Characterization of a Novel Endo-β-galactosidase Specific for Releasing the Disaccharide GlcNAcα1→4Gal from Glycoconjugates[†,‡]

Hisashi Ashida,[§] Karol Maskos,[||] Su-Chen Li,[§] and Yu-Teh Li*[,§]

Department of Biochemistry, Tulane University Health Sciences Center School of Medicine, New Orleans, Louisiana 70112, and Coordinated Instrumentation Facility, Tulane University, New Orleans, Louisiana 70118

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ABSTRACT: In contrast to the β-linked GlcNAc, the α-linked GlcNAc has not been commonly found in glycoconjugates. We have recently revealed the presence of an unusual endo-β-galactosidase (Endo-β-Gal_{GnGa}) in *Clostridium perfringens* capable of releasing GlcNAcα1→4Gal from glycans expressed in the gastric mucous cell-type mucin [Ashida, H., Anderson, K., Nakayama, J., Maskos, K., Chou, C.-W., Cole, R. B., Li, S.-C., and Li, Y.-T. (2001) *J. Biol. Chem. 276,* 28226−28232]. To characterize Endo-β-Gal_{GnGa}, we have cloned its gene, *gngC,* from the genomic DNA library prepared from *C. perfringens* ATCC10543. The gene encodes 420 amino acid residues including a 17-residue signal peptide at the N-terminus. Using pUC18, we were able to prepare 25 mg of the fully active and pure recombinant Endo-β-Gal_{GnGa} from 1 L of *Escherichia coli* DH5α culture, which was 170 times higher than that produced by the original clostridial strain. Endo-β-Gal_{GnGa} shares a low but significant sequence similarity with two other endo-β-galactosidases (16−21% amino acid identity). It also shows some similarity with bacterial 1,3-1,4-β-glucan 4-glucanohydrolases of the glycoside hydrolase family 16. Endo-β-Gal_{GnGa} was found to contain the EXDX(X)E sequence (Glu-168 to Glu-173), that has been identified as the catalytic motif of families 16 and 7 retaining glycoside hydrolases. We have used site-directed mutagenesis to show that Glu-168 and Glu-173 were essential for the Endo-β-Gal_{GnGa} activity. By NMR spectroscopy, Endo-β-Gal_{GnGa} was found to act as a retaining enzyme.

In contrast to the β-linked GlcNAc, the α-linked GlcNAc has not been commonly found in glycoconjugates. In higher animals, except in heparin and heparan sulfate, the α-linked GlcNAc has been found only at the nonreducing end of *O*-glycans. The terminal α-GlcNAc linked to the C4 position of a Gal residue has been mainly detected in *O*-glycans from the gastric and duodenal mucin (*1*−*3*). By using the monoclonal antibody recognizing GlcNAcα1→4Gal (*4*), this disaccharide epitope has been found to be expressed in the gastric gland mucous cells, Brunner's gland of the duodenum, and accessory gland of the pancreaticobiliary tract (*5*, *6*). Using the same antibody, this epitope has been also shown to occur in several tumor tissues, such as gastric adenocarcinoma (*6*), pancreatic ductal carcinoma (*6*), mucinous bronchioalveolar cell carcinoma of the lung (*7*), and the adenoma malignum of the uterine cervix (*8*). The human gene encoding α1,4-*N*-acetylglucosaminyltransferase responsible for the biosynthesis of GlcNAcα1→4Gal-epitope has been cloned from the human stomach cDNA library (*9*). Although the occurrence of this epitope in mammalian tissues has been well documented by immunohistochemical and northern blot analyses, the biological significance of this disaccharide epitope remains to be elucidated.

We have recently revealed the presence of an unusual GlcNAcα1→4Gal-releasing endo-β-galactosidase (Endo-β-Gal_{GnGa})[1] in *Clostridium perfringens* ATCC10543 (*10*). This enzyme specifically releases the disaccharide GlcNAcα1→4Gal from *O*-glycans expressed in the gastric gland mucous cell-type mucin. This enzyme has been shown to hydrolyze the endo-β-galactosyl linkage not only in the GlcNAcα1→4Galβ1→4GlcNAc sequence but also in GlcNAcα1→4Galβ1→3GalNAcα1→Ser/Thr. Endo-β-Gal_{GnGa} is distinct from the hitherto known endo-β-galactosidases because of its strict specificity for releasing the disaccharide GlcNAcα1→4Gal. To characterize Endo-β-Gal_{GnGa}, we have carried out the molecular cloning of this unusual endoglycosidase. This paper describes the cloning, characterization, and overexpression of the gene encoding Endo-β-Gal_{GnGa}, the mutational analyses of the putative catalytic amino acid residues, and the stereochemical specificity of this enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions. C. perfringens ATCC10543 used for the enzyme purification and the

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* Corresponding author. Phone: (504)584-2451. Fax: (504)584-2739. E-mail: yli1@tulane.edu.

[§] Department of Biochemistry.

[||] Coordinated Instrumentation Facility.

[1] Abbreviations: Endo-β-Gal_{GnGa}, GlcNAcα1→4Gal-releasing endo-β-galactosidase; PCR, polymerase chain reaction; PGM, porcine gastric mucin; hexasaccharide-alditol, GlcNAcα1→4Galβ1→4GlcNAcβ1→6-(GlcNAcα1→4Galβ1→3)GalNAc-ol; PAGE, polyacrylamide gel electrophoresis.

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Table 1: Sequences of Four Tryptic Peptides and an N-Terminal Peptide of Endo-β-Gal_{GnGa}

peptide	sequence[c]
tryptic peptide[a]	
T-5	EXAIDYMR
T-7	FLYIEENNDKVSYGDITL
T-9	SSAIMSFDKSXIHNFSG
T-16	NNETGEYLNIENQTGYIEHG
N-terminal[b]	KDFPANPIEKAGYKLDF

[a] The number following T indicates the peak number of the tryptic peptide separated by HPLC. [b] Determined previously (*10*). [c] X means the unidentified amino acid residue.

genomic DNA extraction was cultured in Todd−Hewitt medium under anaerobic conditions (*10*). *Escherichia coli* XL-Blue MRF′ and SOLR (both from Stratagene) were cultured in Luria−Bertani (LB) medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. *E. coli* DH5α was cultured in LB medium with or without 100 μg/mL ampicillin.

