Molecular cloning and catalytic mechanism of a novel glycosphingolipid-degrading β-N-acetylgalactosaminidase from *Paenibacillus* sp. TS12

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We report here the molecular cloning, characterization, and catalytic mechanism of a novel glycosphingolipid-degrading β-N-acetylgalactosaminidase from *Paenibacillus* sp. TS12 (NgaP). NgaP, consisting of 1,034 putative amino acid residues, shares no sequence similarity with known proteins. A recombinant NgaP, expressed in *Escherichia coli*, cleaved the non-reducing terminal β-N-acetylgalactosamine residues of gangliotriaosylceramide and globotetraosylceramide. The enzyme hydrolyzed para-nitrophenyl-β-N-acetylgalactosaminide ~100 times faster than para-nitrophenyl-β-N-acetylglucosaminide.

*N*-Acetylgalactosamine-thiazoline, an analogue of the oxazolinium intermediate and potent inhibitor for enzymes adopting substrate-assisted catalysis, competitively inhibited the enzyme. The *K* <sub>i</sub> value of the enzyme for *N*-acetylgalactosamine-thiazoline was 1.3 nM whereas that for *N*-acetylgluosamine-thiazoline was 46.8 μM. A comparison of secondary structure with that of known enzymes exhibiting substrate-assisted catalysis and a point mutation analysis indicated that NgaP adopts substrate-assisted catalysis and a point mutation analysis indicated that NgaP adopts substrate-assisted catalysis in which Glu608 and Asp607 could function respectively as a proton donor and a stabilizer of the 2-acetamide group of the β-N-acetylgalactosamine at the active site. These results clearly indicated that NgaP is a β-N-acetylgalactosaminidase showing substrate-assisted catalysis. This is the first report describing the molecular cloning of a β-N-acetylgalactosaminidase adopting substrate-assisted catalysis.

β-Hexosaminidase (β-HEX, EC 3.2.1.52) is an enzyme that hydrolyzes non-reducing terminal β-N-acetylglucosamine (β-GlcNAc) and β-N-acetylgalactosamine (β-GalNAc) residues in oligosaccharides, glycoproteins, glycosaminoglycans, and glycosphingolipids (GSLs). A large number of β-HEXs are grouped into GH20 and clan GH-K (clans are classified by three-dimensional structure [2]). Among β-HEXs, an enzyme that hydrolyzes the non-reducing terminal β-GalNAc residue much faster than the β-GlcNAc residue is named β-N-acetylgalactosaminidase (β-NGA, EC 3.2.1.53). β-NGA activities have been detected in bovine brain (3), rat brain (4) and bacteria (5), however β-NGA has not yet been fully characterized. Recently, a nucleocytoplasmic neutral β-HEX (named β-HEX D) was cloned from human and mouse (6) as a homologue of *Caenorhabditis elegans* β-HEX (7). β-HEX D, classified into GH20 with other known β-HEXs, was found to hydrolyze *p*NP-β-GalNAc 4 times faster than *p*NP-β-GlcNAc, suggesting β-HEX D to be a β-NGA although its catalytic mechanism was not clarified.

Many GH family proteins hydrolyze substrates through one of two mechanisms; the inversion or retention of the anomeric configuration of the substrate (8). Inverting glycosidases employ a single-displacement mechanism in which two amino acids function as a general acid and a...
general base, while almost all retaining glycosidases hydrolyze substrates through a double-displacement mechanism in which two amino acids function as a general acid/base and a nucleophile.

Although β-HEX is considered a retaining hydrolase, it hydrolyzes substrates through substrate-assisted catalysis in which the carbonyl oxygen of the C-2 acetamide group of the substrate behaves as a catalytic nucleophile, and therefore only one amino acid is required as a proton donor from the protein side (9).

Previously, we described the isolation and identification of a soil bacterium, *Paenibacillus* sp. TS12, that efficiently degrades various GSLs when added to cultures by producing a series of exoglycosidases (10). Subsequently, the molecular cloning of the TS12 glucocerebrosidase (11) and two β-HEXs (12), all of which are capable of hydrolyzing GSLs, was described.

In this paper, we report the molecular cloning, characterization and catalytic mechanism of a novel GSL-degrading β-NGA (tentatively named NgaP) of *Paenibacillus* sp. TS12. This study revealed that the sequence of NgaP is not shared by any known GH family proteins and NgaP specifically cleaves the terminal non-reducing β-GalNAc residue through substrate-assisted catalysis.

**Experimental Procedures**

**Materials**- *E. coli* strains DH5α and BL21(DE3), and Pyrobest DNA polymerase were purchased from Takara Bio Inc, Japan. Plasmids pET23a and pBluescript II SK(+) were from Novagen and Stratagene, respectively. Restriction enzymes, T4 DNA ligase, globotetraosylceramide (Gb4Cer), and globotriaosylceramide (Gb3Cer) were from Wako Pure Chemical In. Ltd, Japan. The crude ganglioside mixture was prepared from bovine brain as described previously (13), and GM2, asialo GM2 (GA2), asialo GM1 (GA1), lactosylceramide (LacCer), and glucosylceramide (GlcCer) were prepared from the crude ganglioside mixture using GSL-degrading enzymes from *Paenibacillus* sp. TS12. Precoated Silica Gel 60 TLC plates were purchased from Merck. 4-methylumbelliferyl (4MU)-β-GalNAc and various *para*-nitorophenyl (pNP)-glycopyranosides were from Sigma. All other reagents were of the highest purity available.

