Comparison of the $N$-linked glycosylation of human $eta 1,3N$-acetylglucosaminyltransferase 2 expressed in insect cells and silkworm larvae

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Abstract:

N-glycosylation of human β1,3N-acetylglucosaminyltransferase 2 (β3GnT2) is essential for its biological function. β3GnT2 fused to GFPuv (GFPuv-β3GnT2) was produced by non-virus expression systems in stably transformed insect cells and silkworm larvae using a recombinant BmNPV bacmid, and purified for analysis of N-glycosylation. The N-glycan structure of β3GnT2 was identified by glycoamidase A digestion, labeling with 2-aminopyridine (PA), and HPLC mapping. The paucimannosidic N-glycan structure (73.2%) was predominant in stably transformed Trichoplusia ni cells. In contrast, N-glycan with Gal (21.3%) and GlcNAc (16.2%) terminal residues linked to Manα(1,3) branch were detected on β3GnT2 expressed in silkworm larvae. The presence of terminal Gal and bisecting GlcNAc residues such as Galβ1, 4GlcNAcβ1, 2Manα1,3(GlcNAcβ1,4)(Manα1,6)Manβ1, 4GlcNAc is not typical structure for lepidopteran insect N-glycosylation. Although allergenic α1,3-fucose residues have been found in T. ni cells, only α1,6-fucose residues were attached to the β3GnT2 glycan in silkworm larvae. Therefore, silkworm larvae might be a useful host for producing human glycoproteins.

Keywords: silkworm larvae; BmNPV bacmid; insect cell, β1,3N-acetylglucosaminyltransferase 2; N-glycosylation
1. Introduction

Glycosylation plays an important role in a number of therapeutic proteins (Werner et al., 2007) and is one of the most common post-translational modifications to proteins in eukaryotic cells (Jenkins et al., 1994). The carbohydrate moiety of glycoproteins influences solubility, stability, enzyme activity, secretion and cellular processing (Varki et al., 1993). The structure of glycan depends on the glycosylating enzymes in the endoplasmic reticulum and the Golgi apparatus of the eukaryotic host cell (Werner et al., 2007).

Insect cell/larvae systems have been used as hosts for production of many kinds of proteins. These systems can produce heterologous proteins at high-levels, in a cost-effective way, in addition to posttranslationally modifying proteins. In general, the baculovirus-insect cell/larvae system transiently expresses the desired foreign gene during the immediately early phase of infection. Subsequently, the host cell moves to a cell lysis phase, resulting in release of intracellular contents into the cell broth. In this process, the secreted protein might have insufficient post-translational modifications, such as glycosylation. Typically, N-linked glycan structures obtained from insect tissue cells do not contain terminal GlcNAc, Gal or sialic acid residues (Jenkins et al., 1996), but high mannosidic and paucimannosidic structures.

The gene for β1,3N-acetylgalactosaminyltransferase 2 (β3GnT2) was cloned and expressed in the Sf9 insect cell line (Shiraishi et al., 2001), and recently, its production level has been improved using Trichoplusia ni insect cells (Kato et al., 2003, Kato et al.,
β3GnT is indispensable for synthesizing the lactose-\(N\)-triose II (GlcNAc\(\beta\)1-3Gal\(\beta\)1-4Glc) that is converted into lacto-\(N\)-tetraose (Gal\(\beta\)1-3GlcNAc\(\beta\)1-3GalNAc\(\beta\)1-4Glc) and lacto-\(N\)-neotetraose (Gal\(\beta\)1-4GlcNAc\(\beta\)1-3GalNAc\(\beta\)1-4Glc) (Murata et al., 1995). These oligosaccharides are believed to be prebiotics, immunomodulators, and inhibitors in human infection (Murata et al., 2006). The β3GnT2 has five potential \(N\)-glycosylation sites, Asn79, Asn89, Asn127, Asn173 and Asn219. Site-directed mutagenesis revealed that the \(N\)-glycan position at Asn219 is necessary for β3GnT2 activity and that at Asn127 and Asn219 are critical for efficient protein secretion (Kato et al., 2005b).

