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# Heterologous expression, purification and characterization of human β-1,2-*N*-acetylglucosaminyltransferase II using a silkworm–BmNPV bacmid system

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**Short title:** Characterization of human GnTII expressed in silkworm

**Abstract**

β-1,2-*N*-Acetylglucosaminyltransferase II (GnTII, EC 2.4.1.143) is a Golgi-localized type II transmembrane enzyme that catalyzes the transfer of *N*-acetylglucosamine to the 6-arm of the trimanosyl core of *N*-glycans, an essential step in the conversion of oligomannose-type to complex-type *N*-glycans. Despite its physiological importance, there have been only a few reports on the heterologous expression and structure-function relationship of this enzyme. Here, we constructed a silkworm-based *Bombyx mori* nucleopolyhedrovirus bacmid expression system and expressed human GnTII (hGnTII) lacking the N-terminal cytosolic tail and transmembrane region. The recombinant hGnTII was purified from silkworm larval hemolymph in two steps by using tandem affinity purification tags, with a yield of approximately 120 μg from 10 mL hemolymph, and exhibited glycosyltransferase activity and strict substrate specificity. The enzyme was found to be *N*-glycosylated by the enzymatic cleavage of glycans, while hGnTII expressed in insect cells had not been reported to be glycosylated. Although insects typically produce pauci-mannosidic-type glycans, the structure of *N*-glycans in the recombinant hGnTII was suggested to be of the complex type, and the removal of the glycans did not affect the enzymatic activity.

**Keywords:** *N*-glycan, complex type, glycosyltransferase, silkworm, *Bombyx mori* nucleopolyhedrovirus bacmid

Protein glycosylation is one of the most common co- and post-translational modifications that affects many physiological roles of glycoproteins produced in eukaryotic cells. *N*-Glycosylation is involved in a broad range of biological functions such as protein folding, stability, oligomerization, trafficking, and intracellular communication (1,2). In the early steps of *N*-glycosylation, a tetradecasaccharide $Glc_3Man_9GlcNAc_2$ (Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine) precursor is transferred *en bloc* from the dolichol pyrophosphate donor to an asparagine residue of the Asn-Xaa-Ser/Thr (Xaa can be any amino acid except Pro) motif in the nascent polypeptides in the endoplasmic reticulum (ER). Subsequently, ER-resident glycosidases trim glucose and mannose residues to form the oligomannosidic structure, which is the pathway conserved across almost all eukaryotes (3,4). By contrast, in later steps, several glycosidases and glycosyltransferases located in the Golgi apparatus modify the structure of *N*-glycans, which diverge significantly among different species and kingdoms (5). The complex type, which is widely found in mammalian cells, has GlcNAc, galactose, and sialic acid residues at the non-reducing ends of the glycans that are sequentially attached by *N*-acetylglucosaminyltransferases, galactosyltransferases, and sialyltransferases, respectively. In contrast, yeast produces high-mannose-type *N*-glycans with a high degree of mannose polymerization, and insects predominantly synthesize pauci-mannosidic *N*-glycans because they lack the active enzymes necessary for complex *N*-glycan synthesis (6,7).

β-1,2-*N*-Acetylglucosaminyltransferase II (GnTII, MGAT2, EC 2.4.1.143) is a *medial*-Golgi resident glycosyltransferase that catalyzes the transfer GlcNAc from a uridine diphosphate (UDP)-GlcNAc donor to the Manα1→6Manβ- arm of GlcNAcβ1→2Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAcβ1→Asn acceptor (8). This reaction is a key step in the biosynthesis of complex-type *N*-glycan containing two or more branches. Mutations in the catalytic domain of GnTII cause one of the congenital disorders of glycosylation (CDG), MGAT2-CDG (previously known as CDG-IIa), and MGAT2-CDG patients display growth retardation, mental retardation, and

facial dysmorphy (9,10). However, there is no treatment available for this disease, and the relationship between the point mutations within the catalytic domain and the enzymatic function is not known. GnTII belongs to the glycosyltransferase family 16 (GT16) in the CAZy database (http://www.cazy.org/) (11,12), and its genes are found in the genome of animals and plants. GnTII cDNAs from humans (13), rats (14), pigs (15), frogs (16), insects (17,18), *Caenorhabditis elegans* (19), and *Arabidopsis thaliana* (20) were cloned, and their products were shown to exhibit the same enzymatic activity. Because no three-dimensional structure of GT16 proteins, or of GnTII is available to date, the structure-function relationship of these enzymes is not clear. Only a few reports are available on the heterologous expression systems of recombinant GnTII, and therefore, efficient expression systems have been desired for mutational and structural studies of this enzyme.

Baculovirus-insect cell expression systems have been widely used for the production of mammalian glycoproteins because of their productivity and proper posttranslational modifications such as glycosylation (21). We previously developed a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid, which is a shuttle vector for *Escherichia coli* and *B. mori*, to facilitate heterologous protein production in silkworm cultured cells, larvae and pupae without baculovirus construction or amplification (22–24). The silkworm-based BmNPV bacmid expression system enabled us to produce several human proteins, including *N*-glycoproteins, although the *N*-glycan structure of the glycoproteins produced in silkworm is different from the mammalian complex-type structure (25,26). The structure of *N*-glycans produced in insect cells has been modified using the co-expression of mammalian glycosyltransferases necessary for complex-type glycan biosynthesis (27). We also successfully converted the glycan structure produced in silkworm pupae by the co-expression of human GnTII (hGnTII) and β-1,4-galactosyltransferase (28). In this study, the hGnTII was expressed in a secreted form in silkworm larval hemolymph using the BmNPV bacmid expression system. The tandem affinity purification tags enabled the efficient purification of the recombinant protein from

hemolymph, and the activity and the *N*-glycan structure of the recombinant hGnTII were evaluated.