**Construction of a genomic DNA library of *Paenibacillus* sp. TS12**- Genomic DNA was prepared from *Paenibacillus* sp. TS12 as described in (14) and partially digested with Sau3AI. The Sau3AI-fragments (2-10 kbp) were gel-purified and ligated to BamHI-digested pBluescript II SK (+) DNA. The plasmids were used for the transformation of *E. coli* DH5α.

**Expression cloning of the gene encoding β-NGA**- *E. coli* DH5α cells transformed with the plasmids containing *Paenibacillus* DNA fragments were seeded (approximately 400 colonies / 9.2-cm plate) on LB agar plates supplemented with 100 μg/ml of ampicillin and incubated at 37°C for 16 h. Colonies were transferred from the plates onto nylon membranes (Biodyne A), which were then incubated with 0.3 mM 4MU-β-GalNAc in 200 μl of 10 mM sodium acetate buffer, pH 5.5. Following incubation at 37°C for 30 min, positive colonies, visualized under a UV transilluminator, were picked out with a sterilized toothpick and transferred into 5 ml of LB medium. Following incubation at 37°C for 16 h with shaking, cells were harvested by centrifugation, suspended in 500 μl of 10 mM sodium acetate buffer, pH 5.5, and lysed by sonication. The cell lysate was centrifuged at 8,000g for 10 min, and the supernatant obtained was used as the crude enzyme solution. The activity of β-NGA was measured using GA2 as a substrate as described below. The positive clone obtained was designated pNgaP.

**DNA sequencing and sequence analysis**- Nucleotide sequences were determined by the dideoxynucleotide chain termination method with a BigDye Terminator Cycle Sequencing Ready Reaction Kit, Ver.3 and a DNA sequencer (Applied Biosystems, model 377). Computer analyses were performed using DNASIS, and the homology search of deduced amino acid sequences was performed with DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

**Construction of expression vectors**- The following primers were used for PCR: UNgaP (5’-ATA GGA TCC ATG GTG AAT AGA AAA CAG AAG ACA-3’) and LNgaP (5’- ATT CTC...
GAG TTC AAT AAT TTT TTT GGC GAT
TTC-3'). UNgaP and LNgaP contained a BamHI site (underlined) and a XhoI site (double underlined), respectively. PCR was performed in 50 μl of a reaction mixture containing each primer at 0.2 μM, 50 ng of template DNA (pNgaP), 0.2 mM dNTPs (dATP, dCTP, dGTP, and dTTP), and 2 U of Pyrobest DNA polymerase using a T-personal 48 (Biometra, Germany) for 30 cycles (each consisting of denaturation at 98°C for 10 sec and annealing/extension at 68°C for 2.5 min). PCR products were extracted from 0.7% agarose gel, and the amplified products were digested with BamHI and XhoI. The BamHI/XhoI fragments were cloned into the BamHI/XhoI-digested pET23a. The recombinant plasmid was designated pETNgaP.

Expression and purification of the recombinant β-NGA- E. coli BL21 cells transformed with pETNgaP (or recombinant mutant plasmids) were grown at 25°C for 16 h in 5 ml of medium A (LB medium containing 100 μg/ml of ampicillin) with shaking. The culture was transferred into a 2-L flask containing 1 L of medium A and incubated at 25°C for 16 h with shaking. Then, isopropylthio-β-galactopyranoside (IPTG) was added to the culture at a final concentration of 0.1 mM to cause transcription. After an additional 3 h at 25°C, cells were harvested by centrifugation, and suspended in 50 ml of buffer A (25 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM β-mercaptoethanol and 50 mM imidazole) containing a protease inhibitor cocktail (Roche). After sonication, cell debris was removed by centrifugation (8,000 g for 10 min), and the supernatant obtained was loaded on a HisTrap HP column (5 ml, GE Healthcare), pre-equilibrated with buffer A. The column was washed with 50 ml of buffer A and the β-NGA was eluted with buffer B (25 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM β-mercaptoethanol and 100 mM imidazole), and buffer C (25 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 5 mM β-mercaptoethanol and 300 mM imidazole). The active fractions were pooled, dialyzed against 10 mM Tris-HCl, pH 7.5, and used for the characterization of the enzyme.

Protein assay and polyacrylamide gel electrophoresis- Protein content was determined by the bicinchoninic acid method (Pierce) or SDS-PAGE using bovine serum albumin as the standard. SDS-PAGE was carried out according to the method of Laemmli (15). The proteins on the SDS-PAGE gel were visualized by staining with Coomassie brilliant blue.

Western blotting- The proteins, separated by 10% SDS-PAGE, were transferred onto a PVDF membrane using a semi-dry blotter (BIO-RAD). The membrane was then incubated with anti-polyhistidine tag mouse IgG monoclonal antibody (Invitrogen) for 6 h at room temperature. The bands were visualized with HRP-labeled anti-mouse IgG antibody and a peroxidase-staining kit (Nacalai tesque, Japan).

