Appl Microbiol Biotechnol (2015) 99:3941–3948
DOI 10.1007/s00253-014-6201-x

α-*N*-Acetylglucosaminidase from *Bifidobacterium bifidum* specifically hydrolyzes α-linked *N*-acetylglucosamine at nonreducing terminus of *O*-glycan on gastric mucin

Yoshimi Shimada · Yuka Watanabe · Takura Wakinaka · Yoshihisa Funeno · Masayuki Kubota · Thida Chaiwangsri · Shin Kurihara · Kenji Yamamoto · Takane Katayama · Hisashi Ashida

Received: 4 October 2014 / Revised: 26 October 2014 / Accepted: 29 October 2014 / Published online: 11 November 2014

Abstract α-Linked *N*-acetylglucosamine is one of the major glyco-epitopes in *O*-glycan of gastroduodenal mucin. Here, we identified glycoside hydrolase (GH) family 89 α-*N*-acetylglucosaminidase, termed AgnB, from *Bifidobacterium bifidum* JCM 1254, which is essentially specific to GlcNAcα1-4Gal structure. AgnB is a membrane-anchored extracellular enzyme consisting of a GH89 domain and four carbohydrate-binding module (CBM) 32 domains. Among four CBM32 domains, three tandem ones at C-terminus showed to bind porcine gastric mucin, suggesting that these domains enhance the enzyme activity by increasing affinity for multivalent substrates. AgnB might be important for assimilation of gastroduodenal mucin by *B. bifidum* and also applicable to production of prebiotic oligosaccharides from porcine gastric mucin.

Keywords α-*N*-Acetylglucosaminidase · *Bifidobacterium bifidum* · CBM32 · GH89 · Mucin · Probiotics

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-6201-x) contains supplementary material, which is available to authorized users.

Y. Shimada · Y. Funeno · M. Kubota · H. Ashida (✉)
Faculty of Biology-Oriented Science and Technology, Kinki University, Kinokawa, Wakayama 649-6493, Japan
e-mail: ashida@waka.kindai.ac.jp

Y. Watanabe · T. Wakinaka
Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

T. Chaiwangsri
School of Medical Science, University of Phayao, Phayao 56000, Thailand

S. Kurihara · T. Katayama
Host-Microbe Interaction Research Laboratory, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan

K. Yamamoto
Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan

Introduction

Bifidobacteria including *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium breve*, and *Bifidobacterium animalis* subsp. *lactis* are recognized as probiotics that are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." Bifidobacteria produce both lactic acid and acetic acid from sugars via hetero lactic acid fermentation and thereby lower the intestinal pH to prevent the growth of harmful bacteria (Fukuda et al. 2011). In addition, they stimulate the intestinal immunity to enhance antiviral and antibacterial activities (Picard et al. 2005; Trebichavsky et al. 2009). Since they originally reside in the lower intestines of mammals where the sugars are highly limited, they possess various glycosidases to hydrolyze indigestible glycans, such as *O*-glycans of mucin produced by epithelial cells of the gastrointestinal tract. *B. bifidum* is one of the most frequently found bacteria in the intestines of newborn infants (Turroni et al. 2012). This bacterium also resides in intestines of adults, although the population diminishes over time (Turroni et al. 2012). We previously reported that *B. bifidum* possesses unique enzymes acting on the core structures in mucin *O*-glycans: glycoside hydrolase (GH) family 101 endo-α-*N*-acetylgalactosaminidase (EngBF) specific for core 1 structure, also called T-antigen (Galβ1-3GalNAcα1-Ser/Thr) (Fujita et al. 2005), and GH129 α-*N*-

acetylgalactosaminidase (NagBb) specific for Tn-antigen (GalNAcα1-Ser/Thr) (Kiyohara et al. 2012). Furthermore, this bacterium expresses a series of glycosidases to uncover these core structures of mucin *O*-glycan (Katayama et al. 2004; Wada et al. 2008; Ashida et al. 2009; Miwa et al. 2010; Kiyohara et al. 2011; Sakurama et al. 2012, 2013). Whole genomic analysis also revealed that *B. bifidum* is highly adapted to acquire nutrients from *O*-glycans in mucin by producing related glycosidases (Turroni et al. 2010). The nonreducing termini of *O*-glycans on gastrointestinal mucin are usually covered with various glyco-epitopes, which confer resistance to digestive enzymes of the host and of general commensal bacteria. Terminal α-linked GlcNAc is one of the major and unique glyco-epitopes on gastroduodenal mucin (Fig. 1a) and is implicated as a host defense mechanism against colonization of *Helicobacter pylori* by inhibiting the synthesis of cholesteryl-α-glucopyranoside, an essential component of the cell membrane (Kawakubo et al. 2004). Gastroduodenal mucin flowing into the intestines becomes nutrients to some kind of commensal bacteria including bifidobacteria. To assimilate this type of mucin glycans, the first elimination of α-linked GlcNAc is essential. Therefore, we assessed the enzyme activity of α-*N*-acetylglucosaminidase (EC 3.2.1.50) in various bifidobacteria and found that several strains possess this enzyme. In this paper, we describe the cloning and characterization of α-*N*-acetylglucosaminidase from *B. bifidum*.