## MATERIALS AND METHODS

### Construction of the BmNPV bacmid of the recombinant hGnTII

The transmembrane region (Ile12–Gly29) of hGnTII was predicted by using the TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (29). A secretion signal peptide derived from bombyxin was added to the N-terminus, and sequential hexahistidine ($His_6$)-tag and FLAG-tag (DYKDDDDK) were added to the N-terminus of hGnTII (HF-hGnTIIΔ29) or to the C-terminus of the luminal catalytic region (Arg30–Gln447, hGnTIIΔ29) of hGnTII (hGnTIIΔ29-FH). KOD-Plus-Neo DNA polymerase (Toyobo, Otsu, Japan) was used for PCR, and the oligonucleotides used in this study are listed in Table 1. The DNA fragments encoding hGnTIIΔ29 was amplified by PCR using the pFastBac1 plasmid (Thermo Fisher Scientific K.K., Yokohama, Japan) harboring a full-length hGnTII gene (28) as a template and the primer pair of hGnTIIΔ29_NdeI_F and hGnTII_HindIII_R (Table 1), followed by subcloning into the pET28a vector (Merck, Darmstadt, Germany). The gene encoding hGnTIIΔ29 containing the N-terminal $His_6$-tag derived from pET28a was amplified by PCR using the primer pair bx-His_BamHI_F and hGnTII_HindIII_R (Table 1) and then ligated into the pFastBac1 vector using the BamHI and HindIII restriction sites. To replace the thrombin cleavage site following the $His_6$-tag with FLAG-tag, PCR was carried out using the resultant plasmid as a template and the primer pair of His-FLAG_hGnTIIΔ29_F and His-FLAG_hGnTIIΔ29_R (Table 1), resulting in the donor plasmid for HF-hGnTIIΔ29 (pFB-HF-hGnTIIΔ29). To construct the donor plasmid for hGnTIIΔ29-FH (pFB-hGnTIIΔ29-FH), the gene encoding hGnTIIΔ29-FH was amplified using pFB-HF-hGnTIIΔ29 as a template and the primer pair bx-hGnTIIΔ29_BamHI_F and hGnTII-FLAG-

His_HindIII_R (Table 1), followed by ligation into the pFastBac1 vector. The constructs were verified by DNA sequencing. *Escherichia coli* BmDH10Bac-*CP*$^{-}$-*Chi*$^{-}$ competent cells, which contain the cysteine protease- and chitinase-deficient BmNPV bacmid (23), were transformed with pFB-HF-hGnTIIΔ29 or pFB-hGnTIIΔ29-FH and cultured on LB agar plate medium containing 50 μg/mL kanamycin, 7 μg/mL gentamycin, 10 μg/mL tetracycline, 40 μg/mL isopropyl β-D-1-thiogalactopyranoside, and 100 μg/mL 5-bromo-4-chloro-3-indolyl-4-galactopyranoside at 37°C for 18 h. The recombinant BmNPV bacmid containing the desired gene was extracted from a white positive colony after blue-white selection.

**Expression and purification of recombinant hGnTII**

To produce the recombinant HF-hGnTIIΔ29 and hGnTIIΔ29-FH, chitosan/BmNPV bacmid nanocomplexes were prepared as described previously (30) and then injected into fifth-instar silkworm larvae. The bacmid-injected larvae were reared on an artificial diet (Silkmate S2, Nohsan Corporation, Yokohama, Japan) at 26ºC for 6 days. The hemolymph was collected by cutting a caudal leg in a tube containing 1 mM 1-phenyl-2-thiourea and stored at −80ºC until further analysis. The hemolymph was diluted with 5 volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole and applied to 1 mL Ni Sepharose excel resin (GE Healthcare, Buckinghamshire, UK) equilibrated with the same buffer. The column was washed with the same buffer, and then the recombinant hGnTIIΔ29 was eluted with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. The fraction containing the protein was dialyzed against phosphate-buffered saline (PBS, pH 7.4) and subjected to FLAG-tag affinity chromatography using a DDDDK-tagged Protein Purification Gel (Medical and Biological Laboratories, Nagoya, Japan). The column was washed with PBS, and the recombinant protein was eluted with PBS containing 100 μg/mL DYKDDDDK peptide (Medical and Biological Laboratories). The eluted fractions were concentrated using a Vivaspin 500 ultrafiltration device (30 kDa molecular weight

cut-off; Sartorius, Goettingen, Germany). Protein expression and purity were confirmed by SDS-PAGE with Coomassie brilliant blue (CBB) staining. Protein concentration was determined by measuring the absorbance at 280 nm based on theoretical molar absorption coefficients calculated using the ExPASy ProtParam server (http://web.expasy.org/protparam/).

**Enzyme assay**

The fluorescent pyridylaminated (PA) glycans used were purchased from Masuda Chemical Industries (Takamatsu, Japan). Their structures and abbreviations are listed in Figure 1. MGn-PA was also prepared using non-labeled MGn glycan (N030; Chemily Glycoscience, Atlanta, GA, USA) and a Pyridylamination Manual Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. To measure the glycosyltransferase activity of the recombinant protein, a reaction mixture containing 5 μg/mL HF-hGnTIIΔ29 or hGnTIIΔ29-FH, 1 μM MGn-PA, 2 mM UDP-GlcNAc, 10 mM $MnCl_2$, and 50 mM HEPES-NaOH (pH 7.0) was prepared and incubated at 37ºC. To examine the pH effect on HF-hGnTIIΔ29 activity, the same reaction mixtures, with the exception that 50 mM MES-NaOH (pH 6.0–6.5), HEPES-NaOH (pH 6.8–7.4), and Tricine-NaOH (pH 8.0–9.0) buffers were used, were incubated at 37ºC for 10 min. The effect of temperature was assayed at 20–60ºC using 50 mM HEPES-NaOH buffer (pH 7.0). To test thermostability, the enzyme was incubated at 25ºC–60ºC for 30 min and then the remaining activity was examined under the standard condition described above. The metal dependency was investigated at 37ºC in 50 mM HEPES-NaOH buffer (pH 7.0) with 10 mM $MgCl_2$, $CaCl_2$, $FeCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, or $ZnCl_2$ instead of $MnCl_2$. After incubation, the reaction mixtures were boiled for 3 min to stop the enzymatic reaction. The amounts of PA-glycans were measured by reverse phase high-performance liquid chromatography (HPLC) using TSKgel ODS-80$T_M$ (4.6 mm × 250 mm, Tosoh, Tokyo, Japan) and an HPLC system equipped with LC-10AD VP pumps and an RF-10A XL fluorescence detector (Shimadzu, Kyoto, Japan) at 320 nm excitation and 400 nm emission. The samples were eluted under

isocratic conditions at 1.0 mL/min using 100 mM ammonium acetate buffer (pH 4.0) and a column temperature of 40ºC.