Enzyme assay- The activity of β-NGA was measured by two methods. Assay I (pNP-β-GalNAc as a substrate); the reaction mixture contained 100 nmol of pNP-β-GalNAc and an appropriate amount of the enzyme in 100 μl of 25 mM sodium acetate buffer, pH 6.0. Following incubation at 37°C for a specified period, the reaction was stopped by adding 100 μl of 1 M NaOH, and absorbance was measured at 405 nm. One unit of the enzyme was defined as the amount which catalyzes the release of 1 μmol of p-nitrophenol per min from pNP-β-GalNAc under the conditions used. Assay II (GA2 or Gb4Cer as a substrate); the reaction mixture contained 5 nmol of GA2 or Gb4Cer and an appropriate amount of the enzyme in 20 μl of 25 mM sodium acetate buffer, pH 6.0, containing 0.2% (w/v) taurodeoxycholate (TDC). Following incubation at 37°C for a given period, the reaction was stopped by heating in a boiling water bath for 5 min. The sample was dried with a Speed Vac SC110 (Savant Ins), and the residue was dissolved in 10 μl of chloroform-methanol (2/1, v/v), and applied to a TLC plate, which was then developed with chloroform-methanol-0.02% CaCl2 (5/4/1, v/v/v, solvent I). The remaining substrate (GA2 or Gb4Cer) and released product (LacCer or Gb3Cer) after incubation with the enzyme were visualized with orcinol-H2SO4 (16), and quantified by a Shimadzu CS-9300PC chromatoscanner with the reflection mode set at 540 nm.

pH-kcat/Km profile- The pH profile was measured using pNP-β-GalNAc as a substrate under the
conditions described in the legend of Fig. 2. pK_a and pK_b values were calculated by using GraFit (17).

Construction of Asp607 and Glu608 mutants - Mutagenesis was performed using KOD polymerase (TOYOBO, Japan), with the following oligonucleotide primers: D607E, 5'-ACC TAT ATG GCT AAT GAG AGA GCG CTG AAC-3' and 5'-GTT CAG CGC TCT CTC ATT AGC CAT ATA GGT-3': D607N, 5'-ACC TAT ATG GCT AAT GAG AGA GCG CTG AAC-3' and 5'-GTT CAG CGC TCT CTC ATT AGC CAT ATA GGT-3': E608D, 5'-TAT ATG GCT AAT GAT GAC AGA GCG CTG AAC GAC-3' and 5'-GTC GTT CAG CGC TCT GCT ATC ATT AGC CAT ATA-3': E608Q, 5'-TAT ATG GCT AAT GAT CA GAA GCG CTG AAC GAC-3' and 5'-GTC GTT CAG CGC TCT TGG ATC ATT AGC CAT ATA-3' (underlines show the location of the mutation). PCR products were digested with DpnI and introduced into E. coli DH5α. The recombinant mutant plasmids were designated pETD607E, pETD607N, pETE608D and pETE608Q.

Determination of Ki value - The Ki values of NgaP for GalNAc-thiazoline and GlcNAc-thiazoline were determined by Morrison’s equation (18) and Dixon plots (19), respectively.

RESULTS

Molecular cloning, sequencing, and alignment of β-NGA of Paenibacillus sp. TS12. To isolate the gene encoding the β-NGA of Paenibacillus sp. TS12, expression screening was performed using pNP-β-GalNAc as the substrate as described under “Experimental Procedures”. As a result, one positive clone was obtained and designated pNgaP. The open reading frame (ORF) of pNgaP was 3,102 bp long encoding 1,034 amino acids and the gene product was named NgaP (Supplemental Fig. 1A). The molecular weight and pl of NgaP were estimated to be 114,727 and 4.74, respectively, from the deduced amino acid sequence. A hydrophobic region, possibly a signal peptide, was found at the N-terminal of NgaP (Supplemental Fig. 1B). The deduced amino acid sequence of NgaP showed no significant similarity to the GH20 β-HEXs and other members of the GH family. Furthermore, the specific sequence conserved in all GH20 β-HEXs cloned to date (7); His/Asn-Xaa-Gly-Ala/Cys/Gly/Met-Asp-Glu-Ala/Ile/Leu/Val (the catalytic glutamate residue is underlined), was not conserved in NgaP. On the other hand, 29-32% sequence similarity was found for hypothetical proteins of Clostridium perfringens (20), Parabacteroides distasonis (21) and Bacteroides thetaiotaomicron (22) (Fig. 1).

Expression and purification of the recombinant NgaP. NgaP was expressed in E. coli strain BL21(DE3) and purified from the cell lysate. The final preparation gave a single protein band corresponding to a molecular weight of 115 kDa on SDS-PAGE with Coomassie brilliant blue staining (Supplemental Fig. 2A, lane 3), well consistent with the molecular weight estimated from the deduced amino acid sequence. This band corresponded exactly to the band obtained on Western blotting using anti-polyhistidine tag monoclonal antibodies (Supplemental Fig. 2B).

