preferentially consumed by the host and microbes present in the upper intestinal tract. Therefore, to survive in the lower intestinal tract, bifidobacteria seem to produce various kinds of exo- and endo-glycosidases in surface-bound and extracellular forms, by which they utilize diverse carbohydrates (10, 17, 22).

The epithelial cells of the human intestine express and secrete mucin glycoproteins (5). Intestinal mucin glycoproteins possess an abundance of O-glycosidic oligosaccharides(21). The intestinal mucin glycoproteins are believed to be a source of energy for intestinal bacteria. We hypothesized that bifidobacteria might act on mucin glycoproteins in the intestinal tract because we found that many bifidobacteria have the specific endoglycosidase, endo-α-N-acetylgalactosaminidase, which catalyzes the hydrolysis of the O-glycosidic α-linkage between the sugar chain and a serine or threonine residue in mucin glycoprotein (15).

Generally, the intestines of breast-fed infants contain microflora that are dominated by bifidobacteria, in contrast to the contents of the intestines of formula-fed infants (8, 31). It has been widely accepted that oligosaccharides in human milk play a key role in the growth of bifidobacteria (6, 16), because human milk contains a number of different oligosaccharides compared to formula milk. However, it remains unknown what structures constitute the growth-stimulating factors for bifidobacteria in human milk. Human milk is reported to contain more than 100 types...
of oligosaccharide, the building blocks of which contain the following three basic core disaccharides: lactose (Galβ1-4Glc), lacto-N-biose I (LNB; Galβ1-3GlcNAc), and N-acetyllactosamine (LacNAc; Galβ1-4GlcNAc) (26). These oligosaccharides are often modified by sialic acid and/or L-fucose (16). Recently, we found that some bifidobacteria have a unique metabolic pathway specific for LNB and galacto-N-biose (GNB; Galβ1-3GlcNAc). GNB is the common O-glycan core structure in mucin glycoprotein and is the product released by endo-α-N-acetylgalactosaminidase.

In this review, we describe the molecular cloning and characterization of various glycosidases involved in the metabolism of oligosaccharides to provide a better understanding of the catabolism of sugars in bifidobacteria.

**BIFIDOBA
erIAL GLYCOSIDASES INVOLVED IN CATABOLISM OF SUGAR CHAINS IN MUCIN GLYCOPEPTIDES**

In the habitat of bifidobacteria, the intestinal tract, mucin glycoproteins are abundantly present and secreted (21). Mucin glycoproteins contain many O-linked oligosaccharides, and galactosyl β1,3N-acetylgalactosamine (GNB), attached to the hydroxyl group of serine or threonine residues via α-linkage is one of the most abundant core structures present in the intestine (18). These O-linked sugar chains are frequently modified by α-L-fucosyl residues at the non-reducing ends. Such residues are also found in sugar chains of glycolipids, in blood group substances, and in human milk oligosaccharides (24).

**Endo-α-N-acetylgalactosaminidase**

Endo-α-N-acetylgalactosaminidase (endo-α-GalNAc-ase) catalyzes the hydrolysis of the O-glycosidic α-linkage between galactosyl β3N-acetylgalactosamine (GNB) and a serine or threonine residue in mucin glycoproteins from various animal sources (15). This enzyme is a unique endoglycosidase and has been found in the culture fluids of several pathogenic bacteria such as *Clostridium perfringens* (11) and *Streptococcus pneumoniae* (2).

We found endo-α-GalNAc-ase in many bifidobacteria. To elucidate the function of this enzyme in the bacteria, we carried out the molecular cloning, expression, and characterization of the enzyme from *Bifidobacterium longum* JCM 1217 (4). Based on the information obtained from the genome sequencing database of *B. longum* NCC2705 (23), the bacteria has several predicted endoglycosidases such as endo-β-xylanase, endo-β-N-acetylglucosaminidase, endo-α-arabinosidase, and arabinogalactan endo-β-galactosidases, which might be useful for the intake of extracellular carbohydrate polymers. As the enzyme was found in the culture fluids of the bacteria, we searched for an uncharacterized hypothetical protein containing a secretion signal and a transmembrane domain using BLAST and Pfam HMM searches, and found one potential sequence to be an endo-α-GalNAc-ase. Then, using the sequence of the corresponding protein from *B. longum* NCC2705, oligonucleotide primers were designed and a PCR product was amplified from the genomic DNA of *B. longum* JCM1217, which was confirmed to produce endo-α-GalNAc-ase. The upstream and downstream regions of the gene were obtained using colony