**Western blotting and lectin blotting**

Proteins were separated via SDS-PAGE and electroblotted onto a polyvinylidene fluoride (PVDF) membrane with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). For western blotting, the PVDF membrane was probed with an anti-DDDDK-tag monoclonal antibody (Medical and Biological Laboratories) as the primary antibody and an anti-mouse IgG antibody labeled with horseradish peroxidase (Medical and Biological Laboratories) as the secondary antibody. Specific bands were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck, Darmstadt, Germany) and a VersaDoc Imaging system (Bio-Rad). For lectin blotting, the PVDF membrane was incubated with *Griffonia simplicifolia* lectin GS-II Alexa Fluor 488 conjugate (Thermo Fisher Scientific), after which the fluorescent bands were detected with a Molecular Imager PharosFX system (Bio-Rad).

**Glycan digestion**

Endoglycosidase H (Endo H) and Peptide:*N*-glycanase F (PNGase F) were purchased from New England Biolabs (Ipswich, MA, USA) and Takara Bio, respectively. The recombinant proteins were denatured and treated with Endo H or PNGase F according to the manufacturers' protocols.

**RESULTS**

**Expression and purification of recombinant hGnTII**

HF-hGnTIIΔ29 and hGnTIIΔ29-FH were constructed to express the hGnTII protein in a soluble form in silkworm larval hemolymph and to facilitate their purification (Fig.

2A and B). HF-hGnTIIΔ29 and hGnTIIΔ29-FH were successfully secreted into larval hemolymph (Fig. 2C). A few bands with molecular weights around 50 kDa were observed in the fat body cells probably because the recombinant proteins which were not made with a suitable posttranslational modification and/or contained glycoforms with high-mannose-type *N*-glycans remained in the cells. Therefore, the secreted proteins in hemolymph were used for the further analyses. Because it was difficult to purify the recombinant proteins to homogeneity using only Ni affinity chromatography, further purification using FLAG-tag was carried out, which resulted in a single band on SDS-PAGE stained by CBB for both of the recombinant hGnTIIΔ29 proteins (Fig. 2D). The molecular weights of HF-hGnTIIΔ29 and hGnTIIΔ29-FH estimated from SDS-PAGE were 50 and 49 kDa, respectively (Fig. 2E), which were almost identical to those calculated from the amino acid sequences (50.3 and 50.0 kDa, respectively). The yields of purified HF-hGnTIIΔ29 and hGnTIIΔ29-FH were approximately 124 and 128 μg, respectively, from 10 mL of larval hemolymph.

**Enzymatic properties and substrate specificity**

To test whether the recombinant enzymes possessed the glycosyltransferase activity, they were incubated with the substrates UDP-GlcNAc and MGn-PA and the cofactor $Mn^{2+}$ in 50 mM HEPES-NaOH buffer (pH 7.0) at 37ºC, after which the products were analyzed by reverse phase HPLC. The elution profile showed a new peak at 15.7 min, which increased along with the reaction time (Fig. 3). The retention time of the peak was identical to that of a standard GnGn-PA, indicating that the silkworm-expressed hGnTIIΔ29 possessed glycosyltransferase activity that represents the transfer of GlcNAc from UDP-GlcNAc to an α-1,6-mannose residue of MGn-PA. The specific activity of HF-hGnTIIΔ29 (30.1±0.5 nmol/min/mg) was slightly higher than that of hGnTIIΔ29-FH (26.0±1.0 nmol/min/mg), and therefore, HF-hGnTIIΔ29 was used for further analysis.

The effect of pH on HF-hGnTIIΔ29 activity was examined over a pH range of 6.0–8.5. The enzyme exhibited the highest activity at pH 7.0 and more than 90% activity at

pH 6.8–7.4 (Fig. 4A). The effect of temperature was tested at 20–60ºC, and the optimum temperature was determined to be 37°C (Fig. 4B). HF-hGnTIIΔ 29 was stable up to 45ºC during 30 min of incubation and completely lost its activity after incubation at 60ºC (Fig. 4C). The metal dependency was investigated using several divalent metal chlorides. The enzyme had much lower levels of activity when assayed in the presence of all other metals tested, compared with $Mn^{2+}$ (Fig. 4D).

The acceptor substrate specificity of HF-hGnTIIΔ29 was examined using UDP-GlcNAc as a donor substrate and MM-PA, GnM-PA, GnGn-PA, and *p*-nitrophenyl-α-mannopyranoside as acceptor substrates instead of MGn-PA. No new peak was observed in the HPLC profiles of the reaction using MM-PA, GnM-PA, GnGn-PA, and *p*-nitrophenyl-α-mannopyranoside (data not shown).

**Analysis of *N*-glycan attached to HF-hGnTIIΔ29 expressed in silkworm**

The recombinant HF-hGnTIIΔ29 was predicted to be *N*-glycosylated, as it had two potential glycosylation sites, Asn69 (Asn-Val-Ser) and Asn86 (Asn-Leu-Thr). To investigate whether the recombinant enzyme was glycosylated, the protein was digested with PNGase F and Endo H. PNGase F digests most of *N*-glycans while Endo H cleaves high mannose-type and hybrid-type glycans but shows very low or no activity for pauci-mannose-type or complex-type glycans, respectively (31). After deglycosylation with PNGase F, the molecular size of the digested protein was lower than that of the intact protein, indicating that the recombinant protein was *N*-glycosylated (Fig. 5). In contrast, the mobility of Endo H-treated HF-hGnTIIΔ29 did not change compared to that of the intact protein. To predict the structure of *N*-glycan attached to HF-hGnTIIΔ29, lectin blotting was carried out using Alexa Fluor 488-labeled *G. simplicifolia* lectin GS-II, which binds GlcNAc residues at the non-reducing end of glycans (32). A specific band with a molecular weight of 50 kDa was observed in the intact HF-hGnTIIΔ29 (Fig. 5C). The PNGase F-treated HF-hGnTIIΔ29 was also detected by GS-II lectin as a single band with a lower molecular size (45 kDa).