Substrate specificity and enzymatic properties of the recombinant NgaP. The specificity of NgaP was examined using various pNP-glycopyranosides as substrates and 1 mU of the purified recombinant enzyme. The recombinant NgaP hydrolyzed pNP-β-GalNAc, but not pNP-β-GlcNAc, pNP-α-GalNAc, or other pNP-glycosides tested under the conditions used. pNP-β-GlcNAc was found to be hydrolyzed by NgaP very slowly when the amount of enzyme was increased, but at less than 1% of the rate of pNP-β-GalNAc’s hydrolysis (Table I). The time course for the hydrolysis of pNP-β-GalNAc and pNP-β-GlcNAc also indicated the strict specificity of the enzyme for the C4 configuration of β-HexNAc, i.e. pNP-β-GalNAc was completely hydrolyzed by the enzyme after 2 h while less than 5% of pNP-β-GlcNAc was hydrolyzed even after 24 h (Fig. 2A). These results clearly indicated that NgaP is a β-NGA, whose specificity to the C4 configuration of β-HexNAc is extremely rigid. It is worth noting that such strict specificity has not been observed in any other β-HEXs including β-Hex D (6). Paenibacillus sp. TS12 was isolated as a GSL-degrading bacterium (10) and glycosidases produced by TS12 were found to hydrolyze GSLs (11,12). Thus, to elucidate the specificity of the
enzyme, various GSLs were tested as a substrate. Gangliotriaosylceramide (GA2, GalNAcβ1-4Galβ1-4Glcβ1-1′Cer) and globotetraosylceramide (Gb4Cer, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer) were hydrolyzed by NgaP, generating lactosylceramide (LacCer, Galβ1-4Glcβ1-1′Cer) and globotriaosylceramide (Gb3Cer, Galα1-4Galβ1-4Glcβ1-1′Cer), respectively. GM2, GA1, GM1a, Gb3Cer, LacCer, GlcCer, GalCer and sulfatide were completely resistant to hydrolysis by the enzyme (Table I). It is noteworthy that NgaP hydrolyzed GA2 and Gb4Cer in the absence of detergents (Fig. 3, lanes 2 and 7). However, the addition of TDC and Triton X-100 at a concentration of 0.2 % (w/v) increased the hydrolysis of GA2 by NgaP by 8- and 3-fold compared to that in the absence of detergents (Fig. 3, lanes 3 and 4). Activation of the hydrolysis of substrates by detergents was not observed when pNP-β-GalNAc was used as a substrate instead of GSLs, indicating that detergents were required to solubilize the GSL substrates but not enzymes.

The time course for the hydrolysis of GA2 and Gb4Cer by NgaP in the absence of detergent is shown in Fig. 2B. GA2 was hydrolyzed much faster than Gb4Cer by NgaP, suggesting the enzyme prefers the GalNAcβ1-4Galβ1-1′Cer linkage over the GalNAcβ1-3 linkage. The activity of NgaP was maximal at around pH 6.0 when pNP-β-GalNAc (Fig. 2C) or GA2 (data not shown) was used as a substrate. The pH-<i>k</i><sub>cat</sub>/<i>K</i><sub>m</sub> profile indicated that pK<sub>a</sub> and pK<sub>b</sub> values were 4.7 and 7.3, respectively (Fig. 2C). The activity was strongly inhibited by Cu<sup>2+</sup>, Ni<sup>2+</sup>, and Hg<sup>2+</sup> at 5 mM. The <i>K</i><sub>m</sub> and <i>k</i><sub>cat</sub> values of NgaP for pNP-β-GalNAc were 0.35 mM and 7.3 s<sup>-1</sup> of protein, respectively (Table II, Fig. 2D).

**Exploring the catalytic mechanism of NgaP by analysis of secondary structure.** As shown in Table I, the substrates having a C-2 acetamide group, such as pNP-β-GalNAc and β-GalNAc, adopt the substrate-assisted mechanism (23-26). Thus, it was suggested that the carbonyl oxygen of the C-2 acetamide group of the substrate might behave as a catalytic nucleophile, i.e. NgaP could adopt the substrate-assisted mechanism like β-HEX. Here, common features of the active site residue of the enzymes which adopt substrate-assisted catalysis were extracted from GH18, 20, 56 and 84 (23-26). Supplemental Fig. 3 shows ribbon diagrams of the (β/α)<sub>β</sub>-barrel around the substrate-binding pocket and secondary structures of typical enzymes showing the substrate-assisted catalytic mechanism. The proton donor residues of the GH18, 20, 56 and 84 enzymes (Glu140, Glu323, Glu113, and Asp298, respectively) existed in the loop just after strand β4 of the (β/α)<sub>β</sub>-barrel in GH18, 20, and 84 or strand β3 of the (β/α)<sub>β</sub>-barrel in GH56 (Supplemental Fig. 3A). Additionally, an aspartate residue was found one or two residues before the proton donor residue (Supplemental Fig. 3B). The Asp residue was reported to determine the orientation of the 2-acetamide group of the substrate, GlcNAc or GalNAc (27). Although the sequence similarity of GH18, 20, 56, and 84 was very low, the mechanism for recognition of the substrate by these enzymes appears to be very similar (Supplemental Fig. 3). We thus predicted that the region where Asp is adjacent to Glu on the (β/α)<sub>β</sub>-barrel could form an active site in these enzymes.

