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REVIEW

Novel Bifidobacterial Glycosidases Acting on Sugar Chains of Mucin Glycoproteins

TAKANE KATAYAMA,[1] KIYOTAKA FUJITA,[1] AND KENJI YAMAMOTO[1]*

Graduate School of Biostudies, Kyoto University,
Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan[1]

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Bifidobacterium bifidum **was found to produce a specific 1,2-α-L-fucosidase. Its gene (*afcA*) has been cloned and the DNA sequence was determined. The AfcA protein consisting of 1959 amino acid residues with a predicted molecular mass of 205 kDa can be divided into three domains; the N-terminal function-unknown domain (576 aa), the catalytic domain (898 aa), and the C-terminal bacterial Ig-like domain (485 aa). The recombinant catalytic domain specifically hydrolyzed the terminal α-(1→2)-fucosidic linkages of various oligosaccharides and sugar chains of glycoproteins. The primary structure of the catalytic domain exhibited no similarity to those of any glycoside hydrolases but showed similarity to those of several hypothetical proteins in a database, which resulted in establishment of a novel glycoside hydrolase family (GH family 95). Several bifidobacteria were found to produce a specific endo-α-*N*-acetylgalactosaminidase, which is the endoglycosidase liberating the *O*-glycosidically linked galactosyl β1→3 *N*-acetylgalactosamine disaccharide from mucin glycoprotein. The molecular cloning of endo-α-*N*-acetylgalactosaminidase was carried out on *Bifidobacterium longum* based on the information in the database. The gene was found to comprise 1966 amino acid residues with a predicted molecular mass of 210 kDa. The recombinant protein released galactosyl β1→3 *N*-acetylgalactosamine disaccharide from natural glycoproteins. This enzyme of *B. longum* is believed to be involved in the catabolism of oligosaccharide of intestinal mucin glycoproteins. Both 1,2-α-L-fucosidase and endo-α-*N*-acetylgalactosaminidase are novel and specific enzymes acting on oligosaccharides that exist mainly in mucin glycoproteins. Thus, it is reasonable to conclude that bifidobacteria produce these enzymes to preferentially utilize the oligosaccharides present in the intestinal ecosystem.**

[**Key words:** bifidobacteria, *Bifidobacterium bifidum*, *Bifidobacterium longum*, 1,2-α-L-fucosidase, endo-α-*N*-acetylgalactosaminidase, mucin, *O*-linked oligosaccharide]

The human gastrointestinal tract is inhabited by a vast and diverse community of microbes, and a well-balanced microflora is thought to be important for normal digestion and maintenance of the intestinal ecosystem (1). In the complex intestinal microflora, bifidobacteria, which are gram-positive obligate anaerobes, are considered to be key commensals that promote a healthy intestinal tract because of their many beneficial effects on the host, such as regulation of the state of the intestine, reduction of harmful bacteria and toxic compounds, immuno modulation, and anticarcinogenic activity (2–5). Therefore, bifidobacteria have attracted a great deal of attention.

Bifidobacteria naturally colonize the lower intestinal tract, an environment poor in mono- and disaccharides since such sugars are preferentially consumed by the host and microbes present in the upper intestinal tract. Therefore, in order to survive in the lower intestinal tract, bifidobacteria produce various kinds of exo- and endoglycosidases in surface-bound and/or extracellular forms, by which they can utilize diverse carbohydrates (6, 7). Recent genome sequence analysis of *Bifidobacterium longum* NCC2705 revealed that more than 8.5% of the total predicted proteins were involved in the degradation of oligo- and polysaccharides, perhaps reflecting the superior ability of this organism to adapt to its environment (8).

The epithelial cells of the human intestine express and/or secrete mucin glycoproteins (9), which are assumed to play an important role in protecting enterocytes from chemical and physical damage as well as in preventing invasion by pathogens. Intestinal mucin glycoproteins contain a lot of *O*-linked oligosaccharides, on the non-reducing ends of which α-linked L-fucosyl residues are frequently found (10, 11). Such α-L-fucosyl residues are also present in glycolipids on the cell surface, submaxillary mucin, blood group substances, and oligosaccharides in human milk (11, 12).

* Corresponding author. e-mail: yamamotk@kais.kyoto-u.ac.jp
phone: +81-(0)75-753-6277 fax: +81-(0)75-753-6275

Taking into consideration that several fecal mucin-degrading bacteria such as bifidobacteria, clostridia, and bacteroides are known to produce α-L-fucosidase (13–15), it was envisaged that α-L-fucosidase might be responsible for the selective colonization of the intestine by these bacteria.

Meanwhile, intestinal mucin glycoproteins possess an abundant amount of *O*-glycosidic oligosaccharides linked glycosidically to the hydroxyl groups of serine or threonine residues of the protein (16). These oligosaccharides are also found sparsely in soluble glycoproteins. They are characterized by a core structure consisting of a galactosyl β1→3 *N*-acetylgalactosamine α1-serine/threonine residue, and are termed mucin type oligosaccharides. Endo-α-*N*-acetylgalactosaminidase (endo-α-GalNAc-ase; EC 3.2.1.97) is an endoglycosidase that catalyzes the hydrolysis of the *O*-glycosidic α-linkage between galactosyl β1→3 *N*-acetylgalactosamine (Galβ1-3GalNAc) and a serine or threonine residue in mucin glycoproteins of various animal sources (17).