**Effect of *N*-glycan on the activity**

To investigate whether the *N*-linked oligosaccharides affected the enzyme activity, non-denatured HF-hGnTIIΔ29 was deglycosylated with PNGase F, and its activity was measured. After deglycosylation under the native condition for 1 h, most of the glycans were removed (Fig. 5D). HF-hGnTIIΔ29 incubated without PNGase F showed an activity of 28.4±0.1, while the activity of the enzyme treated with PNGase F was 30.2±0.1 nmol/min/mg (Fig. 5E). The optimum pH and temperature of the deglycosylated HF-hGnTIIΔ29 were 7.0 and 37ºC, respectively, which were identical to those of intact HF-hGnTIIΔ29 (Figs. 4A and 4B). The thermostability of the deglycosylated enzyme was similar to that of the intact enzyme (Fig. 4C).

**DISCUSSION**

In this study, we expressed soluble forms of hGnTII in silkworm using the BmNPV bacmid expression system. The yields and activities of HF-hGnTIIΔ29 and hGnTIIΔ29-FH were almost identical, suggesting that neither of the N- and C-terminal affinity tags affected the folding, secretion process or activity of the recombinant enzyme. Although the optimum pH and temperature of hGnTII have not been reported in detail to our knowledge, the recombinant hGnTII containing the transmembrane region was previously measured at 37ºC and pH 6.5 (13). Porcine GnTII had an optimum pH between 6.0 and 6.5 (33), and the activity of those enzymes from other organisms was measured at pH 6.0–7.0 (8,16,34,35). HF-hGnTIIΔ29 had the highest activity in the presence of $MnCl_2$, compared with the other metal ions tested, and did not catalyze the transfer to GnM-PA, MM-PA, GnGn-PA, or *p*-nitrophenyl-α-mannopyranoside. Therefore, HF-hGnTIIΔ29 and the reported GnTII enzymes displayed similar enzymatic properties.

hGnTII has two potential *N*-glycosylation sites, and the recombinant enzyme was predicted to be an *N*-glycoprotein. Tan et al. reported that according to the experimental

molecular weights estimated by SDS-PAGE, the full-length hGnTII expressed in Sf9 insect cells was not glycosylated (13). PNGase F cleaved the *N*-glycan of HF-hGnTIIΔ29, while Endo H did not, suggesting that the recombinant enzyme was *N*-glycosylated and that the *N*-glycan structure was the complex type or the pauci-mannosidic type. Considering together with the lectin blotting using a GlcNAc-binding lectin GS-II, the N-glycan attached to HF-hGnTIIΔ29 was likely to be GlcNAcylated. GnTII activity in lepidopteran cells was reported to be much lower than that in mammalian cells (36) and most of the glycoproteins heterologously expressed in insect cells and silkworm have pauci-mannosidic oligosaccharides (5). Co-expression with heterologous GnTII induced the production of terminally GlcNAcylated glycans in insect cells and silkworm (7,27,28). Although the hGnTII expressed in this study was a soluble form, the enzyme was likely to catalyze the transfer GlcNAc to the *N*-glycan on the enzyme itself during the Golgi pathway. The PNGase F-treated HF-hGnTIIΔ29 was also detected by the GS-II lectin in spite of a decrease in the molecular weight. PNGase F has a broad substrate specificity for the *N*-glycan structures except for core α-1,3-fucosylated one (37), which is commonly found in insects (28,38). Therefore, either *N*-glycan attached to Asn69 or Asn86 of HF-hGnTIIΔ29 was likely to be α-1,3-fucosylated and could not be cleaved by PNGase F.

*N*-glycosylation contributes to the folding, stability and activity of glycoproteins (1,2,39,40). The enzymatic properties of HF-hGnTIIΔ29 treated with PNGase F was similar to that of intact HF-hGnTIIΔ29, indicating that *N*-linked oligosaccharides on HF-hGnTIIΔ29 did not affect the enzymatic activity and thermal stability. The multiple sequence alignment showed that the sequons corresponding to Asn69-Val-Ser and Asn86-Leu-Thr were conserved in mammalian GnTII but not in other phyla and plants (Fig. 6). Golgi-resident enzymes share the common topology of type II membrane proteins, consisting of an N-terminal cytoplasmic tail, a single transmembrane region, and a stem region of variable length followed by a C-terminal catalytic domain (41). While the N-terminal region (Met1–Leu118 of hGnTII) had low sequence identity, the C-terminal

region exhibited high similarity to the other GnTII, suggesting that the catalytic domain might begin around Val119. The number and location of potential sequons are diverse among the reported GnTII proteins, but mammalian GnTII and hGnTII have two conserved sites in the variable N-terminal region, which is predicted to be a non-catalytic stem region. These facts suggested that the *N*-glycans of hGnTII might not directly influence the catalytic activity. Several glycosyltransferases have been reported to be proteolyzed at the stem regions and released retaining their activity (42–47), resulting in a decrease in glycan processing function (44,48,49). Although the existence of *N*-glycans in native hGnTII remains to be studied, the *N*-glycans may protect hGnTII from proteolysis and contribute to its functional stability in the *medial*-Golgi.

In summary, the N-terminally truncated hGnTII was expressed using a silkworm-based BmNPV bacmid expression system, purified to homogeneity, and enzymatically characterized. The recombinant hGnTII exhibited the glycosyltransferase activity with a strict substrate specificity. The structure of the *N*-glycans attached to the recombinant enzyme was not the pauci-mannose type but was instead terminally GlcNAcylated. The *N*-glycans were attached to the conserved sequons among mammalian GnTII, and the removal of the glycans did not affect the glycosyltransferase activity and thermal stability. This expression system will facilitate studies on the structure-function relationship, such as mutational analysis and X-ray crystallography, and will be a tool for the *in vitro* synthesis of glycans.