In this study, we investigated the \(N\)-glycan structures on β3GnT2 expressed in insect cell and silkworm larvae. In order to overcome cell lysis by baculovirus infection, we used non-virus expression and bacmid-based expression systems in stably transformed cells and in silkworm larvae, respectively. The \(N\)-glycan was released by glycoamidase A from purified GFPuv-β3GnT2 and analyzed by HPLC mapping. The purified GFPuv-β3GnT2 could be used as a sample for \(N\)-glycan analysis on β3GnT2, because the GFPuv and linker regions have no potential \(N\)-glycosylation sites that confirmed previously (Kato et al., 2005b).

2. Materials and methods

2.1. Cell lines, medium, silkworm larvae and bacmids
Tn-pXme11CNX6 cells stably co-express GFPuv-β3GnT2 under control of the *Bombyx mori* actin promoter and human Calnexin under control of the OpMNPV IE2 promoter (Kato et al., 2005a). The *B. mori* actin promoter is widely used for gene expression of stably transformed insect cell line, which was constructed by inserting the GFPuv-β3GnT2 gene into pXINSECT-DEST38 (Invitogen). Suspension cultures were grown in 250-ml flasks with 50 ml of SF900II serum-free medium (Invitrogen, San Diego, USA) supplemented with 1% antibiotic-antimycotic (Invitrogen) and 30 mM glutamine.

*B. mori* fifth-instar silkworm larvae (Fuyoutsukubane, Ehime Sansyu, Yahatahama, Japan) were used in this study. The larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 25°C. The recombinant BmNPV bacmid in which the cysteine protease gene was deleted (rBmNPV-CP bacmid) was used for expression of β3GnT2 under control of polyhedrin promoter in silkworm larvae (Park et al., 2007). The β3GnT2 gene fusion construct of Tn-pXme11CNX6 and rBmNPV-CP′ bacmid consists of a hexahistidine affinity tag (His6), the gfpuv gene, and an enterokinase cleavage site, followed by the β3GnT2 gene from the N- to C-terminus.

### 2.2. Expression of GFPuv-β3GnT2 in stable cell lines and silkworm larvae

Tn-pXme11CNX6 cells at a density of $5 \times 10^5$ cells/ml were cultivated in a rotary shaker with an agitation rate of 100 revolutions per minutes (rpm) at 27°C, for 5 d. After the culture cell broth was centrifuged at 7870 × g for 5 min at 4°C, the supernatant was used for GFPuv-β3GnT2 purification.
For silkworm larvae, rBmNPV bacmid DNA was injected directly into the first day of fifth-star silkworm larvae. Forty μg of rBmNPV bacmid was suspended in 5 μl of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE)-C (Invitrogen) as a transfection regent and placed at room temperature for 45 min. The resultant mixture was diluted to a final volume of 50 μl with PBS, and 20 μl of the bacmid mixture was injected into the dorsum of the larvae using a syringe with a 26-gauge beveled needle. The silkworm larvae were reared in a 25°C breeding incubator. The larvae were harvested at 6 days post injection (d.p.i.) and the hemolymph was collected by cutting the caudal leg in a tube containing 5 μl of 200 mM 1-phenyl-2-thiourea, and centrifuging at 10000 × g for 10 min at 4°C. The supernatant samples were immediately frozen at -80°C for GFPuv-β3GnT2 purification. The β3GnT2 activity of hemolymph supernatant was lost after a few days of storage at 4°C.