Amino Acid Sequencing of Tryptic Peptides. The native Endo-β-Gal_{GnGa} (60 μg) purified from the culture supernatant of *C. perfringens* as described previously (*10*) was denatured at 100 °C for 5 min in 60 μL of 100 mM Tris-HCl, pH 8.5, containing 0.02% SDS. The denatured Endo-β-Gal_{GnGa} was subsequently incubated with 0.6 μg of trypsin (Roche) at 30 °C for 16 h. The reaction mixture was applied onto a reverse-phase column (Hi-Pore, 4.6 × 250 mm, Bio-Rad) using a Waters 600E HPLC system. Elution was initially carried out with 20% acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min for 10 min, and then with a linear gradient of 20−50% acetonitrile for 30 min. The effluent was monitored by the absorbance at 214 nm, and peptide fractions were manually collected and evaporated to dryness. Four peptides were subjected to sequence analysis by the Core Facility of Louisiana State University, Health Sciences Center, New Orleans, LA. The sequences of the four tryptic peptides together with the previously determined 17-residue N-terminal sequence (*10*) are shown in Table 1.

Degenerate PCR. PCR with degenerate oligonucleotides was used for the amplification of a DNA fragment encoding Endo-β-Gal_{GnGa}. A sense primer F1 (5′-AARGAYTTYC-CIGCIAAYCCNAT-3′) and an antisense primer R1 (5′-ACYTTRTCRTTRTTYTCYTC-3′) were designed using the N-terminal and the tryptic peptide T-7 sequences (see Table 1), respectively. PCR was performed for 30 cycles using the genomic DNA prepared from *C. perfringens* as a template and *Taq* DNA polymerase (Gibco BRL). Each cycle consisted of denaturation at 94 °C for 40 s, annealing at 50 °C for 40 s, and extension at 72 °C for 2 min. A 850-bp PCR product generated was subsequently cloned into the pGEM-T Easy vector (Promega) and sequenced with an ABI Prism 3100 DNA sequencer using Big Dye Version 1 (Applied Biosystems) at the Center for Gene Therapy, Tulane University Health Sciences Center. This 850-bp DNA fragment was designated as probe A and used for screening the genomic DNA library prepared from *C. perfringens*.

Cloning of the Gene Encoding Endo-β-Gal_{GnGa}. The genomic DNA from *C. perfringens* (80 μg) was digested with 200 units of *Eco*RI and fractionated by 0.9% agarose gel electorophoresis. The DNA fragments between 4 and 9 kbp were extracted from the gel, ligated into the λZAP II predigested *Eco*RI/CIAP-treated vector (Stratagene), and in vitro packaged using the Gigapack Gold III packaging extract (Stratagene) to construct a genomic DNA library.

The phage plaques containing the genomic DNA library that appeared on a lawn of *E. coli* XL-Blue MRF′ were transferred to Hybond-N+ membranes (Amersham Pharmacia). After fixing the DNA, the membranes were hybridized with the ECL-labeled probe A at 42 °C for 16 h in the ECL Gold hybridization buffer (Amersham Pharmacia). The membranes were washed twice with 6 M urea and 0.4% SDS in 0.5× SSC for 20 min at 42 °C, and then washed twice with 2× SSC for 5 min at 25 °C. The washed membranes were soaked into the ECL substrate solution and exposed to X-ray films. One positive cloned phage was selected and in vivo excised using ExAssist helper phage and *E. coli* SOLR according to the instruction manual (Stratagene). The phagemid pBluescript SK(−) containing a 5.5-kbp *Eco*RI fragment was obtained. The flanking regions of probe A as well as the probe itself were sequenced. The cloned phagemid was found to contain the full-length open reading frame of the gene (*gngC*) encoding Endo-β-Gal_{GnGa}.

Construction of the Expression Plasmid. To amplify the DNA fragment encoding the mature Endo-β-Gal_{GnGa}, we designed a pair of primers, F2 (5′-AGAATTCGAAG-GATTTTCCAGCAAATCCAATTG-3′) and R2 (5′-AACT-GCAGTCCCTCTTTTGATTCATATATAGGTC-3′), that contained *Eco*RI and *Pst*I restriction sites, respectively (underlined sequences). PCR was performed with the genomic DNA as a template for 25 cycles using *Pfu* DNA polymerase (Stratagene). Each cycle consisted of denaturation at 94 °C for 40 s, annealing at 52 °C for 1 min, and extension at 72 °C for 3 min. The 1.4-kbp fragment produced was treated with *Eco*RI and *Pst*I, and then inserted into the corresponding sites of pUC18 (Sigma) to generate pUC18/*gngC*. Then, *E. coli* DH5α was transformed with pUC18/*gngC*, and the transformant was selected from an LB/ampicillin plate. The sequence of *gngC* was confirmed by DNA sequencing and used for producing the recombinant Endo-β-Gal_{GnGa}.

Site-Directed Mutagenesis of the Amino Acid Residues in the Putative Active Site. To prepare plasmids encoding one of the following mutant enzymes, E168Q, D170N, and E173Q, the mutagenic PCR were carried out by combining the universal reverse primer RM1 (5′-phosphorylated-TC-CTGTTTGTTTTGAATTGAACCAATC-3′) and one of the mutagenic forward primers, FM1 (5′-CAAATTGATATATT-AGAAACTTTCTTTAGT-3′), FM2 (5′-GAAATTAATATATT-AGAAACTTTCTTTAGT-3′), and FM3 (5′-GAAATTGAT-ATATTACAAACTTTCTTTAGT-3′) (mutagenetic codons are underlined). PCR was performed with pUC18/*gngC* as a template for 20 cycles using *Pfu* DNA polymerase. Each cycle consisted of denaturation at 94 °C for 40 s, annealing at 52 °C for 1 min, and extension at 72 °C for 8 min. Each reaction mixture was treated with *Dpn*I in order to digest the template DNA. Then, the PCR product was self-ligated and amplified in *E. coli* DH5α. Each construct with correct DNA sequence was selected and used for further study.

Purification of the Recombinant Endo-β-Gal_{GnGa}. E. coli DH5α carrying pUC18/*gngC* was cultured in 1 L of LB medium containing 100 μg/mL ampicillin at 30 °C for 20 h with shaking. The cells were harvested by centrifugation

(12000*g*, 15 min) and resuspended in 100 mL of 20 mM sodium acetate buffer, pH 6.0. The cells were lysed by using a French press and centrifuged (27000*g*, 30 min) to remove cell debris. The crude cell extract was further purified by affinity chromatography using Sephacryl S-200 HR as described previously (*10*).