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## FIGURE LEGENDS

**Figure 1. Structures and abbreviations of PA-glycans used in this study.**

**Figure 2. hGnTII constructs, expression and purification. (A)** Deletion of the N-terminal region (residues 1–29) containing the transmembrane (T) region. Two *N*-glycosylation sites (Asn69 and Asn86) are indicated. **(B)** Construction of the expression vectors for HF-hGnTIIΔ29 (upper) and hGnTIIΔ29-FH (lower). Abbreviations: *polh*, polyhedrin promoter; bx, bombyxin secretion signal; H, $His_6$-tag; F, FLAG-tag. **(C)** Western blotting analysis of the expression of HF-hGnTIIΔ29 (HF-Δ29) and hGnTIIΔ29-FH (Δ29-FH) in silkworm hemolymph (H) and fat body (FB) using an anti-FLAG-tag antibody. **(D)** SDS-PAGE analysis of the two-step purification of HF-hGnTIIΔ29 from hemolymph using Ni Sepharose excel and anti-FLAG-tag antibody agarose resins. **(E)** SDS-PAGE analysis of the purified enzymes after two-step affinity purification. The bands for the recombinant proteins are indicated with black arrows. M, molecular weight marker.

**Figure 3. HPLC analysis of the reaction products generated by HF-hGnTIIΔ29.** Aliquots of the reaction mixtures were removed at given sampling times and analyzed by reverse phase HPLC. The lowest profile shows a standard of GnGn-PA.

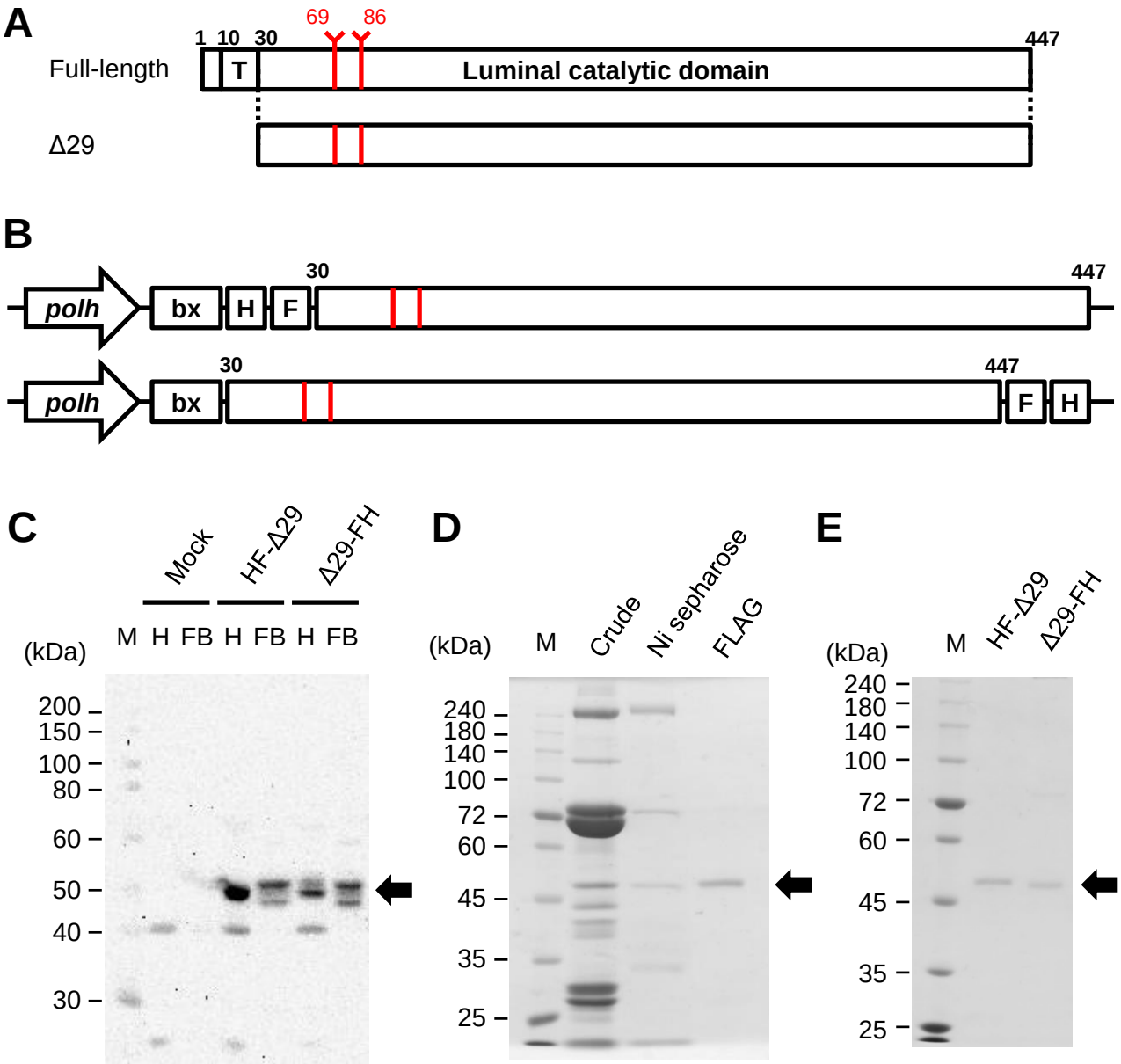
**Figure 4. General enzymatic properties of HF-hGnTIIΔ29. (A)** pH dependence, **(B)** temperature dependence, **(C)** thermostability, and **(D)** metal ions dependence. The pH dependence was measured at 37ºC using 50 mM MES-NaOH buffer (pH 6.0–6.5), 50 mM HEPES-NaOH buffer (pH 6.8–7.4), and 50 mM Tricine-NaOH buffer (pH 8.0–9.0). The temperature dependence was measured at 20–60ºC using 50 mM HEPES-NaOH (pH 7.0). For thermal stability assay, the enzyme was preincubated at 25–60ºC for 30 min.The effect of divalent metal ions was investigated at 37ºC using 50 mM HEPES-NaOH buffer

(pH 7.0) containing 10 mM of each metal chloride. Closed and open circles indicate the intact and PNGase F-treated HF-hGnTII**Δ**29 enzymes, respectively **(A–C)**.