Next, a search for the active site of NgaP was made. First, using the deduced amino acid sequence of NgaP, the enzyme’s secondary structure was constructed with the PSIPRED protein Structure Prediction Server (http://bioinf.cs.ucl.ac.uk/psopred) (Fig. 1). As a result, Asp607 and Glu608 in the putative (β/α)<sub>β</sub>-barrel emerged as candidates on the loop after strand β4. To elucidate whether Asp607 and Glu608 are important for the reaction of NgaP, four mutants, in which Asp607 was replaced by Glu (D607E) or Asn (D607N), and Glu608 was replaced by Asp (E608D) or Gln (E608Q), were constructed by site-directed mutagenesis. Table II shows the <i>K</i><sub>m</sub>, <i>k</i><sub>cat</sub>, and <i>k</i><sub>cat</sub>/<i>K</i><sub>m</sub> values of the wild-type and four mutants of NgaP toward pNP-β-GalNAc as a substrate. In the mutant D607E, the <i>K</i><sub>m</sub> was increased by 5-fold whereas the <i>k</i><sub>cat</sub> was decreased to 1/70. The activity of the mutant D607N was completely abolished. In the
mutants E608D and E608Q, the $K_m$ values were decreased to about 1/2 and 1/4, and the $k_{cat}$ values were decreased to about 1/20 and 1/90, respectively. The $k_{cat}/K_m$ of the D607E, E608D and E608Q mutants were decreased to about 1/20 and 1/90, respectively. The $k_{cat}/K_m$ of the D607E, E608D and E608Q mutants were decreased to about 0.28, 9.0, and 4.2%, respectively, compared to the wild-type enzyme. These results indicate that Asp607 and Glu608 of NgaP are integral for the hydrolysis of the terminal $\beta$-GalNAc residue of pNP-GalNAc.

Inhibition of NgaP by GalNAc-thiazoline and GlcNAc-thiazoline. A comparison of the secondary structure of NgaP with that of known enzymes adopting substrate-assisted catalysis and a point mutation analysis strongly suggested that the enzyme exhibits substrate-assisted catalysis. Thus, we examined whether GalNAc-thiazoline and GlcNAc-thiazoline inhibit the activity of NgaP. GlcNAc-thiazoline ([3αR,5R,6S,7R,7αR]-5-[hydroxymethyl]-2-methyl-5,6,7,7a-tetrahydropyrano[3,2-d]thiazole-6,7-diol), a structural analogue of the oxazolinium intermediate, is known to be a potent inhibitor of enzymes adopting substrate-assisted catalysis such as $\beta$-HEX (28). The inhibition of GalNAc-thiazoline was observed at nanomolar range, in contrast, that of GlcNAc-thiazoline, at micromolar range (Fig. 4A). Thus, we employed Henderson plot (29) and Lineweaver-Burk plot (30) for inhibition model of the enzyme by GalNAc-thiazoline and GlcNAc-thiazoline, respectively. GalNAc-thiazoline and GlcNAc-thiazoline were found to competitively inhibit the enzymatic activity of NgaP (Fig. 4B, C). The $K_i$ values of the enzyme for GalNAc-thiazoline and GlcNAc-thiazoline were calculated by Morrison equation (18) and Dixon plot (19), respectively. As a result, it was found that $K_i$ value of NgaP for GalNAc-thiazoline was 1.3 nM whereas that for GlcNAc-thiazoline was 46.8 μM (Fig. 4D, E), indicating the inhibition of NgaP by GalNAc-thiazoline to be 36,000 times stronger than that by GlcNAc-thiazoline. These results clearly indicated that NgaP is a $\beta$-N-acetylgalactosaminidase exhibiting substrate-assisted catalysis.

DISCUSSION

Paenibacillus sp. TS12 was found to decompose various GSLs added to culture (10). For example, polysialogangliosides, GSLs having several NeuAc, were sequentially degraded in the culture of TS12 as follows: polysialogangliosides→GM1a→GA1→GA2→ LacCer→GlcCer→Cer. This may indicate that Paenibacillus sp. TS12 produces a series of GSL-degrading exoglycosidases such as sialidase, $\beta$-galactosidase, $\beta$-HEX, and glucocerebrosidase. Three enzymes that hydrolyze 4MU-$\beta$-GalNAc were cloned by expression cloning using a TS12 genomic library. Two of the three were found to hydrolyze not only 4MU-$\beta$-GalNAc but also 4MU-$\beta$-GlcNAc efficiently, indicating that they are $\beta$-HEX (12). The sequences of the two enzymes were homologous to those of GH20 $\beta$-HEXs. The remaining enzyme, tentatively designated NgaP in this study, hydrolyzed 4MU-$\beta$-GalNAc, but not 4MU-$\beta$-GlcNAc, under the conditions used.