Enzyme Assay. The Endo-β-Gal$_{GnGa}$ activity was determined using porcine gastric mucin (PGM) as the substrate as previously described (*10*). Enzyme assay was also carried out using 2.5 mM GlcNAcα1→4Galβ1→4GlcNAcβ1→6-(GlcNAcα1→4Galβ1→3)GalNAc-ol (hexasaccharide-alditol) prepared from PGM as the substrate according to the method described previously (*10*).

Determination of the Stereochemical Course of the Hydrolysis of Hexasaccharide-alditol by Endo-β-Gal$_{GnGa}$ Using NMR Spectroscopy. Hexasaccharide-alditol (1.5 mg; 1.3 μmol) dissolved in 600 μL of 5 mM sodium phosphate buffer, pH 6.0, was repeatedly exchanged with D_2O, with intermediate lyophilization, and then dissolved in 600 μL of D_2O (2.2 mM). NMR spectroscopy was carried out by using a 500 MHz Bruker DRX 500 spectrometer, at 25 °C in a 5 mm tube. A Bruker 5 mm TXI probe was used for all experiments. After recording the ^{1}H NMR spectrum of the substrate hexasaccharide-alditol, 5 μg (0.24 unit) of the pure recombinant Endo-β-Gal$_{GnGa}$ dissolved in 5 μL of D_2O was added, and the NMR tube was then placed back into the magnet and the reaction was monitored as a function of time. The spectrum was recorded every 2.5 min over 325 min. The HOD signal was suppressed with a low-power pulse of 2.0 s during the relaxation delay. Spectra were acquired with 16K data points over a spectral width of 3255 Hz with 32 scans. One-bond proton carbon coupling constants ($^1J_{CH}$) were measured from the ^{1}H-detected ^{1}H-^{13}C heteronuclear single quantum coherence spectra acquired without decoupling during acquisition.

Other Methods. SDS−PAGE was performed in a 10% polyacrylamide gel containing 0.1% SDS, and proteins were stained with Coomassie brilliant blue R. The protein concentration was determined using the BCA protein assay reagent (Pierce) with bovine serum albumin as a standard.

RESULTS

Cloning of the Gene Encoding Endo-β-Gal$_{GnGa}$. Table 1 shows the amino acid sequences of 4 tryptic peptides and the previously reported 17-residue N-terminal sequence of the native Endo-β-Gal$_{GnGa}$ (*10*). The deduced amino acid sequence of the 850-bp PCR product (probe A) generated from the genomic DNA with the degenerate primers F1 and R1, respectively designed from the peptide sequences of the N-terminal peptide and tryptic peptide T-7, was found to contain tryptic peptides T-5 and T-9. Thus, this DNA fragment was a part of the gene encoding Endo-β-Gal$_{GnGa}$. When the ECL-labeled probe A was used to screen the λZAP II genomic DNA library prepared from *C. perfringens*, about 30 positive plaques out of 12 000 recombinant phages were detected. One positive cloned phage was selected and in vivo excised to generate the phagemid pBEGA31 that contained a 5.5-kbp *Eco*RI fragment. The entire open reading frame of the Endo-β-Gal$_{GnGa}$ gene designated as *gngC* was found in this fragment. The nucleotide and the deduced amino acid sequences of *gngC* are shown in Figure 1. The gene (*gngC*)

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      AAGTTCACACAAGAAAATTTATAAAGTTTTGATATTTATAATTTTTTTATAAATATATTT  -61
      TATAGGTCAAACTTTTAAATTGGAGGGGTATTTTTGAATAGAAAAGGTGTATTAAGATTC   -1
      ATGTTTGTCTTTATGTTACTATTGCTGCTACCGTTTACTATTTCAAAAGCAAAGGATTTT   60
    1 M  F  V  F  M  L  L  L  L  L  P  F  T  I  S  K  A  K  D  F
                                                      N-terminal
      CCAGCAAATCCAATTGAAAAAGCTGGATATAAATTAGACTTTTCTGATGAATTTAATGGT  120
   21 P  A  N  P  I  E  K  A  G  Y  K  L  D  F  S  D  E  F  N  G
      peptide
      CCTACATTAGATAGAGAAAAATGGACTGATTATTATTTACCACATTGGTGCAAGGATCCT  180
   41 P  T  L  D  R  E  K  W  T  D  Y  Y  L  P  H  W  C  K  D  P

      GAAAGTGCTAAGGCTAATTATCGTTTTGAAAATGGATCACTTGTTGAATATATAACTGAA  240
   61 E  S  A  K  A  N  Y  R  F  E  N  G  S  L  V  E  Y  I  T  E

      GATCAGAAACCATGGTGCCCAGAGCATGATGGAACTGTTAGATCATCTGCCATAATGTCT  300
   81 D  Q  K  P  W  C  P  E  H  D  G  T  V  R  S  S  A  I  M  S
                                                 peptide T-9
      TTTGATAAAAGTTGGATACATAATTTTAGTGGAACAACTGATAACCATGAAAGAAATGAG  360
  101 F  D  K  S  W  I  H  N  F  S  G  T  T  D  N  H  E  R  N  E

      TGGAGAGGTTATACAACTAAATATGGATACTTTGAAATTCGTGCTAAGCTATCTAACACA  420
  121 W  R  G  Y  T  T  K  Y  G  Y  F  E  I  R  A  K  L  S  N  T

      GGTGGTGGAGGTCATCAAGCTTGGTGGATGGTTGGTATGCAGGATGATACTAATGATTGG  480
  141 G  G  G  G  H  Q  A  W  W  M  V  G  M  Q  D  D  T  N  D  W

      TTCAATTCAAAACAAACAGGAGAAATTGATATATTAGAAACTTTCTTTAGTAAAAAAGAT  540
  161 F  N  S  K  Q  T  G  E  I  D  I  L  E  T  F  F  S  K  K  D

      ACATGGAGAATCGCTGCATATGGATGGAATGATCCAAACTTCCAAACATCTTGGACTATT  600
  181 T  W  R  I  A  A  Y  G  W  N  D  P  N  F  Q  T  S  W  T  I

      TCAGAAGATAAAGTTCCATCAGGAGATCCAACTTCTGAATATCATATTTATGCAATGGAA  660
  201 S  E  D  K  V  P  S  G  D  P  T  S  E  Y  H  I  Y  A  M  E