### Table 1. Substrate specificity of *B. longum* endo-α-N-acetylgalactosaminidase

| Substrate Type       | Relative Activity (%) |
|----------------------|-----------------------|
| GalNAc-β-pNP         | 100                   |
| Galβ1-3GalNAc-β-pNP  | 90                    |
| Galβ1-3(GlcNAcβ1-6)GalNAc-β-pNP | 50         |
| GlcNAcβ1-3GalNAc-β-pNP | 30        |
| GlcNAcβ1-3GalNAc-β-pNP | 20        |
| GalNAcβ1-6GalNAc-β-pNP | 10        |
| Galβ1-3GalNAc-β-pNP  | 50                    |
| Glcβ1-3GalNAc-β-pNP  | 50                    |
| Glcβ1-6GalNAc-β-pNP  | 50                    |
| GalNAcβ1-3GalNAc-β-pNP | 35.3     |
| Galβ1-6GlcNAc-β-pNP  | 13.3                  |
| Galβ1-3GalNAc-β-pNP  | 17.3                  |
| Galβ1-3GlcNAc-β-pNP  | 17.3                  |
hybridization and PCR, respectively. The gene comprises an ORF of 5,901 bp encoding a protein of 1,966 amino acids. The coding sequence of endo-α-GalNAc-ase had no homologous domains to known Glycoside Hydrolase (GH) families in the Carbohydrate-Active Enzyme (CAZy) database. Therefore, a novel GH family 101 was established.

We expressed a full-length His-tagged enzyme in *Escherichia coli* BL21(DE3) and the soluble cell lysate exhibited endo-α-GalNAc-ase activity. The His-tagged endo-α-GalNAc-ase was purified and the enzyme migrated as a single band on SDS-PAGE. We examined the substrate specificity of the recombinant enzyme using synthetic pNP substrates and natural glycoproteins (Table 1). Analysis of the reaction mixtures indicated that Galβ1-3GalNAc disaccharide was liberated from asialofetuin, which is a natural glycoprotein, and a pNP substrate containing the core 1 substrate (Galβ1-3GalNAcα1-). The enzyme also released unnatural Glcβ1-3GalNAc and GalNacβ1-3GalNAc from pNP-substrates, but never acted on other pNP-core substrates (core 2–8) (30) or sialofetuin. Recently, we solved the crystal structure of the catalytic domain of this enzyme and revealed the structural basis for substrate recognition (25, 26).

**1,2-α-L-Fucosidase**

The human intestinal tract secretes mucin glycoproteins including blood group substances. The ABO-type blood group substances include an α-1,2-fucosyl residue in common (20). The mucin glycoproteins on the intestinal wall also seem to be rich in many α-1,2-fucosyl residues. We assumed that bifidobacteria might have an ability to degrade blood group substances in the mucin glycoproteins on the intestinal wall because bifidobacteria colonize the lower intestinal tract, which is poor in saccharides.

We found an 1,2-α-L-fucosidase activity, cleaving the α-1,2-fucosyl linkage, in various strains of bifidobacteria, and cloned the gene by screening a genomic library of *B. bifidum* JCM 1254 for the ability to hydrolyze 2′-fucosyllactose (12). The enzyme, when expressed in *E.coli*, was a huge protein with 1,959 amino acids, possessing a secretory signal sequence at the N-terminus and a membrane-anchoring motif at the C-terminus. The truncated recombinant protein selectively hydrolyzed α-1,2-linked L-fucosyl residues at the non-reducing end. It effectively liberated L-fucose from glycoproteins, such as porcine stomach mucin, as well as blood-type antigens and human milk oligosaccharides such as lacto-α-fucopentaose I. The deduced amino acid sequence of this enzyme was completely different from previously identified α-L-fucosidases. In the CAZy database, this enzyme was classified as GH family 95, a new family created for this enzyme.

The crystal structure of the catalytic domain of the enzyme was also determined (19). The overall structure consists of four regions: an N-terminal β-region, a helical linker region, a helical barrel (α/α)6 domain, and a C-terminal β-region. The helical barrel (α/α)6 domain forms a catalytic pocket, in which the fucosylα1-2galactose moiety of 2′-fucosyllactose may be buried (Fig. 1).

