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Heterologous expression, purification and characterization of human β-1,2-*N*-acetylglucosaminyltransferase II using a silkworm-based *Bombyx mori* nucleopolyhedrovirus bacmid expression system

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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.

[**Key words:** *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

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TABLE 1. Primers used in this study.

Primer	Sequence
hGnTIIΔ29_NdeI_F	5′-TTTTCATATGCGACAAAGGAAGAACGAGG CCCTCG-3′
hGnTII_HindIII_R	5′-TTTTAAGCTTTCACTGCAGTCTTCTATAA CTTTTA-3′
bx-His_BamHI_F	5′-TTTGGATCCACCATGAAGATACTCCTTGCT ATTGCATTAATGTTGTCAACAGTAATGTG GGTGTCAACAGGCAGCAGCCATCATCA TCATCATCAC-3′
His-FLAG_hGnTIIΔ29_ F	5′-CACGACTACAAGGATGACGATGACAAGCG ACAAAGGAAGAACGAGGC-3′
His-FLAG_hGnTIIΔ29_ R	5′-TCGCTTGTCATCGTCATCCTTGTAGTCG TGATGATGATGATGATGGC-3′
bx-hGnTIIΔ29_BamHI_F	5′-TTTGGATCCACCATGAAGATACTCCTTG CTATTGCATTAATGTTGTCAACAGTAATG TGGGTGTCAACACGACAAAGGAAGAACG AGGCCCTCG-3′
hGnTII-FLAG-His_HindIII_R	5′-TTTTAAGCTTTCAGTGATGATGATGATG ATGCTTGTCATCGTCATCCTTGTAGTCC TGCAGTCTTCTATAACTTTTACAG-3′

The restriction enzyme sites are underlined.

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-

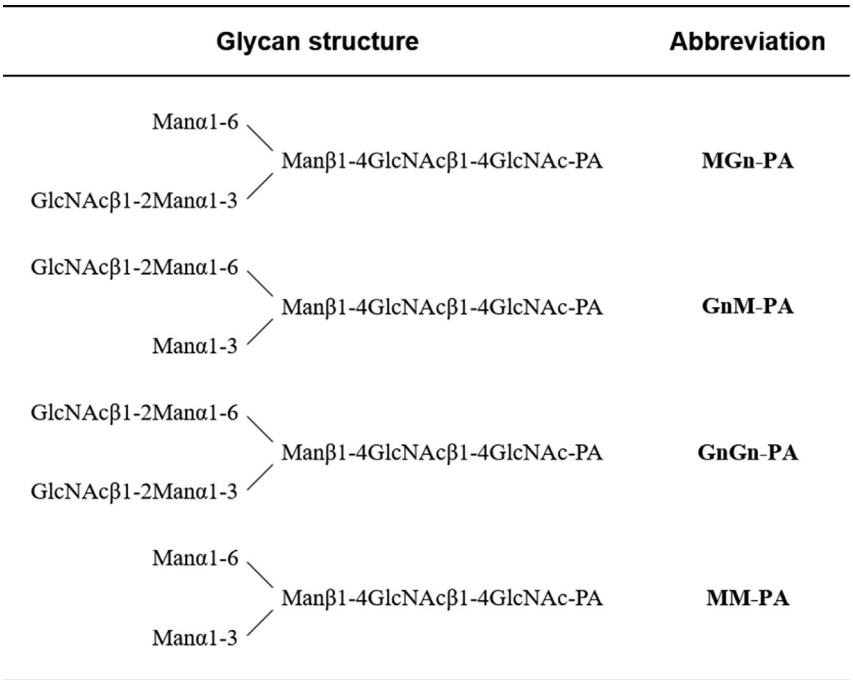

FIG. 1. Structures and abbreviations of PA-glycans used in this study.