      TGGACTCCTACTGCTTTGAAATTCTATTATGATAATGAATTATTTAAGGTTATATATGGT  720
  221 W  T  P  T  A  L  K  F  Y  Y  D  N  E  L  F  K  V  I  Y  G

      TCACCAGACTATGAAATGGGGACAATTTTAAATATATACACAGATGCAGGTTCAGGTGCT  780
  241 S  P  D  Y  E  M  G  T  I  L  N  I  Y  T  D  A  G  S  G  A

      CATAATGATGTTTGGCCTAAGGAATGGGCAATTGATTATATGAGAGTTTGGAAACCAGTA  840
  261 H  N  D  V  W  P  K  E  W  A  I  D  Y  M  R  V  W  K  P  V
                              peptide T-5
      GATGGATATAAAGAGAGTGAAAGTTTAAATAATTACTTAATAAGAAATAGACAAACAGGA  900
  281 D  G  Y  K  E  S  E  S  L  N  N  Y  L  I  R  N  R  Q  T  G

      AAATTCCTTTATATTGAAGAAAATAATGATAAAGTGTCTTATGGGGACATAACTTTAAAA  960
  301 K  F  L  Y  I  E  E  N  N  D  K  V  S  Y  G  D  I  T  L  K
           peptide T-7
      AATGAAAAAAATGCAAAATGGAGTAAAGAATATAGAGATGGATACACTTTATTAAAGAAT 1020
  321 N  E  K  N  A  K  W  S  K  E  Y  R  D  G  Y  T  L  L  K  N

      AATGAAACAGGAGAATATTTAAATATAGAAAATCAAACTGGATATATAGAACATGGTAAG 1080
  341 N  E  T  G  E  Y  L  N  I  E  N  Q  T  G  Y  I  E  H  G  K
      peptide T-16
      GTTCCAAAAACTTGGTGGAGCGCTCAATGGAGTGAAGTACCAGTAGATGGATATACAAGG 1140
  361 V  P  K  T  W  W  S  A  Q  W  S  E  V  P  V  D  G  Y  T  R

      TTTGTTAACAGATGGAAGCCTAATATGTCAATACATACAGAAAGTTATGAAGGCGTTTTA 1200
  381 F  V  N  R  W  K  P  N  M  S  I  H  T  E  S  Y  E  G  V  L

      CAGTATGGAAATGTTCCAAATACTTATTGGACAAGTCAATGGCAACTTATTCCTGTAGAA 1260
  401 Q  Y  G  N  V  P  N  T  Y  W  T  S  Q  W  Q  L  I  P  V  E

      TAAAAATAAAATTTTAAAAGTGTTTTATTAAAAAATTAAGTTTGACCTATATATGAATCA 1320
      *
      AAAGAGGGAGTTTTATTTTAAGTAAAGCTCTCTCTTTTTGTTATTTTATAATCTCTTTAT 1380
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FIGURE 1: Nucleotide and deduced amino acid sequences of Endo-β-Gal$_{GnGa}$. The deduced amino acid sequence of Endo-β-Gal$_{GnGa}$ is shown in single-letter code below the nucleotide sequence, and the amino acid residues are numbered beginning with the first Met. The peptide sequences obtained from the native enzyme shown in Table 1 are underlined; the putative Shine−Dalgarno sequence is double-underlined; the asterisk denotes the translation termination codon.

consists of a 1260-bp open reading frame that encodes 420 amino acid residues. A putative Shine−Dalgarno sequence, AGAAAAGG (nucleotides −21 to −14), was detected upstream from the initiation codon. The N-terminal sequence of the purified native enzyme is found between Lys-18 and Phe-34. By hydropathy analysis (data not shown), the 17 residues of the N-terminal region (Met-1 to Ala-17) were found to be highly hydrophobic, which could be the signal sequence required for secretion of the enzyme to the culture medium. All four internal tryptic peptide sequences shown in Table 1 were also found in the deduced amino acid sequence (Figure 1). Based on the DNA sequence, the mature protein consists of 403 amino acids with a calculated molecular mass of 47 410 Da, which is very close to the molecular mass of the purified native enzyme, 46 kDa, determined by SDS−PAGE.

Expression of Endo-β-Gal$_{GnGa}$ in E. coli. More than 97% of the total enzyme activity expressed by pUC18/*gngC* in

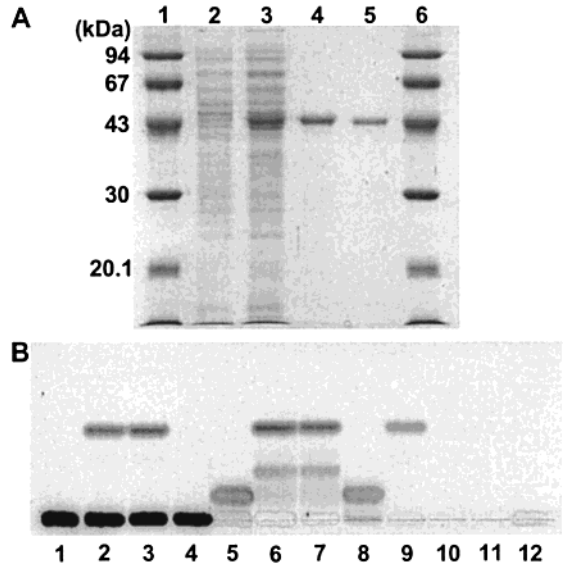

FIGURE 2: Expression of Endo-β-Gal_{GnGa} in *E. coli*. (A) SDS−PAGE of the recombinant Endo-β-Gal_{GnGa}. Lanes 1 and 6, molecular mass markers; lane 2, cell extract (20 μg) of *E. coli* DH5α (pUC18); lane 3, cell extract (20 μg) of *E. coli* DH5α (pUC18/*gngC*); lane 4, the recombinant Endo-β-Gal_{GnGa} (2.5 μg) purified by Sephacryl S-200 HR affinity chromatography; lane 5, purified native Endo-β-Gal_{GnGa} (1.5 μg). (B) TLC analysis showing the hydrolysis of PGM and hexasaccharide-alditol (Hex-ol) by the native and the recombinant Endo-β-Gal_{GnGa}. Lane 1, PGM; lane 2, PGM + purified native enzyme (5.8 ng); lane 3, PGM + purified recombinant enzyme (5.8 ng); lane 4, PGM + cell extract (8 μg) of *E. coli* DH5α (pUC18); lane 5, Hex-ol; lane 6, Hex-ol + purified native enzyme (5.8 ng); lane 7, Hex-ol + purified recombinant enzyme (5.8 ng); lane 8, Hex-ol + cell extract (8 μg) of *E. coli* DH5α (pUC18); lane 9, authentic GlcNAcα1→4Gal; lane 10, purified native enzyme (5.8 ng); lane 11, purified recombinant enzyme (5.8 ng); lane 12, cell extract (8 μg) of *E. coli* DH5α (pUC18). Incubation was carried out at 37 °C for 15 h according to the assay conditions described in the text.