**Figure 5. Analysis of *N*-glycans attached to HF-hGnTIIΔ29. (A)** SDS-PAGE analysis of recombinant HF-hGnTIIΔ29 treated with PNGase F or Endo H in the denatured condition using CBB staining. **(B)** Western blotting with anti-FLAG-tag antibody. **(C)** Lectin blotting with a GlcNAc-specific lectin GS-II. **(D)** SDS-PAGE analysis of HF-hGnTIIΔ29 deglycosylated with PNGase F in the native condition. Black and white arrows indicate intact and deglycosylated HF-hGnTIIΔ29, respectively. **(E)** Activity of the enzymes pre-incubated with (+) and without (−) PNGase F at 37ºC for 1 h. The activity of HF-hGnTIIΔ29 without pre-incubation was taken to be 100%.

**Figure 6. Multiple sequence alignment of characterized GnTII.** The alignment was performed using the Clustal Omega server (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sequences used were from humans (GenBank AAH06390.1), rats (AAA86721.1), pigs (CAA70732.1), *Xenopus laevis* (CAD56909.1), *Drosophila melanogaster* (AAX53005.1), *Spodoptera frugiperda* (AEX00083.1), *Caenorhabditis elegans* (AAF71273.1), and *Arabidopsis thaliana* (CAC08806.1). The amino acid residues conserved among all sequences are highlighted by black-and-white inversion, and the residues with similar properties are in gray boxes. The potential *N*-glycosylation sites are highlighted in red. The predicted transmembrane region of hGnTII is underlined.

| Glycan structure | Abbreviation |
| --- | --- |
| Manα1-6<br>Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2Manα1-3 | **MGn-PA** |
| GlcNAcβ1-2Manα1-6<br>Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Manα1-3 | **GnM-PA** |
| GlcNAcβ1-2Manα1-6<br>Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>GlcNAcβ1-2Manα1-3 | **GnGn-PA** |
| Manα1-6<br>Manβ1-4GlcNAcβ1-4GlcNAc-PA<br>Manα1-3 | **MM-PA** |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Full-length
Δ29
1 10 30
69 86
447
T
Luminal catalytic domain
B
polh
bx
H
F
30
447
C
Mock
HF-Δ29
Δ29-FH
M H FB H FB H FB
(kDa)
200
150
100
80
60
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D
(kDa)
M
Crude
Ni sepharose
FLAG
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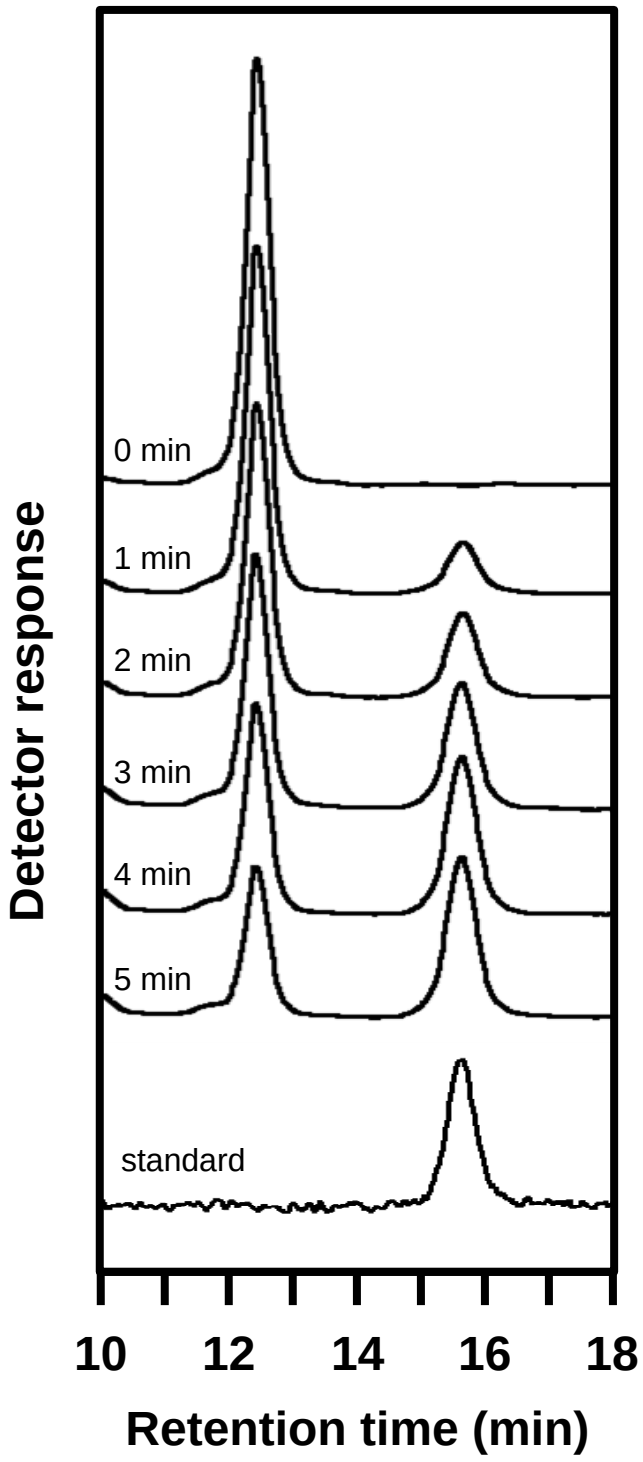
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standard
10
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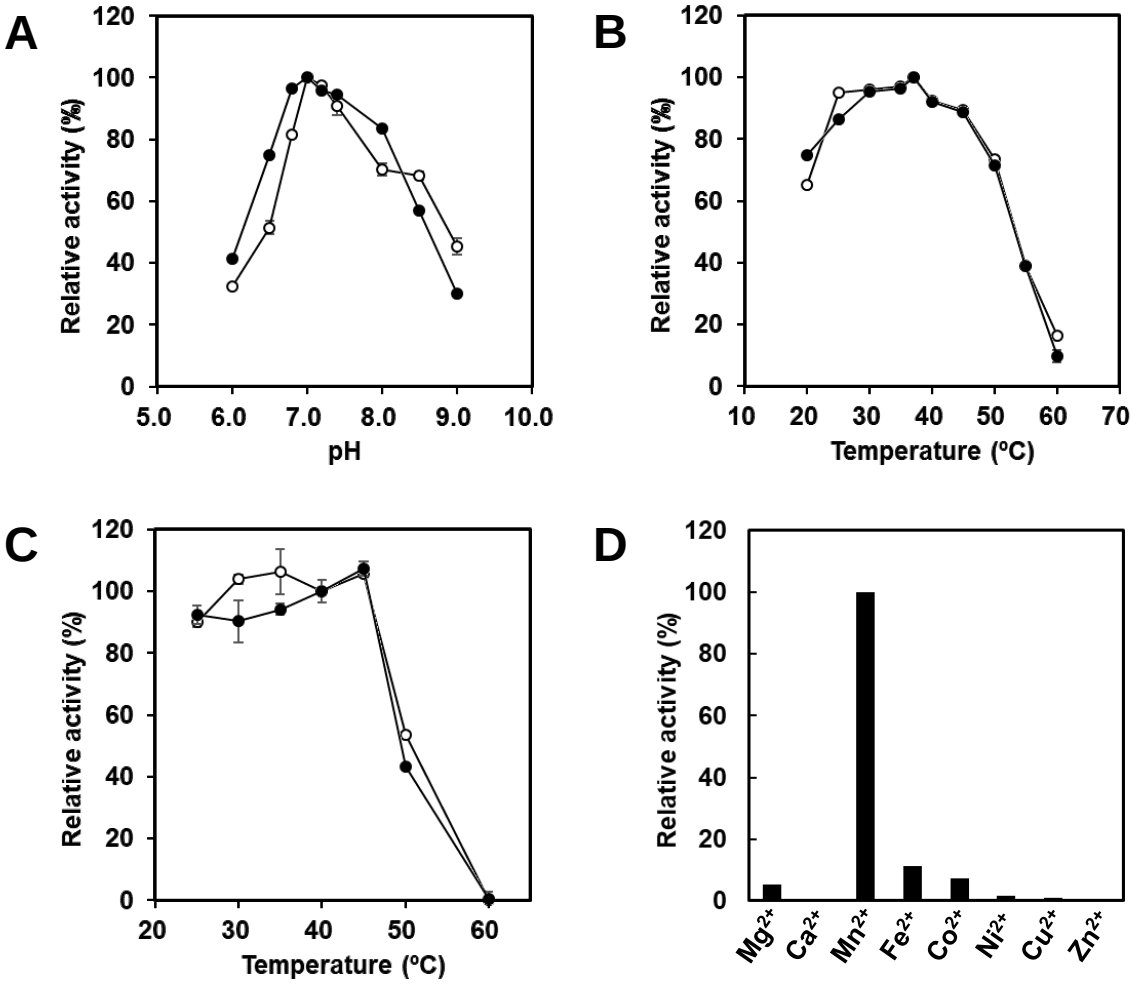
Retention time (min)