**BIFIDOBACTERIAL GLYCOSIDASES INVOLVED IN DEGRADATION OF HUMAN MILK OLIGOSACCHARIDES**

Cow’s milk contains lactose as the sole saccharide, and the major carbohydrate present in human milk is also lactose, but it also contains many oligosaccharides. Human milk oligosaccharides (HMOs) are composed of 12 core structures (7) (Table 2). These core structures are often modified with α-L-fucosyl and/or α-sialyl residues. To date, at least 93 kinds of molecule have been identified. Core structures of HMOs other than lactose are classified into type I and II, based on the disaccharide unit at the non-reducing end. The building blocks are three basic core disaccharides, such as lactose, lacto-N-biose, and N-acetyllactosamine, as well as modifying monosaccharides, such as sialic acid and L-fucose. Type I and II sugars contain lacto-N-biose I (LNB, Gaβ1-3GalNAc) and N-acetyllactosamine (LacNAc, Galβ1-4GlcNAc) structures at the non-reducing end, respectively. Type I oligosaccharides are predominant in HMOs and type II oligosaccharides are minor.
components. Among them, 2'-fucosyllactose (Fucα1-2Galβ1-4Glc, 2'FL), lacto-N-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNT), and lacto-N-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNFPI) are the most abundant components of HMOs, and the latter two are type I oligosaccharides. The domain of type I milk oligosaccharides is a specific feature of human milk, which is hardly found in other mammals (29).

Oligosaccharides in human milk, other than lactose, play a key role in the growth of bifidobacteria because the intestinal flora of breast-fed infants is predominantly bifidobacteria, whereas bottle-fed infants do not show such a rapid colonization (33). The mechanism whereby HMOs promote the selective growth of bifidobacteria has long been unsolved because of the difficulty created by the complicated content of HMOs.

Lacto-N-biosidase

We studied how bifidobacteria degrade HMOs and, using cell suspensions of various bifidobacteria, we found that several bifidobacteria strains have an enzyme releasing lacto-N-biose (LNB) from lacto-N-tetraose (LNT), which is a major component of HMOs and includes a type I chain (31). Among bifidobacteria species known to be infant intestinal colonizers, B. bifidum and B. longum showed this enzyme activity, but no activities were detected in the other bifidobacteria or in the other enteric bacteria, such as clostridia, bacteroides, and lactobacilli. This shows that only bifidobacteria that colonize infant intestines seem to have such enzyme activity. Therefore, we cloned the gene encoding the enzyme (lacto-N-biosidase) from B. bifidum JCM1254 (31). Analysis of the primary structure of this enzyme revealed the presence of a signal peptide and a membrane anchor at the N-terminus and C-terminus, respectively. The protein consists of 1,112 amino acids. To estimate the substrate preference of this enzyme, we used various pNP-sugars as substrates and found that the enzyme was most active towards pNP-β-lacto-N-biose I (pNP-β-LNB) and less active towards pNP-β-galacto-N-biose (pNP-β-GNB) (30% compared with pNP-β-LNB). The enzyme did not hydrolyze α-linked disaccharides, it did not act on any β-linked pNP-monosaccharides including pNP-β-GlcNAc and pNP-β-GalNAc, and also could not hydrolyze the fucosylated forms of LNT (lacto-N-fucopentaose I and II) or lacto-N-neotetraose (including type II chain) (Table 3). These results reveal that the lacto-N-biosidase from B. bifidum has a substrate preference for unmodified β-linked lacto-N-biose I.

Table 2. Structure of core oligosaccharides composed of human milk oligosaccharides

| Oligosaccharide | Structure |
|-----------------|-----------|
| Lactose         | Gal(β1-4)Glc |
| Lacto-N-tetraose (LNT) | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc |
| (Type I LNB)    |           |
| Lacto-N-neotetraose | Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc |
| (Type II LacNAc) |           |
| Lacto-N-hexaose | Gal(β1-4)GlcNAc(β1-6) |
|                 | Gal(β1-4)Glc |
|                 | Gal(β1-3)GlcNAc(β1-3) |
| Lacto-N-neohexaose | Gal(β1-4)GlcNAc(β1-6) |
|                 | Gal(β1-4)Glc |
|                 | Gal(β1-3)GlcNAc(β1-3) |
| Paralacto-N-hexaose | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc |
|                 | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc |
| Paralacto-N-neohexaose | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc |
|                 | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc |
| Paralacto-N-octaose | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc |
BIFIDOBACTERIAL GLYCOSIDASES FOR METABOLISM OF OLIGOSACCHARIDES