Table 2: Purification of the Recombinant Endo-β-Gal_{GnGa} from a 1-L Culture of *E. coli* DH5α Carrying pUC18/*gngC*

step	protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)	purification (*x*-fold)
cell extract	490	1690	3.45	100	1
Sephacryl S-200 HR	25.1	1210	48.2	71.6	14.0

E. coli DH5α was detected in the intracellular fraction. The mock transfectant carrying pUC18 without *gngC* showed no Endo-β-Gal_{GnGa} activity (Figure 2B, lanes 4 and 8), indicating that the observed enzyme activity was in fact due to the expression of the cloned *gngC* gene. The enzyme was expressed at a level of 35 mg/L of culture, which was 170 times higher than that produced by the original clostridial strain. Isopropyl-β-D-galactopyranoside did not have any effect on the level of expression of the enzyme. As in the case of the native enzyme, we were able to purify the recombinant enzyme by one-step affinity chromatography using a Sephacryl S-200 HR column (Figure 2A, lane 4). As shown in Table 2, 25 mg of the pure recombinant enzyme was obtained with a recovery of 70% from a 1-L culture of *E. coli*. The specific activity of the recombinant enzyme was 48.2 units/mg, which is identical to that of the native enzyme (47.9 units/mg) (*10*). These results indicate that the recombinant enzyme produced in *E. coli* is in a fully active form. In addition, the native and recombinant enzymes showed the same electrophoretic mobility (Figure 2A, lanes 4 and 5).

Under control of the *lac* promoter, pUC18/*gngC* should express the recombinant enzyme in *E. coli* DH5α as a fusion protein containing the seven amino acid residues, MT-MITNS, from the N-terminus of β-galactosidase α-peptide. The N-terminal amino acid sequence of the recombinant Endo-β-Gal_{GnGa} was found to be MITNSKDFPANPI, confirming that the enzyme expressed in *E. coli* was a fusion protein carrying five amino acid residues, MITNS (without Met and Thr), from the N-terminal part of the β-galactosidase α-peptide. This may be due to the occurrence of an atypical initiation at the second AUG codon, or due to the processing of the two amino acids, Met and Thr, after translation. As in the case of the native enzyme (Figure 2B, lanes 2 and 6), the recombinant enzyme released the disaccharide GlcNAcα1→4Gal from PGM (Figure 2B, lane 3), and also hydrolyzed hexasaccharide-alditol to produce GlcNAcα1→4Gal and GlcNAcβ1→6(GlcNAcα1→4Galβ1→3)GalNAc-ol (Figure 2B, lane 7). The optimum pH and the stability of the recombinant enzyme were found to be identical to those of native enzyme (data not shown).

Comparison of Endo-β-Gal_{GnGa} with Other Related Enzymes. Searching the databases of FASTA (*11*) and BLAST (*12*) revealed that Endo-β-Gal_{GnGa} shares a low but significant similarity with endo-β-galactosidase from *Flavobacterium keratolyticus* (*13*) and endo-β-galactosidase C from *C. perfringens* ATCC10873 (*14*) (16% and 21% amino acid identity, respectively). While the molecular size of Endo-β-Gal_{GnGa} is similar to that of *Flavobacterium* endo-β-galactosidase (420 vs 422 amino acids), endo-β-galactosidase C is much larger in size (845 amino acids). Figure 3A shows the multiple alignment of Endo-β-Gal_{GnGa} with these two endo-β-galactosidases using the Clustal W program (*15*). Two-thirds of the sequence from the N-terminus of Endo-β-Gal_{GnGa} shares several conserved regions with these two endo-β-galactosidases: Leu-32 to Thr-49, Tyr-128 to Met-150, Glu-168 to Glu-173, Thr-211 to Asp-231, and Ile-271 to Lys-278 (boxed in Figure 3A). One-third of the sequence from the C-terminus, on the other hand, shares no significant similarity with any other known proteins. Endo-β-Gal_{GnGa} also shares some similarity (∼18% amino acid identity) with bacterial endo-β-glucanases of the glycoside hydrolase family 16 (*16*, *17*), such as 1,3-1,4-β-glucan 4-glucanohydrolase (EC 3.2.1.73), 1,3-β-glucan 3-glucanohydrolase (EC 3.2.1.39), and 1,3(1,3;1,4)-β-glucan 3(4)-glucanohydrolase (EC 3.2.1.6). These endo-β-glucanases were found to possess a common motif, EXDX(X)E (*18*), and the two Glu residues in this motif have been shown to be the active site catalytic residues of 1,3-1,4-β-glucan 4-glucanohydrolase from *Bacillus* (*18*−*20*). This motif was also found in the fungal cellulases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91) of the glycoside hydrolase family 7, formerly called cellulase family C (*21*, *22*). Interestingly, as shown in Figure 3B, the EXDX(X)E motif was found to be conserved in Endo-β-Gal_{GnGa} as well as in *C. perfringens* endo-β-galactosidase C and *F. keratolyticus* endo-β-galactosidase.