A
Relative activity (%)
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100
80
60
40
20
0
5.0
6.0
7.0
8.0
9.0
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pH
B
Relative activity (%)
120
100
80
60
40
20
0
10
20
30
40
50
60
70
Temperature (°C)
C
Relative activity (%)
120
100
80
60
40
20
0
20
30
40
50
60
Temperature (°C)
D
Relative activity (%)
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100
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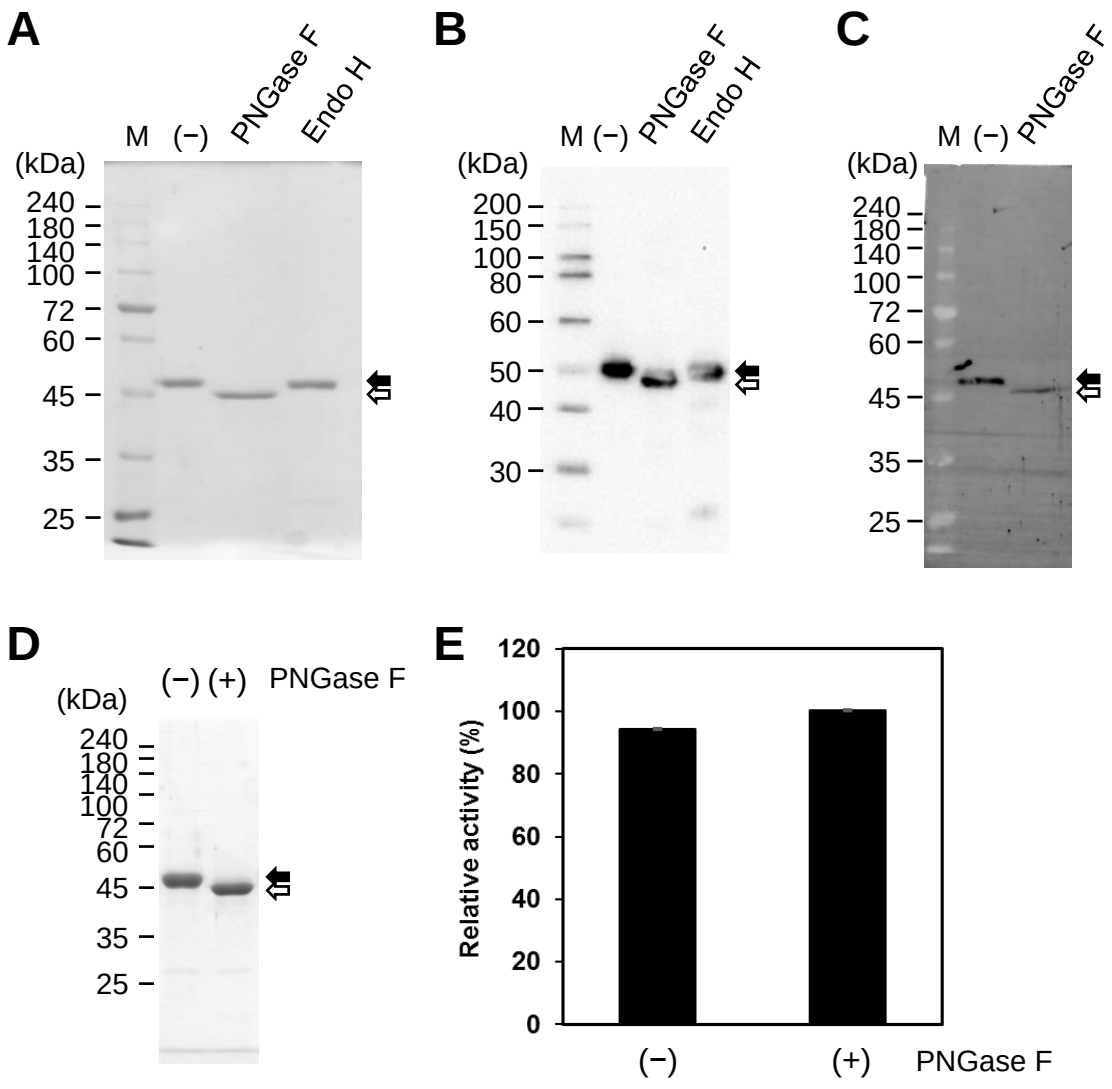
Fe2+
Co2+
Ni2+
Cu2+
Zn2+