NgaP hydrolyzed the terminal non-reducing GalNAc residue of GA2 but not the internal GalNAc residue of GA1 (Table I), indicating that NgaP is an exo-type glycosidase. NgaP hydrolyzed GA2 more rapidly than Gb4Cer, suggesting that the $\beta$1-4 GalNAc linkage is more susceptible to NgaP than the $\beta$1-3 GalNAc linkage. NgaP hydrolyzed not only 4MU-$\beta$-GalNAc but also pNP-$\beta$-GalNAc. However, the enzyme did not hydrolyze pNP-$\alpha$-GalNAc, pNP-$\beta$-GlcNAc, or pNP-$\beta$-Gal. Thus, NgaP appears to strictly recognize 1-OH, 4-OH and C2-acetamide groups of the terminal sugar at the non-reducing end.

NgaP was not likely to belong to any existent GH families because its deduced amino acid sequence showed no similarity to any GH family member including $\beta$-HEX. This is quite remarkable because almost all O-glycoside hydrolases reported so far have been classified into a GH family based on amino acid similarity. Thus, we propose a new group which includes NgaP and hypothetical proteins of C. perfringens (EDS79974.1), P. distasonis (ABR44152.1) and B. thetaiotaomicron (AAO77857.1) showing high sequence similarity to NgaP but whose functions are unknown. It remains to be clarified whether these hypothetical proteins have $\beta$-NGA activity. Through X-ray structural analyses of various glycosidases, information about the reaction
mechanism of the enzymes has been accumulated. GH families are further grouped into ‘clans’ which display the same folding and catalytic mechanisms. The structures of the GH18, 20, 56, and 84 proteins have been uncovered. As a result, GH18 and GH20 were placed in clan GH-K while GH56 and GH84 were not. However, all glycosidases belonging to these four GH families hydrolyze substrates having a C2-acetamide group such as β-GlcNAc and β-GalNAc through substrate-assisted catalysis. Comparison of the amino acid sequences of these enzymes revealed that the arrangement of important residues around the active site is remarkably similar (Supplemental Fig. 3A). Since NgaP hydrolyzes the β-GalNAc linkage at the non-reducing end, NgaP may use substrate-assisted catalysis like β-HEX. In substrate-assisted catalysis, the C2 acetamide group of the substrate behaves as a nucleophile and the proton donor is present on the loop just after the β strand of the (β/α) barrel, and the Asp residue is present one or two residues before the proton donor. This Asp residue was found to assist the correct orientation of the 2-acetamide group at the active site (27). 

Williams et al. reported the structure of two Asp variants (D313N and D313A) of a GH20 β-HEX from S. plicatus in a complex with β-GlcNAc (27). According to their data, the 2-acetamide group of β-GlcNAc was rotated in the D313N mutant and consequently the carbonyl oxygen of the 2-acetamide group could not function as a nucleophile during the formation of an oxazolinium ion intermediate (27). This result indicated that Asp313 was crucial for the substrate-assisted catalysis of β-HEX. Although the structure of NgaP has yet to be solved, we predicted the position of the proton donor (E608) and crucial Asp residue (D607) based on the observation by Williams et al (27). The results of point mutations of these two amino acids are consistent with the prediction. GalNAc-thiazoline competitively inhibited NgaP and the inhibition was 36,000-fold that by GlcNAc-thiazoline. Collectively, these results indicate that NgaP is a β-N-acetylgalactosaminidase adopting substrate-assisted catalysis in which E608 and Asp607 could function as a proton donor and a stabilizer of the 2-acetamide group of β-GalNAc at the active site, respectively. The X-ray crystal analysis of NgaP would provide more precise information about the catalytic mechanism of this novel glycosidase.

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**FOOTNOTES**

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The abbreviations used are: GA1, asialo GM1; GA2, gangliotriaosylceramide (asialo GM2); GalCer, galactosylceramide; \( \beta \)-GalNAc, \( \beta \)-N-acetylgalactosamine; Gb3Cer, globotriaosylceramide; Gb4Cer, globotetraosylceramide; GH, glycoside hydrolase; GlcCer, glucosylceramide; \( \beta \)-GlcNAc, \( \beta \)-N-acetylgalcosamine; GSL, glycosphingolipid; \( \beta \)-HEX, \( \beta \)-hexosaminidase; LacCer, lactosylceramide; 4MU, 4-methylumbelliferyl; \( \beta \)-NGA, \( \beta \)-N-acetylgalactosaminidase; NgaP, \( \beta \)-N-acetylgalactosaminidase from *Paenibacillus* sp. TS12; pNP, para-nitorophenyl; TDC, taurodeoxycholate. The structures of GSLs are presented in Table 1.

**FIGURE LEGENDS**

Fig. 1. Sequence alignment of NgaP and other unknown proteins and predicted secondary structure of NgaP. NgaP is aligned with other unknown proteins. Residues conserved in all the proteins are shown on a colored background. The alignment was made with Clustal W (31). The predicted secondary structural elements are indicated above the alignment. The putative \((\beta/\alpha)_8\) barrel region is in blue box. The putative essential carboxylic amino acid residues (*) are indicated below the sequence.