Materials and methods

Bacterial strains and culture

The bifidobacterial strains (Table 1) were obtained from the Japan Collection of Microorganisms (JCM, RIKEN Bioresource Center, Japan). The bacteria were cultured in Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical, Japan) for 16 h at 37 °C under anaerobic conditions using AnaeroPack-Anaero (Mitsubishi Gas Chemical, Japan).

Genome sequence of *B. bifidum* JCM 1254

Draft sequencing of the genome of *B. bifidum* JCM 1254 was performed using a Genome Sequencer 20 System (Roche Applied Science, IN, USA). The details will be reported elsewhere.

Cloning and expression of α-*N*-acetylglucosaminidase in *Escherichia coli*

To construct the α-*N*-acetylglucosaminidase (AgnB) expression vector, a DNA fragment encoding amino acids (aa) 51-1926 (without an N-terminal signal peptide and C-terminal transmembrane region) was amplified by high-fidelity DNA polymerase (PrimeSTAR Max DNA Polymerase, Takara Bio, Japan) using genomic DNA from *B. bifidum* JCM 1254 as a template and the primers (AgnB-F and AgnB-R, Table S1), digested with *Eco*RI and *Xho*I, and ligated into pET23b(+). The nucleotide sequence was confirmed by sequencing. *E. coli* BL21(λDE3)Δ*lacZ* (Miwa et al. 2010) was transformed with pET23b/*agnb* and cultured in Luria-Bertani liquid medium containing 100 μg/mL ampicillin at 37 °C until the optical density at 600 nm reached 0.5. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM, and the culture was continued for more 3 h at 37 °C. Cells were harvested and lysed by BugBuster Protein Extraction Reagent (Novagen, Germany). After centrifugation, the supernatant was applied to a HisTrap

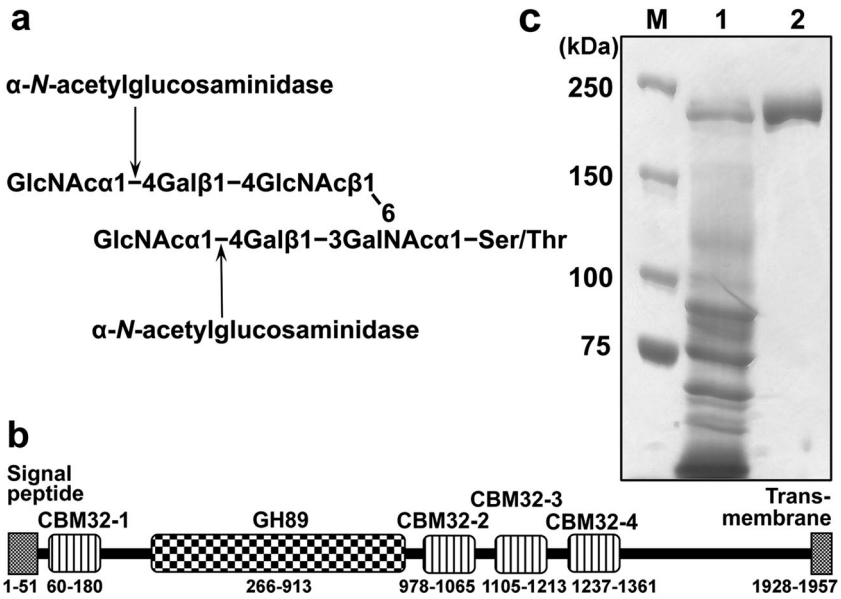

Fig. 1 AgnB α-*N*-acetylglucosaminidase from *B. bifidum* JCM 1254. **a** Typical hexasaccharide structure of gastric mucin *O*-glycan and the action of α-*N*-acetylglucosaminidase. **b** Domain structure of AgnB from *B. bifidum* JCM 1254. **c** SDS-PAGE of the recombinant AgnB expressed in *E. coli*. *M*, protein marker; *lane 1*, crude extract; *lane 2*, affinity purified AgnB

Table 1 α-*N*-Acetylglucosaminidase activity in various bifidobacterial cells

B. adolescentis JCM 1275	–
B. adolescentis JCM 7046	–
B. angulatum JCM 7096	–
B. animalis subsp. *lactis* JCM 10602	–
B. bifidum JCM 1254	+
B. bifidum JCM 1255	–
B. bifidum JCM 7004	+
B. breve JCM 1192	–
B. catenulatum JCM 1194	–
B. dentium JCM 1195	–
B. gallicum JCM 8224	–
B. longum subsp. *longum* JCM 1217	–
B. longum subsp. *longum* JCM 7054	–
B. longum subsp. *infantis* JCM 1210	–
B. longum subsp. *infantis* JCM 1222	–
B. pseudocatenulatum JCM 1200	–
B. pseudolongum subsp. *pseudolongum* JCM 1205	–
B. scardovii JCM 12489	+

Bifidobacterial cells cultured in GAM were washed with PBS and incubated with GlcNAcα1-MU. +, detected; −, not detected

HP column (1 mL, GE Healthcare, UK). The column was washed with 5 mM imidazole in 50 mM sodium phosphate buffer, pH 7.0, containing 250 mM NaCl, and then the adsorbed proteins were eluted by 250 mM imidazole in the same buffer. The active fraction was collected, concentrated, and desalted using an Amicon Ultra 30K (Merck Millipore, MA, USA). Protein concentration was determined by Protein Assay (Bio-Rad, CA, USA) using bovine serum albumin as a standard.