2.3. Purification of GFPuv-β3GnT2

His-tagged GFPuv-β3GnT2 was purified by affinity chromatography using a Ni2+ immobilized resin (5.0 ml bed volume, Ni Sepharose 6 FF, GE Healthcare, Piscataway, NJ, USA). The sample was applied to equilibrated column with start buffer (20 mM phosphate buffer, 0.5 M NaCl and 20 mM imidazole, pH 7.4). The column was re-washed with start buffer, followed by elution with a linear gradient of imidazole from 20 mM to 500 mM. One ml fractions were collected and assayed for β3GnT2 activity and SDS-PAGE.
For silkworm larvae, the hemolymph sample was diluted 5 times with 20 mM phosphate buffer (pH 7.4) and mixed with 40% saturated ammonium sulfate (pH 7.4) by vigorous stirring for 1.5 h. The preparation was centrifuged at 20000 × g for 30 min, and the supernatant was mixed with 50% saturated ammonium sulfate (pH 7.4) for 1.5 h and centrifuged again. The precipitate including GFPuv-β3GnT2 was dissolved in 20 mM phosphate buffer (pH 7.4), followed by dialysis overnight. The dialyzed sample was applied to the immobilized Ni²⁺ affinity column (His Trap HP 5ml, GE Healthcare). The column was then eluted at 0.5ml/min by stepwise gradient of 100, 250 and 500 mM imidazole in 20 mM phosphate buffer containing 0.5 M NaCl, monitored by both UV detection at 280 nm and fluorescence intensity using a fluorescence detector (RF-10AXL, Shimadzu, Kyoto, Japan) at an excitation wavelength of 395 nm and emission at 509 nm. The fractions with GFPuv-β3GnT2 were concentrated by Amicon Ultra Centrifugal Filter Devices (Millipore Co., Billerica, MA, USA) followed by loading onto a Hi Prep Sephacryl S-200 HR column (24 ml, GE Healthcare). The proteins were eluted with 20 mM phosphate buffer at flow rate of 1 ml/min. Chromatography was carried out using an AKTA 10S unit controlled by UNICORN software version 5.11 (GE Healthcare). The elution was collected in 0.5 ml-fractions with monitoring UV at 280 nm and fluorescence intensity as above. All purification steps were performed either on ice or in a refrigerated cabinet at 4°C.

2.4. SDS-PAGE and lectin blot analysis
Protein samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN II system (Bio-Rad, Hercules, USA). Bands were detected using Coomasie Brilliant Blue (CBB). For the detection of fluorescent GFPuv fusion protein by SDS-PAGE, bands were detected by Molecular Image FX (Bio-Rad).

The glycosylation status of β3GnT2 was assayed by lectin blot with Concanavalin A (ConA, Sigma), which detects specifically linked Manα1-3(Manα1-6)Man. The purified sample was run on SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with FITC-conjugated ConA at room temperature, followed by three washes with TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 5 min each. The bands were analyzed in a Fluor-S/MAX multi-image (Bio-Rad).

Protein concentration was measured with a Bradford protein assay kit (Bio-Rad).

2.5. β3GnT2 activity assay

The β3GnT2 activity assay was carried out in 50 mM Tris-HCl, pH 8.0, 15 mM MnCl₂, 19 mM UDP-GlcNAc, 22 mM Galβ1-4GlcNAcβ-clicked-PNP, and 5 μl of the enzyme solution (total volume 25 μl). The reaction started by the addition of the β3GnT2 sample to the reaction mixture. For each sampling, 5 μl of the reaction mixture were added to 195 μl distilled water, and the mixture was boiled for 5 min. The resulting solution was filtered with a 0.45-μm nitrocellulose filter (Millipore, Bedford, Massachusetts, USA) and then the filtrates were analyzed by HPLC (LC-VP, Shimadzu, Kyoto, Japan).
Mightysil RP-18(H) GP 150-4.6 (Kanto Chem., Tokyo, Japan) was used as a column. The reaction products were eluted with 10% methanol and detected at an absorbance of 300 nm. HPLC was performed at 40°C at flow rate of 1 ml/min. One unit of enzyme activity was defined as the amount of enzyme capable of catalyzing the transfer of 1 μmol GlcNAc per minute.

2.6. Structural analysis of N-glycans

All experimental procedures, including the chromatographic conditions and glycosidase treatments, have been described previously (Tomiya et al., 1987, Nakagawa et al., 1995, Takahashi et al., 1995). The purified β3GnT2 was proteolyzed with a chymotrypsin and trypsin mixture, and was further digested with glycoamidase A to release N-glycans. After the removal of the peptide materials, the reducing ends of the N-glycans were derivatized with 2-aminopyridine (PA) (Wako, Osaka, Japan). The mixture of PA-derivatives of the N-glycans was individually separated and identified sequentially on a Shim-pack HRC-ODS column (Shimadzu) and on a TSK-gel Amide-80 column (Tosoh, Tokyo, Japan). The identification of N-glycan structures was based on their elution positions on two kinds of columns in comparison with PA-glycans in the GALAXY database (Takahashi et al., 2003). The N-glycans were confirmed by co-chromatography and MALDI-TOF-MS analysis (Yagi et al., 2005).