Fig. 2. Specificity and general properties of NgaP. (A), Time course for the hydrolysis of
pNP-β-HexNAc by NgaP. Aliquots of 50 nmol of pNP-β-GalNAc (■) and pNP-β-GlcNAc (●) were incubated with 1 mU of the enzyme at 37°C for the periods indicated in 100 μl of 25 mM acetate buffer, pH 6.0. The hydrolysis of pNP-substrates was examined as described under "Experimental Procedures". 

(B) Time course of the hydrolysis of GSLs by NgaP. Aliquots of 5 nmol of GA2 (○) and Gb4Cer (□) were incubated with 10 mU of the enzyme at 37°C for the times indicated in 20 μl of 25 mM acetate buffer, pH 6.0. The hydrolysis of GSLs was examined as described under "Experimental Procedures".

(C) pH-κcat/Km profile of wild-type NgaP. The reaction mixture containing different amounts of pNP-β-GalNAc (12.5 - 200 nmol) and an appropriate amount of enzyme in 100 μl of various 25 mM buffers was incubated at 37°C for 30 min. ○, acetate buffer, pH 3.5-6.0; ●, phosphate buffer pH 6.0-7.5; □, Tris-HCl buffer, pH 7.5-9.0. The κcat/Km values were determined by Hanes-Woolf plot. (E) Hanes-Woolf plot for the action of NgaP. The concentration of pNP-β-GalNAc was varied as indicated, and incubation time was 30 min. Values are means of triplicate determinations.

Fig. 3. Hydrolysis of GA2 and Gb4Cer by the recombinant NgaP. TLC showing the hydrolysis of GA2 and Gb4Cer. Aliquots of 5 nmol of GA2 or Gb4Cer were incubated with 10 mU of the enzyme at 37°C for 30 min or 6 h in 20 μl of 25 mM sodium acetate buffer, pH 6.0, containing 0.2% detergent. The hydrolysis of GA2 or Gb4Cer was examined as described under “Experimental Procedures”. Lane 1, GA2 marker; lane 2, NgaP + GA2; lane 3, NgaP + GA2 + 0.2% TDC; lane 4, NgaP + GA2 + 0.2% Triton X-100; lane 5, LacCer marker; lane 6, Gb4Cer marker; lane 7, NgaP + Gb4Cer; lane 8, NgaP + Gb4Cer + 0.2% TDC; lane 9, NgaP + Gb4Cer + 0.2% Triton X-100; lane 10, Gb3Cer marker.

Fig. 4. Inhibition of the NgaP activity by GalNAc-thiazoline and GlcNAc-thiazoline. (A) Aliquots of 50 nmol of pNP-β-GalNAc were incubated with 17 nM of enzyme at 37°C for 30 min in 100 μl of 25 mM acetate buffer, pH 6.0. The concentrations of GalNAc-thiazoline used were 2.5, 5, 10 nM (white bar) and GlcNAc-thiazoline used were 100, 200, 400 μM (grey bar). The type of inhibition was determined by Henderson plot (B) and Lineweaver-Burk plot (C) using GalNAc-thiazoline and GlcNAc-thiazoline, respectively. The reaction mixture containing different amounts of pNP-β-GalNAc and 17 nM of the enzyme were incubated at 37°C for 30 min in 100 μl of 25 mM acetate buffer, pH 6.0. (B) The concentrations of GalNAc-thiazoline used were 5~40 nM and pNP-β-GalNAc (mM) used were 0.25 (●), 0.5 (○) and 0.75 (□). (C) The concentration of GlcNAc-thiazoline (μM) used were 800 (●), 400 (○), 200 (■), 100 (□) and 0 (▲). K_i values of NgaP for GalNAc-thiazoline (D) and GlcNAc-thiazoline (E) were determined by Morrison’s equation (18) and Dixon plots (19), respectively. (D) Aliquots of 50 nmol of pNP-β-GalNAc were incubated with 17 nM of enzyme at 37°C for 30 min in 100 μl of 25 mM acetate buffer, pH 6.0. The ratio of v_i/v_0 was plotted against inhibitor concentration. (E) The concentrations of pNP-β-GalNAc (mM) used were 2 (●), 1 (○), 0.5 (■), 0.25 (□), and 0.167 (▲). The reaction mixture containing different amounts of pNP-β-GalNAc, 17 nM of the enzyme and different amounts of inhibitor in 100 μl of 25 mM acetate buffer, pH 6.0, was incubated at 37°C for 30 min. Values are means of triplicate determinations.
Table I  Substrate specificity of the recombinant NgaP

| pNP-substrate | 1 mU NgaP | 3 mU NgaP | 30 mU NgaP |
|---------------|-----------|-----------|------------|
| pNP-β-GalNAc  | 55.2      | 100       | 100        |
| pNP-β-GlcNAc  | 0         | 0.851     | 2.84       |
| pNP-β-Gal     | -         | -         | 0          |
| pNP-β-Glc     | -         | -         | 0          |
| pNP-β-Fuc     | -         | -         | 0          |
| pNP-β-Xyl     | -         | -         | 0          |
| pNP-α-GalNAc  | -         | -         | 0          |
| pNP-α-GlcNAc  | -         | -         | 0          |
| pNP-α-Gal     | -         | -         | 0          |
| pNP-α-Glc     | -         | -         | 0          |
| pNP-α-Fuc     | -         | -         | 0          |
| pNP-α-Xyl     | -         | -         | 0          |