Preparation of AgnB mutants

To construct AgnBΔC (aa 51-940) expression vector, a DNA fragment was amplified using pET23b/*agnb* as a template and the primer set of AgnB-F and AgnB-N-R (Table S1). For AgnBΔN (aa 913-1926), the same template and the primer set of AgnB-C-F and AgnB-R were used. In both mutant construction, the DNA fragments were digested with *Eco*RI and *Xho*I and ligated into pET23b(+), resulting pET23b/*agnbΔC* and pET23b/*agnbΔN*, respectively. For AgnB(E638A) and AgnB(E638A)ΔC, PCR was performed with pET23b/*agnb* and pET23b/*agnbΔC* as templates, respectively, and the primer set of E638A-F and E638A-R (Table S1). After *Dpn*I digestion, mutant plasmid was used to transform *E. coli* DH5α, and the mutation was confirmed by sequencing. The mutant proteins were expressed in *E. coli* BL21(λDE3)Δ*lacZ* and purified under the same conditions as the wild-type AgnB.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 6 % polyacrylamide gel under reducing condition. Proteins were stained with EZ stain AQua (ATTO, Japan). Precision Plus Protein Dual Color Standards (Bio-Rad) were used as markers.

Enzyme assay

p-Methoxyphenyl (*p*MP)-disaccharides with GlcNAcα1-2/3/4/6Galβ were chemically synthesized as previously described (Fujita et al. 2011). Substrates were incubated with the enzyme at 37 °C for an appropriate time in 50 mM sodium acetate buffer (pH 5.5). The reaction mixture was analyzed by silica-gel TLC (Merck 5553, Germany) with 1-butanol/acetic acid/water (2:1:1, by volume) as a developing solvent and visualized with spraying diphenylamine-aniline-phosphoric acid reagent (0.1 g diphenylamine, 0.1 mL aniline, and 1 mL phosphoric acid dissolved in 10 mL acetone) followed by heating at 140 °C for 15 min. Released GlcNAc was quantified by the Morgan-Elson method after stopping the reaction by heating. In the case of 4-methylumberiferyl-α-GlcNAc (GlcNAcα1-MU, Sigma-Aldrich, MO, USA) as a substrate, the reaction was stopped by addition of 1.5 times volume of 1 M Na_2CO_3 (pH 10.9) and released 4-methylumberiferon was measured fluorimetrically (excitation 365 nm; emission 445 nm). For *p*-nitrophenyl (*p*NP)-glycosides (Sigma-Aldrich) as substrates, the reaction was stopped by addition of 1.5 times volume of 1 M Na_2CO_3 (pH 10.9) and released *p*NP was measured by absorbance at 405 nm.

Preparation of GlcNAcα1-4Gal from porcine gastric mucin

Recombinant GlcNAcα1-4Gal-releasing endo-β-galactosidase (GngC) from *Clostridium perfringens* (Ashida et al. 2001, 2002) was used for preparation of GlcNAcα1-4Gal. Porcine gastric mucin (PGM, 2.0 g, type III, Sigma-Aldrich) previously dialyzed thoroughly against water was incubated with the recombinant GngC (4.75 mg) in 20 mM sodium acetate buffer (pH 5.6). The reaction mixture was dialyzed against water and the outer solution was concentrated to 7.0 mL of 140 mM GlcNAcα1-4Gal. The concentration of the disaccharide was determined with phenol-sulfuric acid method using equal molar mixture solution of GlcNAc and Gal as the standard. The sample (100 μL) was added with equal volume of 5 % phenol aqueous solution and 500 μL concentrated H_2SO_4, and then absorbance at 495 nm was measured.

Dot-blot overlay assay

Either 2 μg PGM or bovine serum albumin (BSA) was blotted onto a nitrocellulose membrane (Protran BA 85, GE Healthcare, UK), followed by blocking with 10 % skim milk (Wako Pure Chemical Industries, Japan) in Tris-buffered saline (TBS), pH 7.5, containing 0.05 % Tween 20 (TBS-T). The membranes were then treated with 1 μM of each of purified AgnB mutant proteins in 2 % skim milk in TBS-T for 2 h at 25 °C. After washing the membrane with TBS-T, subsequent detection was carried out by a rabbit anti-His-probe primary antibody (1/800, MBL, Japan) and horseradish peroxidase-conjugated anti-rabbit IgG (1/5000, Santa Cruz Biotechnology, CA, USA). Visualization was carried out using 0.5 mM of 3,3′,5,5′-tetramethylbenzidine in 0.2 M citrate buffer (pH 4.0) with 0.06 % H_2O_2.

Results

Distribution of α-*N*-acetylglucosaminidase activity in bifidobacteria

To examine whether bifidobacteria possess α-*N*-acetylglucosaminidase, we incubated GlcNAcα1-MU with various bifidobacterial cells cultured in GAM. Among those tested, *B. bifidum* JCM 1254, *B. bifidum* JCM 7004, and *Bifidobacterium scardovii* JCM 12489 degraded the substrate, while the other bifidobacterial species/strains did not hydrolyze it (Table 1). Unexpectedly, *B. bifidum* JCM 1255, the type strain of this species, did not show the activity. The diminished ability of the strain as compared with the other *B. bifidum* strains to utilize host-derived glycans has already been reported (Asakuma et al. 2011). No enzyme activity was detected in the culture supernatant of any strain, suggesting that three positive strains expressed the enzyme on the cell surface or in the cytosol.