3. Results
3.1. Expression of recombinant β3GnT2 in stably transformed cell and silkworm larvae

In the stably transformed cells, Tn-pXme11CNX6 were cultivated at 5d and the supernatant was harvested. For silkworm larvae, the hemolymph was collected at 6 d.p.i. The β3GnT2 activity of hemolymph and supernatant were 11 mU/ml and 32 mU/ml, respectively. The β3GnT2 expressed in silkworm larval hemolymph was 2.9 fold higher than that in supernatant of stably transformed cells culture.

The degraded products of GFPuv-β3GnT2 in supernatant of cell culture and silkworm larval hemolymph were analyzed by SDS-PAGE (Fig. 1A, B and Fig. 2A, B). Although some bands of fluorescent protein with low-molecular-wight were detected in supernatant of cell culture, the fragmentation of GFPuv-β3GnT2 was not observed as in the case of that expressed using baculovirus (Kato et al, 2004).

3.2. Purification of β3GnT2 expressed in a stable cell line

Cell culture supernatants were diluted two times with start buffer and applied to an equilibrated Ni²⁺ affinity column. Approximately 46% of the β3GnT2 activity bound to the column, whereas more than 50% containing protein was washed out. The absorbed GFPuv-β3GnT2 was eluted by linear imidazole gradient. The elution fractions were then analyzed for β3GnT2 activity and SDS-PAGE. The purified sample showed two major bands including the GFPuv-β3GnT2 (75 kDa) and a contaminating protein (Fig. 1A).

The purity of the GFPuv-β3GnT as a pool of N-glycoproteins was assayed by lectin blotting. A single GFPuv-β3GnT2 band was detected by ConA (Fig. 1B), indicating no
contaminants of Man terminal residue on $N$-glycan in the purified sample. Therefore, the purified pool was dialyzed against pure water overnight, followed by lyophilization to analyze $N$-glycans on $\beta$3GnT2. One mg of $\beta$3GnT2 fusion protein was purified from 280 ml culture supernatant and the recovery yield was 8.8%. The specific $\beta$3GnT2 activity indicates a 52-fold enrichment from cell culture supernatant (Table 1).

3.3. Purification of $\beta$3GnT2 expressed in silkworm larval hemolymph

Twenty-five ml of hemolymph was collected and diluted five times with 20 mM phosphate buffer (pH 7.4), followed by precipitation with 40-50% saturated ammonium sulfate. The GFP$_{uv}$-$\beta$3GnT2-containing fraction was dialyzed against 20 mM phosphate buffer (pH 7.4) and loaded on an immobilized Ni$^{2+}$ affinity column. Elution was performed by a stepwise gradient of imidazole concentrations from 100-500 mM. Elution fractions that detected fluorescence were analyzed for $\beta$3GnT2 activity and SDS-PAGE (Fig. 2A). $N$-linked glycoprotein contamination was assayed by lectin blotting, and several bands were detected with ConA (Fig. 2B). Therefore, an additional purification step of size exclusion chromatography was carried out using a Sephacryl S-200 HR column. Fractions eluted with 20 mM phosphate buffer were assayed by SDS-PAGE (Fig. 2C). Fractions with a single GFP$_{uv}$-$\beta$3GnT2 band were dialyzed against pure water and lyophilized for $N$-glycan analysis. In these purification processes, 0.5 mg of $\beta$3GnT2 fusion protein was purified from 25 ml silkworm hemolymph and the recovery yield was 5%. The specific $\beta$3GnT2 activity indicates 155-fold enrichment from hemolymph (Table 2).
3.4. Characterization of N-glycans

The reducing ends of N-glycans released from purified GFPuv-β3GnT2 by glucosamidase A were reductively aminated with PA. The PA-oligosaccharides were separated by ODS column and their profiles are shown in Fig. 3. Each separated fraction (a through k) was then applied to an amide column. Structural assignment was performed by HPLC mapping (Table 3).