Effect of Site-Directed Mutagenesis on the Amino Acid Residues in the Putative Catalytic Site. The amino acid residues that are involved in the catalytic reaction of endo-β-galactosidases have not yet been identified. To determine

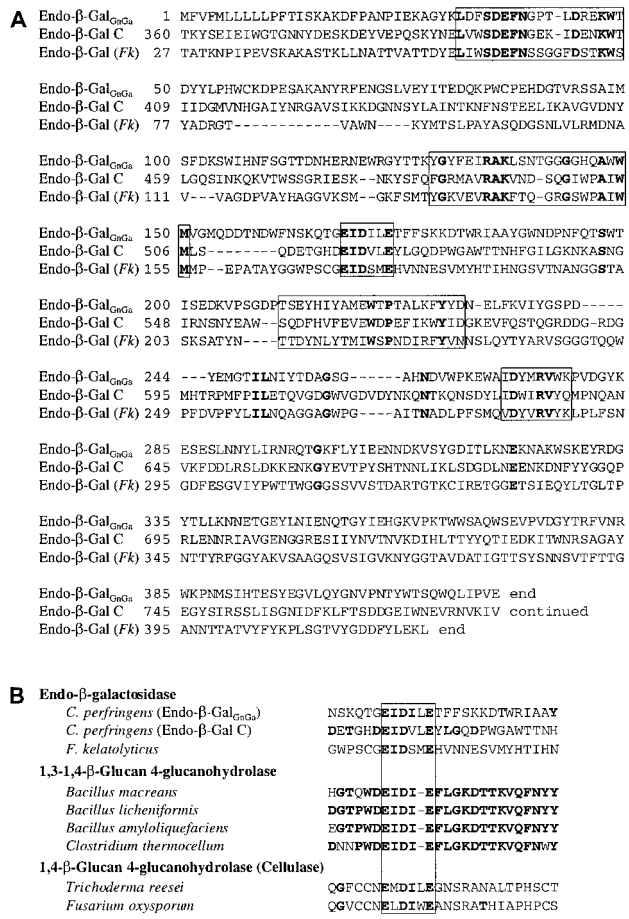

FIGURE 3: Sequence alignment of Endo-β-Gal$_{GnGa}$ with two endo-β-galactosidases and other related enzymes. (A) Alignment of three endo-β-galactosidases using the Clastal W program (*16*). Endo-β-Gal$_{GnGa}$ (accession number AB059351); Endo-β-Gal C, endo-β-galactosidase C from *C. perfringens* (AB038772); Endo-β-Gal (*Fk*), endo-β-galactosidase from *F. keratolyticus* (AF083896). The identical amino acids are in boldface letters. The highly conserved regions are boxed. The gaps inserted into the sequence are indicated by dashed lines. (B) Alignment of the putative active sites of endo-β-galactosidases with other related enzymes. The EXDX(X)E motif is boxed. Accession numbers are as follows: 1,3-1,4-β-glucan 4-glucanohydrolases from *Bacillus macreans* (X55959), *B. licheniformis* (X57279), *B. amyloliquefaciens* (M15674), and *C. thermocellum* (X58392); 1,4-β-glucan 4-glucanohydrolases (cellulases) from *Trichoderma reesei* (M15665) and *Fusarium oxysporum* (L29378).

whether the EIDILE sequence (Glu-168 to Glu-173) of Endo-β-Gal$_{GnGa}$ could be a catalytic motif, we constructed three specific mutants each containing a single amino acid substitution, E168Q, D170N, and E173Q, by site-directed mutagenesis. The mutant proteins were expressed in *E. coli* DH5α. As these three mutant proteins were also retained by the Sephacryl S-200 HR column, we were able to isolate them in homogeneous form from the cell extract by this affinity column (Figure 4A). Based on the yield of the protein, the expression levels of these three mutant proteins were found to be comparable to that of the wild type. However, as shown in Table 3, the enzyme activities toward the hydrolysis of PGM and hexasaccharide-alditol were drastically reduced by the point mutation. Under the standard assay condition (7 ng of enzyme, 10-min incubation), only the wild-type enzyme released GlcNAcα1$\rightarrow$4Gal from PGM (Figure 4B, lane 2). By increasing the enzyme concentration

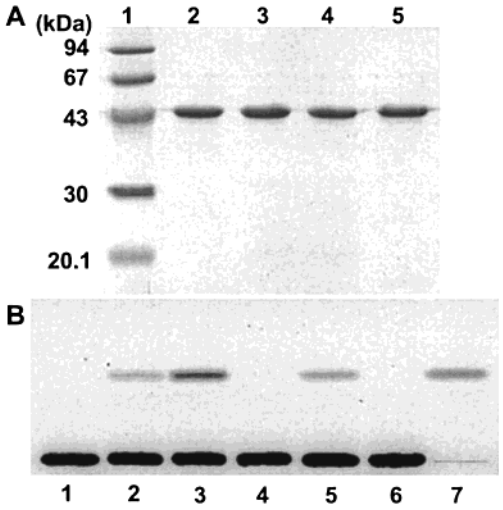

FIGURE 4: Effect of site-directed mutagenesis on the putative catalytic amino acid residues of Endo-β-Gal$_{GnGa}$. (A) SDS$-$PAGE of the mutant enzymes. Lane 1, molecular mass markers; lane 2, recombinant wild type (3.5 μg); lane 3, E168Q (3.5 μg); lane 4, D170N (3.5 μg); lane 5, E173Q (3.5 μg). (B) TLC analysis showing the hydrolysis of PGM by the wild type and the mutants of Endo-β-Gal$_{GnGa}$. Lane 1, PGM; lane 2, PGM + wild type (7 ng of enzyme, 10-min incubation); lane 3, PGM + wild type (700 ng of enzyme, 100-min incubation); lane 4, PGM + E168Q (700 ng of enzyme, 100-min incubation); lane 5, PGM + D170N (700 ng of enzyme, 100-min incubation); lane 6, PGM + E173Q (700 ng of enzyme, 100-min incubation); lane 7, authentic GlcNAcα1$\rightarrow$4Gal.

Table 3: Specific Activity (μmol min^{-1} mg^{-1}) of Each of the Wild-Type and the Three Mutants of Endo-β-Gal$_{GnGa}$ Expressed *in E. coli* DH5α toward the Hydrolysis of PGM and Hexasaccharide-alditol

	PGM	hexasaccharide-alditol
wild type	48.2	36.2
E168Q	0.004	0.003
D170N	0.08	0.06
E173Q	0	0

to 700 ng and prolonging the incubation time to 100 min (Figure 4B, lanes 3$-$6), the wild-type enzyme liberated almost all GlcNAcα1$\rightarrow$4Gal from PGM (Figure 4B, lane 3), while the mutant D170N showed only a low level of hydrolysis (Figure 4B, lane 5). The mutants E168Q and E173Q, on the other hand, did not show any detectable activity under this condition (Figure 4B, lanes 4 and 6, respectively). Only after extending the incubation to 10 h did the mutant E168Q show a trace of activity, whereas E173Q did not show any detectable activity under this condition. These results suggest that Glu-168 and Glu-173 are essential for the enzyme activity and that the side chains of these two Glu residues are involved in the catalytic activity of Endo-β-Gal$_{GnGa}$. The replacement of Asp-170 with Asn also severely attenuated the enzyme activity (Table 3), suggesting that this acidic amino acid residue is also important for the Endo-β-Gal$_{GnGa}$ activity.