A
M
(−)
PNGase F
Endo H
(kDa)
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180
140
100
72
60
45
35
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B
M
(−)
PNGase F
Endo H
(kDa)
200
150
100
80
60
50
40
30
C
M
(−)
PNGase F
(kDa)
240
180
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72
60
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D
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PNGase F
(kDa)
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Relative activity (%)
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PNGase F

# Miyazaki et al. Figure 6

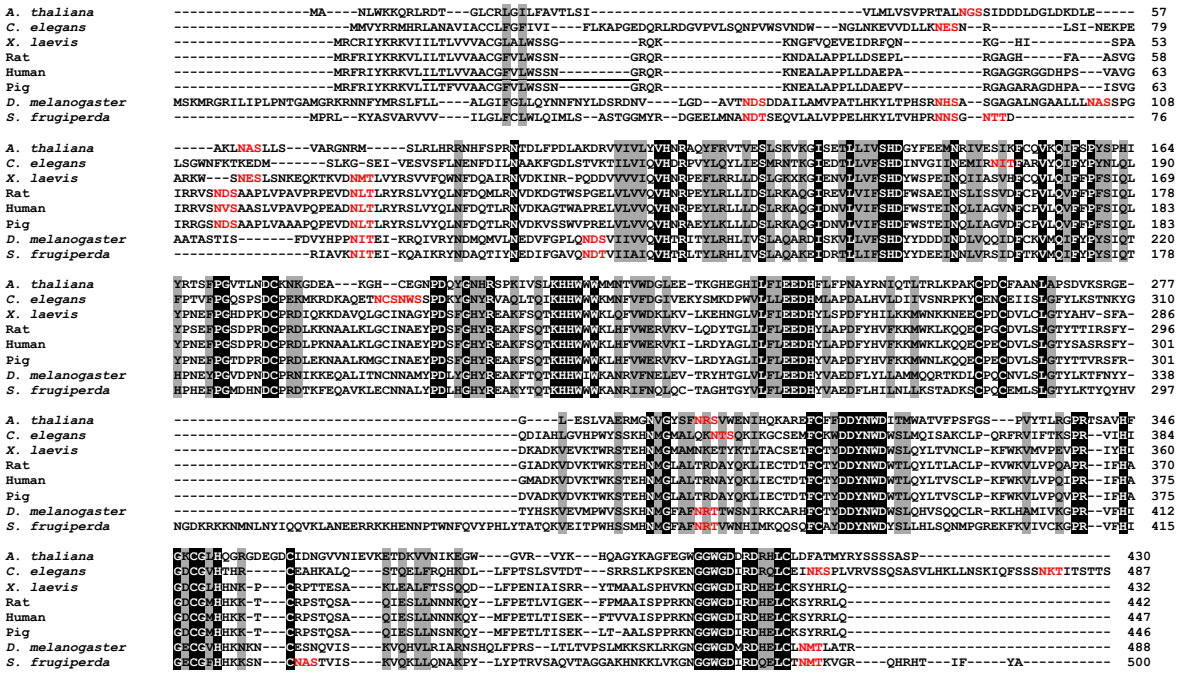

**Table 1. Primers used in this study.**

| Primer | Sequence |
|---|---|
| hGnTIIΔ29_NdeI_F | 5’-TTTTCATATGCGACAAAGGAAGAACGAGGCCCTCG-3’ |
| hGnTII_HindIII_R | 5’-TTTTAAGCTTTCACTGCAGTCTTCTATAACTTTTA-3’ |
| bx-His_BamHI_F | 5’-TTTGGATCCACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACAGGCAGCAGCCATCATCATCATCATCAC-3’ |
| His-FLAG_hGnTIIΔ29_F | 5’-CACGACTACAAGGATGACGATGACAAGCGACAAAGGAAGAACGAGGC-3’ |
| His-FLAG_hGnTIIΔ29_R | 5’-TCGCTTGTCATCGTCATCCTTGTAGTCGTGATGATGATGATGATGGC-3’ |
| bx-hGnTIIΔ29_BamHI_F | 5’-TTTGGATCCACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACACGACAAAGGAAGAACGAGGCCCTCG-3’ |
| hGnTII-FLAG-His_HindIII_R | 5’-TTTTAAGCTTTCAGTGATGATGATGATGATGCTTGTCATCGTCATCCTTGTAGTCCTGCAGTCTTCTATAACTTTTACAG-3’ |

The restriction enzyme sites are underlined.