The purified β3GnT2 sample from silkworm larvae contained

\[ \text{GlcNAcβ1,2Manα1,3(GlcNAcβ1,4)(Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc} \quad (16.2\%) \]

and

\[ \text{Galβ1,4GlcNAcβ1,2Manα1,3(GlcNAcβ1,4)(Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc} \quad (21.3\%) \]

which were not observed in samples from Tn-pXme11CNX6 cells (Table 3).

These data indicate that activities of β1,2N-acetylglucosaminyltransferase I (GlcNAcT I), β1,4N-galactosyltransferase (GalT) and β1,4N-acetylglucosaminyltransferase III (GlcNAcT III) might exist in silkworm larvae but not in Tn-pXme11CNX6 cells.

The N-glycan structure is summarized in Table 4. The different glycoforms were classified as high-mannose (with more than three mannose residues), paucimannose (containing two core GlcNAc residues and three or fewer mannose residues), and a terminal glycan other than mannose, such as GlcNAc or galactose. The percentage of high-mannose structures produced in Tn-pXme11CNX6 and silkworm larvae were 19.4% and 9.3%, respectively. The Tn-pXme11CNX6 sample contained larger amounts of three to seven mannose residues without fucosylation (23.6%), compared to samples
from silkworm larvae (0.9%). Most of the paucimannosidic glycans were different in addition with or without fucose residues. The N-glycans expressed in Tn-pXme11CNX6 were \(\alpha(1,3)\) and \(\alpha(1,6)\) fucosylated (36.0%). In silkworm larvae, the N-glycan structure was \(\alpha(1,6)\) fucosylated (29.4%). The fucosylated N-glycans produced by Tn-pXme11CNX6 and silkworm larvae comprised 58.2% and 29.4%, of total N-glycans respectively.

4. Discussion

The baculovirus-insect cell expression system is widely used for recombinant protein production in insect cells. However, this system has limited commercial use because of a difference in glycosylation between insect cells and mammalian cells. N-glycans from insect cell lines are not usually processed to terminally sialylated complex-type structures but are instead modified to paucimannosidic or oligomannose structures (Tomiya et al., 2004). This is because the terminal residue of N-acetylgalactosamine can be removed by a membrane bound \(\beta1,2\)N-acetylgalactosaminidase (GlcNAcase) during intracellular trafficking in insect cells (Altmann et al., 1995). However, there have been reports of terminal Gal residues on recombinant glycoprotein produced by insect cells (Oganah et al., 1996, Hsu et al., 1997, Joshi et al., 2000).

In this study, we characterized N-glycans of a \(\beta3GnT2\) fusion protein expressed by a non-virus expression system in stably transformed \(T. \text{ni}\) cell and silkworm larvae, by
HPLC mapping. The supernatant of β3GnT2 fusion protein expressed in stably transformed cell was purified in single-step Ni\(^{2+}\) affinity chromatography, but only 46% of total β3GnT2 was recovered. This is because that interference with affinity of the immobilized metal due to existence of complex agents in the medium (Rouslahi et al., 1989). In the case of silkworm larval hemolymph, it contains various kinds of proteins which cause non-specific binding to Ni\(^{2+}\) affinity column. This required to perform several purification steps, ammonium sulfate precipitation, Ni\(^{2+}\) affinity chromatography, and size exclusion chromatography to prepare sample for N-glycan analysis. The Strep-tag (II) might be more useful for one-step affinity purification of recombinant protein from silkworm larval hemolymph (Dojima et al., 2009).