Catalytic Mechanism of Endo-β-Gal$_{GnGa}$ Monitored by NMR Spectroscopy. The glycoside hydrolases have been divided into retaining and inverting enzymes. Each enzyme in these two classes exhibits a unique stereochemical course of reaction resulting in the release of the glycon with either retention or inversion of the original anomeric configuration at the cleavage site. To determine the catalytic mechanism

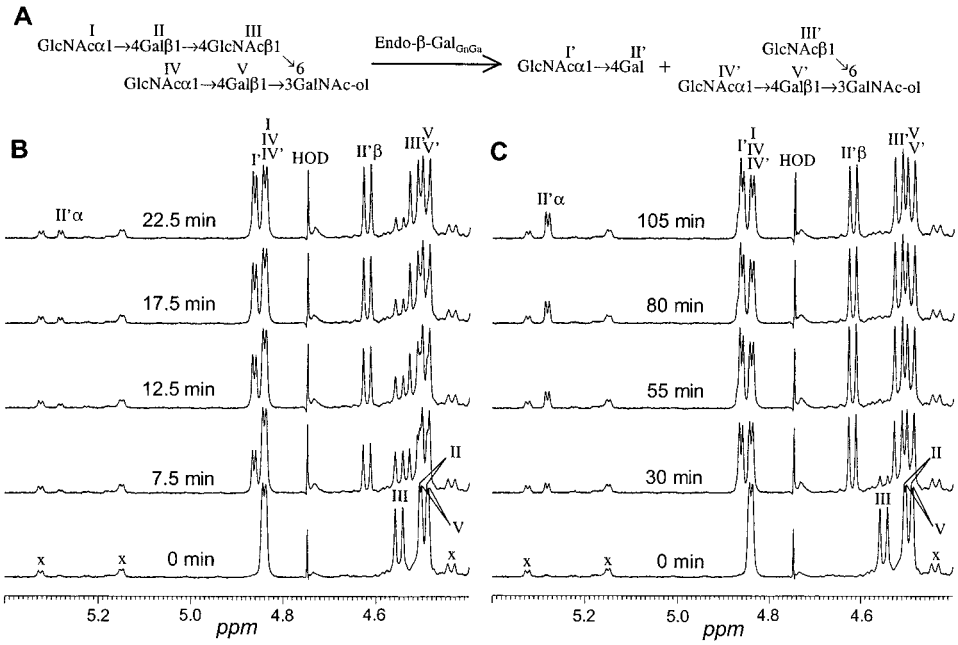

FIGURE 5: ^{1}H NMR spectra of the anomeric proton region showing the stereochemical course of hydrolysis of hexasaccharide-alditol by Endo-β-Gal$_{GnGa}$. (A) Structural representation of the overall reaction. (B) The spectra acquired during the first 22.5 min after the addition of the enzyme, showing the disappearance of I, II, and III, and the appearance of I′, II′β, and III′. (C) The spectra acquired between 30 and 105.5 min showing the conversion of II′β to II′α due to the mutarotation. See the text for the detailed explanation of the chemical shifts. The small resonances at ~5.32, 5.15, and 4.43 ppm (marked x) originate from the minor contaminants present in the substrate.

of Endo-β-Gal$_{GnGa}$, we have used NMR spectroscopy to monitor the hydrolysis of hexasaccharide-alditol. Figure 5 presents the time course of the hydrolysis of hexasaccharide-alditol showing the appearance of the resonance signals for the anomeric protons (H1s) of the released products, the disaccharide GlcNAcα1→4Gal and the tetrasaccharide-alditol GlcNAcβ1→6(GlcNAcα1→4Galβ1→3)GalNAc-ol, as well as the disappearance of several key anomeric protons of the substrate. We have assigned all the ^{1}H and ^{13}C chemical shifts of the hexasaccharide-alditol, and our assignments were consonant with that reported by Ishihara et al. (*4*). After the enzymatic reaction and the mutarotation have been completed, we have also assigned all the ^{1}H and ^{13}C chemical shifts of the two anomers of the disaccharide and the tetrasaccharide-alditol produced in the reaction mixture. The assignments for the two forms of the disaccharide were in full agreement with our previously reported results (*10*). The "0 min" spectrum of hexasaccharide-alditol before adding the enzyme is shown at the bottom (Figure 5B,C). The H1$_{ax}$ resonance of one of the two β-Gal (Figure 5A, V) is seen at 4.494 ppm ($^3J_{HH}$ = 7.7 Hz; $^1J_{CH}$ = 161 Hz) and the other β-Gal (Figure 5A, II) at 4.498 ppm ($^3J_{HH}$ = 7.8 Hz; $^1J_{CH}$ = 161 Hz). The H1$_{ax}$ doublet of β-GlcNAc (Figure 5A, III) appears at 4.549 ppm ($^3J_{HH}$ = 8.2 Hz; $^1J_{CH}$ = 161 Hz), and the H1$_{eq}$ signals of the two α-GlcNAc residues (Figure 5A, I and IV) are observed at 4.841 and 4.844 ppm ($^3J_{HH}$ = 3.4 Hz; $^1J_{CH}$ = 171 Hz), respectively. All subsequent spectra show the time course of the reaction. In the spectrum at 7.5 min shown in Figure 5B, the H1$_{ax}$ resonance of β-Gal (Figure 5B, II′β) of the released disaccharide (GlcNAcα1→4Gal) appeared at 4.619 ppm ($^3J_{HH}$ = 7.8 Hz; $^1J_{CH}$ = 161 Hz). This is consistent with the product retaining the original anomeric configuration found in the substrate. This signal continuously increased for about 37.5 min, and then became slightly lower as mutarotation took place (Figure 5C). The mutarotation started immediately after the liberation of the disaccharide since at 7.5 min a small H1$_{eq}$ signal of α-Gal (Figure 5B, II′α) of the disaccharide was visible at 5.282 ppm ($^3J_{HH}$ = 3.7 Hz; $^1J_{CH}$ = 170 Hz). The mutarotation process lasted for ~150 min until the final equilibrium values of 70% β-Gal and 30% α-Gal were reached, as determined by integration of the two H1 signals. During the reaction, the H1$_{eq}$ signal of the α-GlcNAc at 4.841 ppm (Figure 5B, I) and the H1$_{ax}$ signal of the β-GlcNAc at 4.549 ppm (Figure 5B, III) disappeared and shifted to 4.863 ppm ($^3J_{HH}$ = 3.2 Hz; $^1J_{CH}$ = 171 Hz) (Figure 5B, I′) and 4.520 ppm ($^3J_{HH}$ = 8.5 Hz; $^1J_{CH}$ = 161 Hz) (Figure 5B, III′), respectively. After the removal of GlcNAcα1→4Gal, the chemical shift of the α-GlcNAc in the tetrasaccharide produced (Figure 5B, IV′) was found to be identical to that in the substrate (Figure 5B, IV). These results clearly indicate that the mechanism of Endo-β-Gal$_{GnGa}$ involves stereochemical retention of the anomeric configuration of the product.