Identification of a candidate gene of α-*N*-acetylglucosaminidase in *B. bifidum*

We searched the genome of *B. bifidum* JCM 1254, which was previously sequenced by ourselves, and found a candidate gene encoding a putative GH89 α-*N*-acetylglucosaminidase. The gene, termed *agnb* (accession number AB986539), consists of a 5874-bp open-reading-frame and encodes a polypeptide with 1957 amino acids (aa) containing the following putative sequences/domains: an N-terminal signal sequence (aa 1-51), four carbohydrate-binding module (CBM) 32 domain (aa 60-180, aa 978-1065, aa 1105-1213, aa 1237-1361), a GH89 domain (aa 266-913), and a C-terminal transmembrane region (aa 1928-1957) (Fig. 1b). The presence of an N-terminal signal sequence and a C-terminal transmembrane region indicates that AgnB is a membrane-anchored protein with a large extracellular region that includes GH89 and CBM32 domains. This is consistent with the presence of the major α-*N*-acetylglucosaminidase activity on the cells but not in the culture supernatant of *B. bifidum* JCM 1254.

Expression of AgnB

A DNA fragment of *agnb* lacking the sequences encoding the putative N-terminal signal peptide (aa 1-51) and the C-terminal transmembrane region (aa 1928-1957) was amplified by high-fidelity PCR and ligated into a pET-23b(+) expression vector to produce N-terminally T7-tagged and C-terminally 6×His-tagged AgnB. *E. coli* BL21(λDE3)Δ*lacZ* transformed with pET-23b/*agnb* were cultured, and the expression was induced with IPTG. The 6×His-tagged protein was purified from cell lysate using immobilized Ni^{2+} affinity chromatography. The purified protein migrated as a single protein band of around 200 kDa on reducing SDS-PAGE, which coincides with the calculated molecular mass (212 kDa) (Fig. 1c).

Substrate specificity of AgnB

First, we incubated the purified recombinant enzyme with various *p*NP-monosaccharides (GlcNAcα1-*p*NP, GlcNAcβ1-*p*NP, GalNAcα1-*p*NP, GalNAcβ1-*p*NP, Glcα1-*p*NP, Glcβ1-*p*NP, Galα1-*p*NP, Galβ1-*p*NP, Fucα1-*p*NP, Fucβ1-*p*NP, Manα1-*p*NP, Manβ1-*p*NP, Xylα1-*p*NP, and Xylβ1-*p*NP). Among these, only GlcNAcα1-*p*NP was slowly hydrolyzed by this enzyme (data not shown). We confirmed the release of GlcNAc from GlcNAcα1-*p*NP using TLC, but not from GlcNAcβ1-*p*NP (Fig. 2a). GlcNAcα1-MU was efficiently hydrolyzed under the same condition (Fig. 2b). Then, we incubated the enzyme with *p*MP-disaccharides containing α-GlcNAc with various linkages (Fig. 2c). GlcNAc was readily released from GlcNAcα1-4Galβ1-*p*MP and very slowly from GlcNAcα1-6Galβ1-*p*MP. However, α1,2- and α1,3-linked GlcNAc were resistant to this enzyme. Since GlcNAcα1-4Galβ1-R structure is specifically distributed in *O*-glycans of gastroduodenal mucin, we incubated AgnB with PGM and detected the release of GlcNAc (Fig. 2d). The disaccharide GlcNAcα1-4Gal prepared from PGM using GlcNAcα1-4Gal-releasing endo-β-galactosidase from *C. perfringens* (Ashida et al. 2001, 2002) was also readily hydrolyzed into GlcNAc and Gal (Fig. 2e). These results indicate that AgnB has strict glycone and linkage specificity toward terminal α1,4-linked GlcNAc. The specific activities toward hydrolyzed substrates were determined by measuring released GlcNAc (Table 2). The activities for the substrates containing GlcNAcα1-4Gal structure were nearly the same to each other, whereas those for GlcNAcα1-MU and GlcNAcα1-*p*NP were 20 % and less than 10 %, respectively.