(Sialidases)
In HMOs, there are many sialyl oligosaccharides modified by sialic acid on their non-reducing ends. The lacto-N-biosidase, which is the key enzyme for degrading HMOs, cannot act on sialyl oligosaccharides because sialic acid is a hindrance for this enzyme. Thus, the removal of sialic acid is necessary for the degradation of oligosaccharides by the lacto-N-biosidase. We then discovered two sialidases from \textit{B. bifidum} JCM1254 cloned their genes, and characterized the enzymes.

Sequence analyses revealed that the two sialidases have a signal peptide and a membrane anchor at the N-terminus and C-terminus, respectively. One of these sialidases, SiaBb1, which comprises 1,795 amino acids, contains an esterase-like catalytic domain in addition to the sialidase catalytic domain. SiaBb2 (835 amino acids) must be a primitive form of sialidase, because it contains only a sialidase catalytic domain. SiaBb1 expressed in \textit{E.coli} did have an esterase activity, as determined using \textit{p-NP-acetate} as substrate, confirming that this enzyme is a bi-functional protein. SiaBb1 released an acetyl residue from 9-O-acetyl sialic acid that is one molecular species of sialic acids, suggesting that it has esterase activity. On the other hand, SiaBb2 did not release an acetyl residue. We hypothesize that SiaBb1 changes 9-O-acetyl sialic acid to sialic acid, which is easily degraded by normal sialidase.

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Strain & Activity & Strain & Activity \\
\hline
\textit{Bifidobacterium adolescentis} JCM1275 & – & \textit{Bacteroides ovatus} JCM5824 & – \\
\textit{Bifidobacterium adolescentis} JCM7046 & – & \textit{Bacteroides thetaiotaomicron} JCM5827 & – \\
\textit{Bifidobacterium angulatum} JCM7096 & – & \textit{Clostridium celatum} JCM1394 & – \\
\textit{Bifidobacterium animalis} JCM10602 & – & \textit{Clostridium hylemonae} JCM10539 & – \\
\textit{Bifidobacterium bifidum} JCM1255 & + & \textit{Clostridium perfringens} JCM1290 & – \\
\textit{Bifidobacterium bifidum} JCM7004 & + & \textit{Clostridium perfringens} JCM3816 & – \\
\textit{Bifidobacterium breve} JCM1192 & – & \textit{Clostridium perfringens} JCM3818 & – \\
\textit{Bifidobacterium catenulatum} JCM1194 & – & \textit{Clostridium scindens} JCM3819 & – \\
\textit{Bifidobacterium dentium} JCM1195 & – & \textit{Enterococcus pseudaoavium} JCM8732 & – \\
\textit{Bifidobacterium longum} JCM1210 & – & \textit{Eubacterium elongatum} JCM6421 & – \\
\textit{Bifidobacterium longum} JCM1217 & + & \textit{Lactobacillus casei} JCM1134 & – \\
\textit{Bifidobacterium longum} JCM1222 & – & \textit{Lactobacillus gasseri} JCM1130 & – \\
\textit{Bifidobacterium longum} JCM7054 & + & \textit{Lactobacillus johnsonii} JCM8794 & – \\
\textit{Bifidobacterium pseudocatenulatum} JCM1200 & – & \textit{Lactobacillus paracasei} JCM1181 & – \\
\textit{Bifidobacterium pseudolongum} JCM1205 & – & \textit{Lactobacillus plantarum} JCM1149 & – \\
\textit{Bifidobacterium scardovii} JCM12489 & – & \textit{Lactobacillus reuteri} JCM1112 & – \\
\textit{Bifidobacterium scardovii} JCM12489 & – & \textit{Propionibacterium acnes} JCM6425 & – \\
\hline
\end{tabular}
\caption{Lacto-N-biosidase activities found from various bifidobacteria and intestinal bacteria}
\end{table}

\textit{Fucosidases}
In addition to 1,2-\textit{\alpha}-L-fucosidase and sialidase, bifidobacteria seem to have another cleaving enzyme acting on side-chains of HMOs; an \textit{\alpha}-1,3/4-fucosidase. The enzyme was duly isolated and molecularly cloned from \textit{B. bifidum} JCM1254 (1).