| Glycosphingolipid | Structure                                      | Relative activity (%) |
|-------------------|-----------------------------------------------|-----------------------|
| GA2               | GalNAcβ1-4Galβ1-4Glcβ1-1'Cer                  | 100                   |
| GM2               | GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer       | 0                     |
| GA1               | Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer           | 0                     |
| GM1a              | Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer | 0                     |
| Gb4Cer            | GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1'Cer           | 39.6                  |
| Gb3Cer            | Galα1-4Galβ1-4Glcβ1-1'Cer                     | 0                     |
| LacCer            | Galβ1-4Glcβ1-1'Cer                            | 0                     |
| GlcCer            | Glcβ1-1'Cer                                   | 0                     |
| GalCer            | Galβ1-1'Cer                                   | 0                     |
| Sulfatide         | HSO3-3Galβ1-1'Cer                             | 0                     |

Upper panel; pNP-glycosides (50 nmol) were incubated with the enzyme at 37°C for 30 min in 100 µl of 25 mM sodium acetate buffer, pH 6.0. -, not determined. Values are the means of triplicate determinations. Lower panel; 10 nmol of each GSL was incubated at 37°C for 24 h with 10 mU of the enzyme in 20 µl of 25 mM sodium acetate, pH 6.0. Values are the means of duplicate determinations.
Table II  Kinetic parameters of wild-type and mutants of NgaP

| Enzyme  | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) | $k_{cat}/K_m$ relative |
|---------|------------|----------------------|-----------------------------------|------------------------|
| WT      | 0.35       | 7.3                  | 21.0                              | 100                    |
| D607E   | 1.74       | 0.10                 | 0.06                              | 0.28                   |
| D607N   | ND         | ND                   | ND                                | ND                     |
| E608D   | 0.20       | 0.38                 | 1.9                               | 9.0                    |
| E608Q   | 0.09       | 0.08                 | 0.88                              | 4.2                    |

The reaction mixture containing different amounts of pNP-β-GalNAc (3-250 nmol) was incubated with 0.5 mU of the enzyme at 37°C for 30 min in 100 μl of 25 mM sodium acetate buffer, pH 6.0. Values are the means of triplicate determinations. ND, no products were detected.
Figure 1

Predicted secondary structure

| NgaP                  | Clostridium          | Parabacteroides       | Bacteroides          |
|-----------------------|----------------------|-----------------------|----------------------|
| Predicted 2ndary structure |                      |                       |                      |
| NgaP                  | QLNAAVLPANASNQTIRWSTSDDQVAKIDAQGRLSAEAVGTVQVTATSEDGGFQATRKVVVAPASQYLKGAV |                      |                      |
| Clostridium           |                      |                       |                      |
| Parabacteroides       |                      |                       |                      |
| Bacteroides           |                      |                       |                      |

| NgaP                  | Clostridium          | Parabacteroides       | Bacteroides          |
|-----------------------|----------------------|-----------------------|----------------------|
| Predicted 2ndary structure |                      |                       |                      |
| NgaP                  | RLELYAIKDPRHGIYAVSV  |                      |                      |
| Clostridium           |                      |                       |                      |
| Parabacteroides       |                      |                       |                      |
| Bacteroides           |                      |                       |                      |

| NgaP                  | Clostridium          | Parabacteroides       | Bacteroides          |
|-----------------------|----------------------|-----------------------|----------------------|
| Predicted 2ndary structure |                      |                       |                      |
| NgaP                  | MVNRKQKTISDQVKASIALQAAAQSLAAPAEVVTIQTQ7DSGQP3VTQKRAVPAFVLQAGLTVAVKLAASALLDDMTQAIYVFLRQKE |                      |                      |
| Clostridium           |                      |                       |                      |
| Parabacteroides       |                      |                       |                      |
| Bacteroides           |                      |                       |                      |

| NgaP                  | Clostridium          | Parabacteroides       | Bacteroides          |
|-----------------------|----------------------|-----------------------|----------------------|
| Predicted 2ndary structure |                      |                       |                      |
| NgaP                  | IDVLPYDPRKQSGIGLSSLTSRAHYGTKDQSPDFENMKXADVPRSQRKDIIVDTEYKIE |                      |                      |
| Clostridium           |                      |                       |                      |
| Parabacteroides       |                      |                       |                      |
| Bacteroides           |                      |                       |                      |
Figure 2

(A) Hydrolysis (%) versus Time (h)

(B) Hydrolysis (%) versus Time (h)

(C) $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) versus pH

(D) $S$ (mM) / $V$ (μmol/min/mg protein) versus $S$ (mM)
Figure 3
Figure 4

(A) Relative activity

(B) GalNAc-thiazoline

(C) GlcNAc-thiazoline

(D) $K_i = 1.3 \text{nM}$

(E) $K_i = 46.8 \text{µM}$
Molecular cloning and catalytic mechanism of a novel glycosphingolipid-degrading \( \beta \)-N-acetylgalactosaminidase from Paenibacillus sp. TS12
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