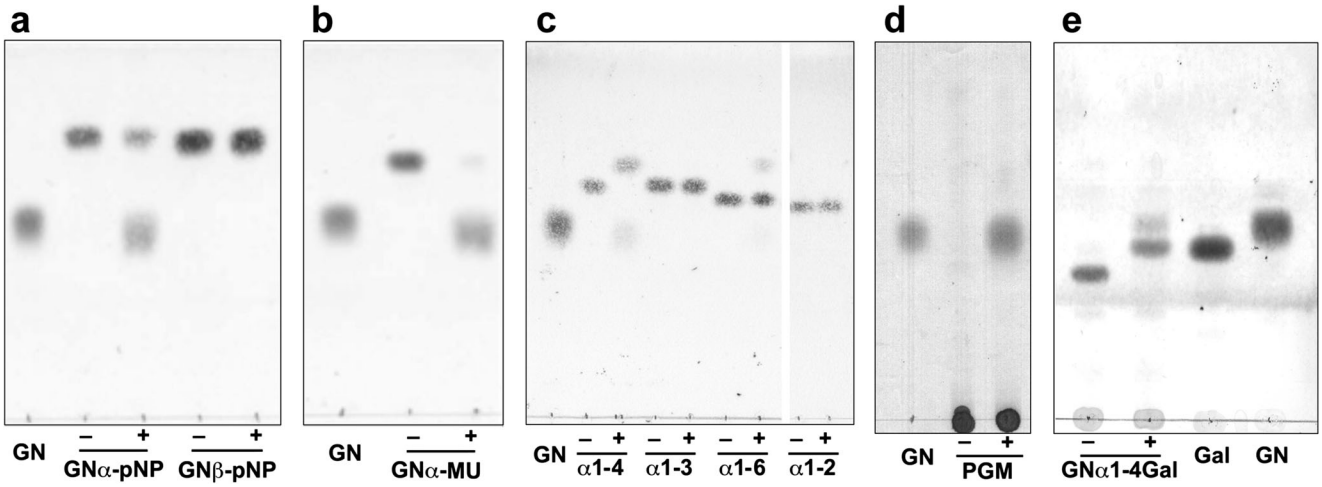

Fig. 2 Substrate specificity of AgnB. Various substrates were incubated with recombinant AgnB and then analyzed by TLC. **a** GlcNAcα1-*p*NP and GlcNAcβ1-*p*NP. **b** GlcNAcα1-MU. **c** GlcNAcα1-4Galβ1-*p*MP (α1-4), GlcNAcα1-3Galβ1-*p*MP (α1-3), GlcNAcα1-6Galβ1-*p*MP (α1-6), and GlcNAcα1-2Galβ1-*p*MP (α1-2). **d** Porcine gastric mucin (PGM). **e** GlcNAcα1-4Gal. *Plus sign* indicates with AgnB; *minus sign* indicates without AgnB; GN, GlcNAc

The activity for GlcNAcα1-6Galβ1-*p*MP was very low compared to those for the other substrates. These results suggest that AgnB is essentially specific to the terminal GlcNAcα1-4Gal with rather strict recognition for the aglycone Gal residue.

General properties of AgnB

The general properties of AgnB were determined using GlcNAcα1-MU as a substrate (Fig. S1). The highest activity was observed at pH 5.5, and more than 80 % activity was detected between pH 4.0 and 6.5. The enzyme was stable between pH 5.0 and 9.5. The optimum temperature was 50 °C and stable up to 40 °C. The divalent cations, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+}, did not affect enzyme activity at 5 mM concentration, whereas 5 mM Cu^{2+} reduced the activity to 10 % (data not shown).

Table 2 Specific activities of AgnB and AgnBΔC toward various substrates

Substrate	AgnB (units/μmol protein)	AgnBΔC (units/μmol protein)
PGM (2.5 mg/mL)	787	164
PGM (1.5 mg/mL)	706	98.0
GlcNAcα1-MU	125	67.1
GlcNAcα1-*p*NP	42.4	14.6
GlcNAcα1-4Galβ1-*p*MP	689	298
GlcNAcα1-6Galβ1-*p*MP	8.7	6.1
GlcNAcα1-4Gal	530	307

Synthetic substrates were used at 1.0 mM. Released GlcNAc was measured by the Morgan-Elson method

CBM32 domains enhance the affinity of AgnB toward mucin

AgnB possesses four CBM32 domains in the molecule: one near N-terminus and tandem three in C-terminal part (Fig. 1b). To analyze the function of these domains, we first deleted C-terminal three CBM32 domains to make AgnBΔC (aa 52-941) (Fig. 3a). We compared the specific activities of full-length AgnB and AgnBΔC toward various substrates

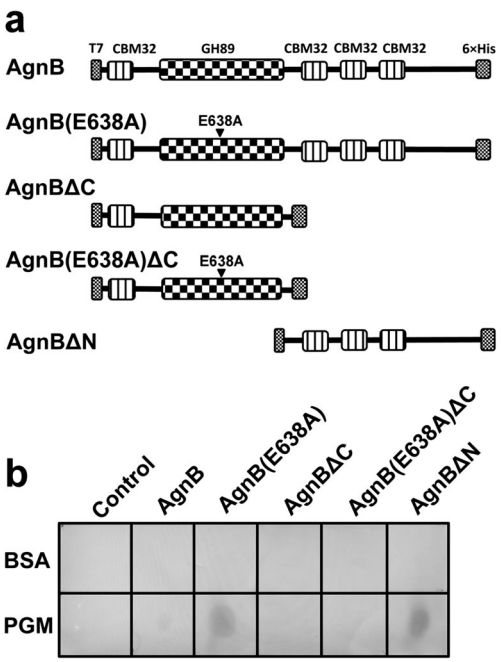

Fig 3 Dot-blot overlay assay of mutant constructs. **a** Schematic representation of mutants. **b** Dot-blot overlay assay. Either PGM or BSA was dot-blotted onto nitrocellulose membrane and then various mutants were overlaid. After washing, bound proteins were detected with anti-His antibody