Recently, we isolated and identified some bifidobacteria-specific enzymes that are involved in the degradation of sugar chains of intestinal mucin. We also revealed that these enzymes belong to novel glycoside hydrolase families. In this review, we describe the molecular cloning and characterization of 1,2-α-L-fucosidase (EC 3.2.1.63) and endo-α-GalNAc-ase from bifidobacteria, an intestinal colonizer strain, in order to obtain a better understanding of the catabolism of sugars in bifidobacteria and to examine the roles of sugar chain-degrading enzymes in intestinal bacteria. These unique enzymes are not normally found in microorganisms.

I. MOLECULAR CLONING OF 1,2-α-L-FUCOSIDASE FROM *BIFIDOBACTERIUM BIFIDUM* JCM1254

To date, α-L-fucosidases that liberate terminal α-linked L-fucose from the oligosaccharides of various glycoconjugates including mucin glycoprotein have been purified from several prokaryotic and eukaryotic sources (14, 15, 18–23), and the enzymes have been divided into two groups (19); one capable of hydrolyzing various types of fucosidic linkages as well as synthetic substrates, and another that is only active on the α-(1→2)-linkage, although a few reports have described enzymes that cannot be classified into either group (20, 21). In contrast to the dozens of studies on the purification of such enzymes, there is a paucity of reports regarding the cloning of α-L-fucosidase genes, and they are all derived from eukaryotic cells such as human and rat livers (24, 25), *Canis familiaris* (26), and *Dictyostelium discoideum* (27), which constitute glycoside hydrolase family 29 (GH family 29). To the best of our knowledge, there have been no reports regarding the isolation of a bacterial α-L-fucosidase gene, although the sequence of the 1,3-/4-α-L-fucosidase gene from *Streptomyces* sp. has been deposited in GenBank (accession no. U39394). We have found that a few bifidobacteria strains produced 1,2-α-L-fucosidase (EC 3.2.1.63) in cell surface-bound and/or extracellular forms, using 2′-fucosyllactose as a substrate. Among these bacteria, we chose *B. bifidum* JCM1254 and attempted to clone the 1,2-α-L-fucosidase gene (designated as *afcA*). The expression cloning was done using *Escherichia coli* DH5α, a non-fucosidase-producing bacterium. A genomic library of *B. bifidum* JCM1254 constructed in *E. coli* DH5α was screened for the ability to hydrolyze the α-(1→2)-linkage of 2′-fucosyllactose. One recombinant, designated SA3, was selected. Sequence analysis of plasmid pSA3 revealed that the cloned gene contained two large truncated open reading frames (designated as ORF1 and ORF2) (Fig. 1). The sense strands of ORF1 and ORF2 overlapped to a large extent in reverse, which is not surprising since such cases are sometimes found in the recently determined genome sequence of *B. longum* NCC2705 (8). In order to determine which ORF actually encodes 1,2-α-L-fucosidase, each ORF (the *Mfe*I-*Afl*II fragment) was placed under the control of the *lac* promoter and its expression was induced by the addition of IPTG. While no increase in 1,2-α-L-fucosidase activity was observed when ORF2 was induced, the activity was significantly elevated when ORF1 was induced, indicating that ORF1 encodes 1,2-α-L-fucosidase. The codon usage of the *afcA* gene was quite similar to that of other genes of *B. bifidum* in the database (Codon Usage Database in Kazusa DNA Research Institute), and Southern hybridization analysis with the 2.3-kb *Kpn*I fragment as a specific probe revealed that the *afcA* gene exists as asingle-copy on the genome of *B. bifidum* JCM1254. Analysis of the primary structure by using the SignalP (28) and PSORT (29) programs revealed the presence of a signal peptide and a membrane anchor at the N-terminus and C-terminus, respectively (Fig. 1). A possible ribosome-binding site was located 6-bp from a probable initiation codon and a promoter-like sequence was also found in the upstream region. The AfcA protein consists of 1959 amino acid residues with a calculated molecular mass of 205 kDa (GenBank accession no. AY303700).

II. DOMAIN STRUCTURE OF 1,2-α-L-FUCOSIDASE FROM *B. BIFIDUM* JCM1254

Since the primary structure of AfcA protein did not exhibit any similarity to those of known glycosidase families, we attempted to localize a catalytic domain that is essential for the hydrolysis of 2′-fucosyllactose. The N-terminal and C-terminal deletion mutants were constructed and expressed, and then their activity was assessed. Consequently, the 576 N-terminal amino acid residues (911–2638 bases) and 485 C-terminal amino acid residues (5333–6790 bases) were found to be removable without loss of fucosidase activity. These results revealed that the region consisting of the 577th–1474th amino acid residues constitutes the catalytic domain (Fig. 1). The region consisting of the 1475th–1728th amino acid residues contained four repetitive sequences with immunoglobulin-like folds, the so-called bacterial Ig-like domain B (Pfam 02368). Although the function of this domain is not clear, it is highly likely that this domain, 254 amino acids in length, at least acts to display the fucosidase domain of AfcA so that it protrudes from the cell surface, thereby enabling *B. bifidum* JCM1254 cells to gain access to and degrade the fucosyl residues present on the glycoconjugates of enterocytes. Neither sequence similarity to other ORFs nor a functional motif was found in the sequence of