DISCUSSION

Four distinct types of microbial endo-β-galactosidases have so far been reported: the polylactosaminoglycan-cleaving endo-β-galactosidase (EC 3.2.1.102) from *E. freundi* (*23*, *24*), *F. keratolyticus* (*13*, *25*), and *Bacteroides fragilis* (*26*); the blood group A and B trisaccharide-releasing endo-β-galactosidase (EC 3.2.1.103) from *Streptococcus pneumoniae* (*27*); the Galα1→3Gal-releasing endo-β-galactosidase C from *C. perfringens* ATCC10873 (*14*); and the GlcNAcα1→4Gal-releasing endo-β-galactosidase, Endo-β-Gal$_{GnGa}$, from *C. perfringens* ATCC10543 (*10*). Among these, only the genes of *F. keratolyticus* endo-β-galactosidase (*13*) and *C. perfringens* endo-β-galactosidase C (*14*) have been previously cloned.

For detailed molecular characterization of Endo-β-Gal$_{GnGa}$, we have cloned the *gngC* gene that encodes this enzyme.

Although the codon usage of *C. perfringens* is quite different from that of *E. coli* (*28*), mainly due to the high A+T content (67% in *gngC*), we have successfully overexpressed *gngC* in *E. coli*. Using the plasmid pUC18, the recombinant enzyme was overproduced in *E. coli* at a level of 35 mg/L of culture. The yield of the pure native enzyme from the culture supernatant of *C. perfringens* ATCC10543 was only 0.1 mg/L despite the availability of a simple purification procedure using Sephacryl S-200 HR affinity chromatography (*10*). We were able to obtain 25 mg of the pure and fully active recombinant enzyme from a 1-L culture of *E. coli* carrying pUC18/*gngC* (Table 2). The availability of the recombinant Endo-β-Gal_{GnGa} should facilitate the study of the structure and function of glycoconjugates containing the GlcNAcα1→4Gal-epitope.

Although Endo-β-Gal_{GnGa} shares only 16−21% identity with the two other endo-β-galactosidases, several conserved regions have been observed (Figure 3A). Notably, the sequence motif of EXDX(X)E was found to be conserved in Endo-β-Gal_{GnGa} as well as in the two other endo-β-galactosidases (Figure 3B). This motif has been shown to be the active site of the glycoside hydrolases of families 16 and 7 (*18*, *21*). Site-directed mutagenesis revealed that Glu-168 and Glu-173 residues in this motif were essential for the Endo-β-Gal_{GnGa} activity (Figure 4B and Table 3). In *Bacillus* 1,3-1,4-β-glucan 4-glucanohydrolases of family 16, the equivalent residues, Glu-134 and Glu-138 (numbering in *B. licheniformis* enzyme), have also been shown to be the catalytic amino acid residues by site-directed mutagenesis (*18*).

It has been postulated that all glycoside hydrolases display either a single-displacement or a double-displacement reaction resulting in inversion or retention, respectively, of the configuration of the anomeric carbon atom in the hydrolyzed glycoside (*29*). The *B. licheniformis* 1,3-1,4-β-glucan 4-glucanohydrolase has been determined to be a retaining enzyme by monitoring the enzymatic depolymerization of barley β-glucan using ^{1}H NMR spectroscopy (*30*). Although glycoside hydrolases of families 16 and 7 are believed to be retaining enzymes, not all of them have been experimentally proven. The existence of the EXDX(X)E motif in Endo-β-Gal_{GnGa} suggests that this endo-β-galactosidase may be a retaining enzyme. In this study, we have used ^{1}H NMR spectroscopy to show that Endo-β-Gal_{GnGa} indeed catalyzes the liberation of GlcNAc1α1→4Gal from hexasaccharide-alditol with a retention of the anomeric configuration of the Gal residue (Figure 5).

In the retaining enzymes, two catalytic amino acid residues act as the general acid/base and the catalytic nucleophile (*29*). By the chemical rescue method using inactive mutants, Glu-134 and Glu-138 of *B. licheniformis* 1,3-1,4-β-glucan 4-glucanohydrolase were shown to act as the catalytic nucleophile and the general acid/base, respectively (*31*). X-ray crystallography of the enzyme−inhibitor complex proved that the inhibitor was bound covalently to the side chain of one of the two Glu residues (*20*). The catalytic nucleophile was also identified in *B. amyloliquefaciens* 1,3-1,4-β-glucan 4-glucanohydrolase by the affinity labeling method using the epoxide-based inhibitor (*19*). Judging from the sequence homology of the catalytic motif, the side chains of Glu-168 and Glu-173 in Endo-β-Gal_{GnGa} are highly likely to act as the catalytic nucleophile and the general acid/base, respectively. In the first step of the enzyme reaction, Glu-168 may attack the glycosidic bond to form a covalent intermediate with the glycon part of the substrate. Glu-173 acts as a general acid (proton donor) during this reaction. In the second step, the intermediate is hydrolyzed, and the glycon departs from the enzyme. Glu-173 acts as a general base (proton acceptor) during the second reaction. The Asp residue between the two Glu residues in the catalytic motif is also conserved in the glycoside hydrolases of families 16 and 7. The substitution of Asp-170 with Asn resulted in a great loss of the Endo-β-Gal_{GnGa} activity (Figure 4B and Table 3). A similar result has been reported for the mutant D136N of *B. licheniformis* 1,3-1,4-β-glucan 4-glucanohydrolase (*18*). This Asp residue has been postulated to be involved in maintaining the appropriate pK_a of the side chains of these two Glu residues during catalysis (*18*). The carboxyl group of Asp-170 of Endo-β-Gal_{GnGa} might have a similar role. Further study of the three-dimensional structure of this unique endoglycosidase by X-ray crystallography is in progress.

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