(Table 2). The specific activities of AgnBΔC toward the monomeric substrates were approximately half of those of the full-length AgnB, whereas the activity toward PGM was dramatically reduced to 1/5–1/7. Since this result suggested that C-terminal CBM32 domains enhance the affinity of AgnB toward multivalent substrate, we further made three mutants: AgnBΔN (aa 914-1927), including only C-terminal CBM32-2, CBM32-3, and CBM32-4 domains, and E638A point mutants of full-length AgnB and AgnBΔC (Fig. 3a). E638 is the putative nucleophilic residue, and the enzymatic inactivation in AgnB(E638A) and AgnB(E638A)ΔC mutants was confirmed (data not shown). Then, we performed dot-blot overlay assay to evaluate the binding activity toward PGM (Fig. 3b). Among the constructs, full-length AgnB(E638A) and AgnBΔN bound PGM, showing that C-terminal CBM32 domains contribute the binding. AgnBΔC and AgnB(E638A)ΔC did not bind PGM, suggesting that N-terminal CBM32-1 does not have binding activity. Interestingly, enzymatically active AgnB hardly bound PGM, probably because active enzyme hydrolyzed α-linked GlcNAc, the binding epitopes of CBM32 domains. Taken together, these results suggest that either of C-terminal CBM32 domains specifically binds GlcNAcα1-4Gal-epitope of *O*-glycans and enhance enzyme activity toward multivalent substrate such as PGM.

Discussion

In this study, we identified GH89 α-*N*-acetylglucosaminidase from bifidobacteria for the first time. The GH89 family that is exclusively composed of α-*N*-acetylglucosaminidase is distributed from bacteria to higher eukaryotes. Human GH89 enzymes have been well characterized as a lysosomal enzyme that is involved in degradation of heparan sulfate (NAGLU, AAB06188) (Weber et al. 2001). In contrast, those from other organisms have been poorly investigated, except for the enzyme from *C. perfringens* (AgnC, BAB80572; *Cp*GH89, ABG84150) (Ficko-Blean et al. 2008; Fujita et al. 2011; Ficko-Blean and Boraston 2012). AgnC has multi-domain structure similar to AgnB; the enzyme consists of a CBM32 domain, a GH89 domain, and five CBM32 domains from its N-terminus in this order. The striking difference is that AgnC does not have C-terminal transmembrane region, indicating that AgnC is an extracellular soluble enzyme. GH89 domain of AgnB shares 57 % amino acid identity with that of AgnC. Acid/base and nucleophilic residues are conserved in both enzymes (E519 and E638 in AgnB, E583 and E601 in AgnC, respectively). Their substrate specificities are also similar to each other: both enzymes essentially specific to GlcNAcα1-4Gal structure. However, AgnB slowly hydrolyzed GlcNAcα1-6Gal, which has unnatural structure and is completely resistant to AgnC. GH89 family contains several uncharacterized enzymes of other intestinal bacteria, such as *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, and *Akkermansia muciniphila*. These GH89 enzymes including AgnB and AgnC may play an important role in obtaining sugars from host's mucin and in microbe-host interaction.

AgnB has four CBM32 domains, while AgnC has six. Among the six CBM32 domains in AgnC, the third and fourth ones (AgnC-CBM32-3 and AgnC-CBM32-4) were shown to bind GlcNAcα1-4Gal as revealed by glycan array and isothermal titration calorimetry. AgnC-CBM32-5 bound Gal residue, which is the most common feature of CBM32 family. AgnC-CBM32-1 and AgnC-CBM32-6 were concluded to be nonfunctional (Ficko-Blean et al. 2012). Phylogenetic analysis of CBM32 domains revealed that AgnB-CBM32-2, AgnB-CBM32-3, and AgnB-CBM32-4 are relatively close to AgnC-CBM32-3 and AgnC-CBM32-4 (Fig. 4). Thus, each of these three CBM32 domains in AgnB might bind α-linked GlcNAc specifically. Since AgnB-CBM32-1 is very close to AgnC-CBM32-1, this may have no binding activity. CBM32 is one of the most diverse CBM families, whose members were reported to recognize Gal, Galβ1-4GlcNAc, GalNAc, GlcNAc, blood group H trisaccharide, and GlcNAcα1-4Gal (Grondin et al. 2014). Among these, the domains recognizing GlcNAcα1-4Gal have so far been found only in GH89 AgnC and AgnB. It is very interesting that these domains have evolved binding specificity complementary to the catalytic specificity.

The core-1 disaccharide of *O*-glycans, Galβ1-3GalNAc, released by bifidobacterial GH101 endo-α-*N*-acetylgalactosaminidase is highly selective bifidogenic factor, because bifidobacteria possess a specific assimilation pathway (Kiyohara et al. 2009). Galβ1-3GalNAc is resistant to general bacterial β-galactosidases (Yoshida et al. 2012). The