The major modifications of the core structures of HMOs are \textit{\alpha}-1,2/3/4-fucosylation and \textit{\alpha}-2,3/6-sialylation; therefore, the presence of these enzymes is beneficial for bifidobacteria to unmask the core structure of HMOs and to further degrade them.

\textit{GNB/LNB TRANSPORTER}
Derensy-Dron et al. characterized a \textit{\beta}-1,3-galactosyl-N-acetylhexosamine phosphorylase (EC 2.4.1.211) in a cell-free extract of \textit{B.bifidum} DSM20082, which has the ability to produce galactose-1-phosphate (Gal-1-P) and GlcNAc from LNB (3). This enzyme could also phosphorylze GNB, which is produced by endo-\textit{\alpha}-GalNAc-ase, to Gal-1-P and GalNAc. Kitaoka et al. purified this enzyme (13) and found that N-terminal and internal amino acid sequences of the enzyme showed high identities with the BL1641 protein of \textit{B.longum} NCC2705 whose genome sequence is available (23). They cloned the homologous gene, \textit{lnpA}, from the type strain \textit{B.longum} JCM1217 and confirmed that the gene encoded a \textit{\beta}-1,3-galactosyl-N-acetylhexosamine phosphorylase (GLNBP) (13). This result suggests that the catabolism of GNB from mucin glycoprotein is catalyzed by endo-\textit{\alpha}-GalNAc-ase, and that of LNB in
HMOs is catalyzed by various HMO-cleaving enzymes, including lacto-N-biosidase in *B. longum* JCM1217. Kitaoka et al. confirmed that three downstream genes encode catalytic enzymes involved in the Leloir-like galactose pathway (13). This suggests that bifidobacteria possess a specific transporter system to incorporate GNB and LNB. We investigated the presence of the ATP-binding cassette (ABC)-type sugar transporter genes located upstream of *lnpA* gene of *B. longum* JCM1217 (27). Generally, the bacterial ABC transporter system includes a solute binding protein that binds to substrates and delivers them to the membrane-associated transporter complex. Thus, we cloned the solute-binding protein gene of *B. longum* JCM1217 (27), expressed it in recombinant *E. coli* and purified the enzyme. The protein (named GNB/LNB-binding protein, GL-BP) specifically binds GNB (Kd = 10 nM) and LNB (Kd = 87 nM) in an enthalpy-driven manner, which was confirmed by isothermal titration calorimetry measurement (Fig. 2). It does not bind to monosaccharides and other disaccharides including LacNac. It possesses the capacity to bind with LNT with a much higher Kd value (11 μM), indicating that LNT is a poor ligand of GL-BP. These results indicate that several bifidobacterial strains can liberate LNB from type I HMOs through the action of lacto-N-biosidase and then import it using a GNB/LNB-specific transporter. We also determined the crystal structure of GL-BP (32) and showed that the interactions between GL-BP and the disaccharide ligands mainly occur through water-mediated hydrogen bonds. In comparison with the LNB complex, one additional hydrogen bond was found in the GNB complex (27).

**CONCLUSION**

Bifidobacteria seem to have a special metabolic pathway for galactosyl β1,3 N-acetylgalactosamine (GNB), which is released from intestinal mucin glycoproteins by endo-α-N-acetylgalactosaminidase. They also have the ability to produce galactosyl β1,3 N-acetylgalcosamine (LNB) from human milk oligosaccharides. The bacteria seem to import the disaccharides released into the cells through a specific transporter. Thus, we hypothesize that human milk oligosaccharides are digested by extracellular sialidases and α-L-fucosidases, and are then cleaved by lacto-N-biosidase to lacto-N-biose that is sequestered by cells using the transporter. The putative metabolic pathway of LNB/GNB is shown in Fig. 3. We also found that LNB is a growth-promoting factor for bifidobacteria (14). Our results provide a basis for the development of new prebiotics in the near future.

**Acknowledgments.** This work was supported in part by
a Grant-in-Aid for the Protein 3,000 project of the Ministry of Education, Culture, Sport, Science and Technology of Japan and also by a Grant-in Aid for the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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