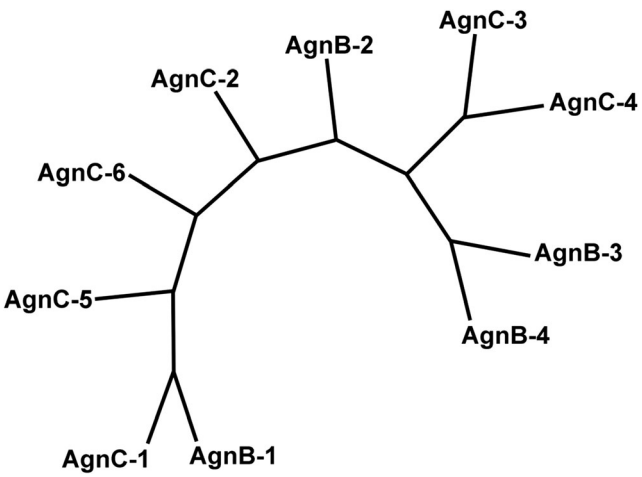

Fig. 4 Phylogenetic tree of CBM32 domains in AgnB and AgnC. Tree was constructed using the ClustalW program using neighbor-joining method. The sequences of CBM32 domains of AgnC (BAB80572) used are as follows: AgnC-1 (aa 26-154), AgnC-2 (aa 918-1057), AgnC-3 (aa 1063-1201), AgnC-4 (aa 1206-1343), AgnC-5 (aa 1356-1493), and AgnC-6 (aa 1496-1621)

disaccharide is, however, transported into the bifidobacterial cells through a specific ABC transporter (Suzuki et al. 2008) and degraded in the cytosol by a specific phosphorylase (Kitaoka et al. 2005). We attempt to produce this disaccharide from PGM, a meat waste, by using a combination of glycosidases and to apply it as a functional food ingredient, so-called prebiotics. For this purpose, bifidobacterial glycosidases, but not clostridial ones, are suitable, because bifidobacteria are safe probiotic bacteria. We have already identified bifidobacterial glycosidases degrading nonreducing terminal glyco-epitopes such as α-sialidase (Kiyohara et al. 2011), 1,2-α-L-fucosidase (Katayama et al. 2004), and blood group B antigen-specific α-galactosidase (Wakinaka et al. 2013). AgnB α-*N*-acetylglucosaminidase might be indispensable for high-yield production of bifidogenic factor Galβ1-3GalNAc from PGM, because a large proportion of *O*-glycans are attached by α-linked GlcNAc.

Acknowledgments This work was supported by JSPS KAKENHI grant number 24580179 (to H.A.) and JSPS Core-to-Core Program (to T.C., K.Y., T.K., and H.A.). We thank Dr. Masaya Fujita (The Noguchi Institute, Tokyo, Japan) for providing *p*MP-disaccharides. Our thanks are due to the late Dr. Masashi Kiyohara for contribution in the initial stage of this study.

References

Asakuma S, Hatakeyama E, Urashima T, Yoshida E, Katayama T, Yamamoto K, Kumagai H, Ashida H, Hirose J, Kitaoka M (2011) Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. J Biol Chem 286:34583–34592

Ashida H, Anderson K, Nakayama J, Maskos K, Chou CW, Cole RB, Li SC, Li YT (2001) A novel endo-β-galactosidase from *Clostridium perfringens* that liberates the disaccharide GlcNAcα1→4Gal from glycans specifically expressed in the gastric gland mucous cell-type mucin. J Biol Chem 276:28226–28232

Ashida H, Maskos K, Li SC, Li YT (2002) Characterization of a novel endo-β-galactosidase specific for releasing the disaccharide GlcNAcα1→4Gal from glycoconjugates. Biochemistry 41:2388–2395

Ashida H, Miyake A, Kiyohara M, Wada J, Yoshida E, Kumagai H, Katayama T, Yamamoto K (2009) Two distinct α-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. Glycobiology 19:1010–1017

Ficko-Blean E, Boraston AB (2012) Structural analysis of a bacterial exo-α-D-*N*-acetylglucosaminidase in complex with an unusual disaccharide found in class III mucin. Glycobiology 22:590–595

Ficko-Blean E, Stubbs KA, Nemirovsky O, Vocadlo DJ, Boraston AB (2008) Structural and mechanistic insight into the basis of mucopolysaccharidosis IIIB. Proc Natl Acad Sci U S A 105:6560–6565

Ficko-Blean E, Stuart CP, Suits MD, Cid M, Tessier M, Woods RJ, Boraston AB (2012) Carbohydrate recognition by an architecturally complex α-*N*-acetylglucosaminidase from *Clostridium perfringens*. PLoS One 7:e33524

Fujita K, Oura F, Nagamine N, Katayama T, Hiratake J, Sakata K, Kumagai H, Yamamoto K (2005) Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type *O*-glycan-specific endo-α-*N*-acetylgalactosaminidase from *Bifidobacterium longum*. J Biol Chem 280:37415–37422

Fujita M, Tsuchida A, Hirata A, Kobayashi N, Goto K, Osumi K, Hirose Y, Nakayama J, Yamanoi T, Ashida H, Mizuno M (2011) Glycoside hydrolase family 89 α-*N*-acetylglucosaminidase from *Clostridium perfringens* specifically acts on GlcNAcα1,4Galβ1R at the non-reducing terminus of *O*-glycans in gastric mucin. J Biol Chem 286:6479–6489

Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T, Taylor TD, Itoh K, Kikuchi J, Morita H, Hattori M, Ohno H (2011) Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469:543–547

Grondin JM, Chitayat S, Ficko-Blean E, Houliston S, Arrowsmith CH, Boraston AB, Smith SP (2014) An unusual mode of galactose recognition by a family 32 carbohydrate-binding module. J Mol Biol 426:869–880

Katayama T, Sakuma A, Kimura T, Makimura Y, Hiratake J, Sakata K, Yamanoi T, Kumagai H, Yamamoto K (2004) Molecular cloning and characterization of *Bifidobacterium bifidum* 1,2-α-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). J Bacteriol 186:4885–4893

Kawakubo M, Ito Y, Okimura Y, Kobayashi M, Sakura K, Kasama S, Fukuda MN, Fukuda M, Katsuyama T, Nakayama J (2004) Natural antibiotic function of a human gastric mucin against *Helicobacter pylori* infection. Science 305:1003–1006

Kitaoka M, Tian J, Nishimoto M (2005) Novel putative galactose operon involving lacto-*N*-biose phosphorylase in *Bifidobacterium longum*. Appl Environ Microbiol 71:3158–3162

Kiyohara M, Tachizawa A, Nishimoto M, Kitaoka M, Ashida H, Yamamoto K (2009) Prebiotic effect of lacto-*N*-biose I on bifidobacterial growth. Biosci Biotechnol Biochem 73:1175–1179

Kiyohara M, Tanigawa K, Chaiwangsri T, Katayama T, Ashida H, Yamamoto K (2011) An exo-α-sialidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates. Glycobiology 21:437–447

Kiyohara M, Nakatomi T, Kurihara S, Fushinobu S, Suzuki H, Tanaka T, Shoda S, Kitaoka M, Katayama T, Yamamoto K, Ashida H (2012) α-*N*-Acetylgalactosaminidase from infant-associated bifidobacteria belonging to novel glycoside hydrolase family 129 is implicated in alternative mucin degradation pathway. J Biol Chem 287:693–700

Miwa M, Horimoto T, Kiyohara M, Katayama T, Kitaoka M, Ashida H, Yamamoto K (2010) Cooperation of β-galactosidase and β-*N*-acetylhexosaminidase from bifidobacteria in assimilation of human milk oligosaccharides with type 2 structure. Glycobiology 20:1402–1409

Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C (2005) Review article: bifidobacteria as probiotic agents—physiological effects and clinical benefits. Aliment Pharmacol Ther 22:495–512

Sakurama H, Fushinobu S, Hidaka M, Yoshida E, Honda Y, Ashida H, Kitaoka M, Kumagai H, Yamamoto K, Katayama T (2012) 1,3-1,4-α-L-Fucosynthase that specifically introduces Lewis a/x antigens into type-1/2 chains. J Biol Chem 287:16709–16719

Sakurama H, Kiyohara M, Wada J, Honda Y, Yamaguchi M, Fukiya S, Yokota A, Ashida H, Kumagai H, Kitaoka M, Yamamoto K, Katayama T (2013) Lacto-*N*-biosidase encoded by a novel gene of *Bifidobacterium longum* subspecies *longum* shows unique substrate specificity and requires a designated chaperone for its active expression. J Biol Chem 288:25194–25206

Suzuki R, Wada J, Katayama T, Fushinobu S, Wakagi T, Shoun H, Sugimoto H, Tanaka A, Kumagai H, Ashida H, Kitaoka M, Yamamoto K (2008) Structural and thermodynamic analyses of solute-binding protein from *Bifidobacterium longum* specific for core 1 disaccharide and lacto-*N*-biose I. J Biol Chem 283:13165–13173

Trebichavsky I, Rada V, Splichalova A, Splichal I (2009) Cross-talk of human gut with bifidobacteria. Nutr Rev 67:77–82

Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, Sanchez B, Bidossi A, Ferrarini A, Giubellini V, Delledonne M, Henrissat B, Coutinho P, Oggioni M, Fitzgerald GF, Mills D, Margolles A, Kelly D, van Sinderen D, Ventura M (2010) Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A 107:19514–19519

Turroni F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, Gueimonde M, Margolles A, De Bellis G, O'Toole PW, van Sinderen D, Marchesi JR, Ventura M (2012) Diversity of bifidobacteria within the infant gut microbiota. PLoS One 7:e36957

Wada J, Ando T, Kiyohara M, Ashida H, Kitaoka M, Yamaguchi M, Kumagai H, Katayama T, Yamamoto K (2008) *Bifidobacterium bifidum* lacto-*N*-biosidase, a critical enzyme for the degradation of human milk oligosaccharides with a type 1 structure. Appl Environ Microbiol 74:3996–4004

Wakinaka T, Kiyohara M, Kurihara S, Hirata A, Chaiwangsri T, Ohnuma T, Fukamizo T, Katayama T, Ashida H, Yamamoto K (2013) Bifidobacterial α-galactosidase with unique carbohydrate-binding module specifically acts on blood group B antigen. Glycobiology 23:232–240

Weber B, Hopwood JJ, Yogalingam G (2001) Expression and characterization of human recombinant and α-*N*-acetylglucosaminidase. Protein Expr Purif 21:251–259

Yoshida E, Sakurama H, Kiyohara M, Nakajima M, Kitaoka M, Ashida H, Hirose J, Katayama T, Yamamoto K, Kumagai H (2012) *Bifidobacterium longum* subsp. *infantis* uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides. Glycobiology 22:361–368