Most of the glycans produced by stably transformed T. ni cells were paucimannosidic forms (73.2%). This indicates that glycans were properly processed in the endoplasmic reticulum and Golgi apparatus, but underwent degradation by GlcNAcase (Altmann et al., 1999). Remarkably, stably transformed T. ni cells produced the allergenic α1,3-fucose residue in an α1,6-fucosylated trimannosidic structure (36.0%). In contrast, Galβ1,4GlcNAcβ1,2 residues (21.3%) and GlcNAc residues (16.2%) linked to the Manα(1,3) branch with bisecting GlcNAc residue were detected on β3GnT2 produced by silkworm larvae. This indicates that the activity of GlcNAcT I and GalT might exist in the silkworm.

Kulakosky et al. investigated variability in glycosylation of a recombinant protein in different insect cells (Kulakosky et al., 1998). Using the baculovirus expression system...
with *B. mori* larvae, the secreted alkaline phosphatase contained significant quantities of fucosylated oligosaccharides containing dimannose and linear trimannose, with virtually no terminal α1,3-linked mannose. On recombinant interleukin-3, the predominant oligosaccharide was Manα1,6Manβ1,4GlcNAc1,4(Fucα1,6)GlcNAc (Hogeland et al., 1994). When BmNPV bacmids were used, recombinant IgG contained oligosaccharides that were also predominantly Manα1,6Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc (77.5%) (Park et al., 2009). These previous studies suggest that *B. mori* larvae cells may express high levels of hydrolyzing enzyme that can catalyze a terminal α1,3-linked mannose residue. In this study, a Man$_2$GlcNAc(Fuc)GlcNAc structure was detected on 29.4% of β3GnT2 produced by silkworms.

In general, the galactosylated glycans are not a substrate for GlcNAcase, while paucimannosidic forms are not a substrate for GalT. A balance of competitive rates between both enzymes should be a key factor for obtaining complex glycans from insect cells (Altmann et al., 1999). Additionally, we can find the Gal terminal residue on Manα(1,3) branches with bisecting GlcNAc residues in silkworm larvae. However, there were no biantennary complex-type structures that contained N-glycans of GlcNAc residues on the Manα(1,6) branches, despite the availability of an accepter substrate GlcNAc for GlcNAcT II in silkworm larvae. In N-glycosylation biosynthesis, GlcNAcT III can also play a regulatory role, as addition of the bisecting GlcNAc eliminates the potential for α-mannosidase II, GlcNAcT II and core FucT in mammalian cells (Pristal et al., 1997). Therefore, GlcNAcase activity also might interfere with the β3GnT2 glycosylation process by GlcNAcT III in silkworm larvae.
From these analytical data, we summarize and propose the $N$-linked glycosylation pathway illustrated in Fig. 4. The initial processing of $N$-glycans in the endoplasmic reticulum and the Golgi complex of $T. ni$ and silkworm is similar to in mammalian cell lines. Man$_9$GlcNAc$_2$ is processed by $\alpha$-mannosidase I to generate the Man$_5$GlcNAc$_2$ structure. In the general pathway, GlcNAc is added to the $\alpha$1,3-Man branch of Man$_5$GlcNAc$_2$ by GlcNAcT I, after which two Man residues are removed by $\alpha$-mannosidase II to GlcNAcMan$_3$GlcNAc$_2$. However, alternative pathways may exist in stably transformed $T. ni$ cells and silkworm larvae. $N$-glycan intermediates with terminal GlcNAc residues linked to the Man$\alpha$(1,3) branch without bisecting GlcNAc residues such as GlcNAcMan$_3$GlcNAc$_2$, GlcNAcMan$_3$GlcNAc$_2$, and GlcNAcMan$_3$GlcNAc(Fuc)GlcNAc, could not be detected by the HPLC mapping method used in this study. This suggests the existence of substantial amounts of insect-specific GlcNAcase that removes terminal $N$-acetylg glucosamine residues. Significant levels of non-fucosylated Man$_3$GlcNAc$_2$ and Man$_2$GlcNAc$_2$ exist in stably transformed $T. ni$ cells and silkworm larvae, respectively. Recently, $\alpha$-mannosidase III was isolated from Sf9 insect cells by Kawar et al (2001), which catalyzes Man$_5$GlcNAc$_2$ to Man$_2$GlcNAc$_2$ without the prior addition of a terminal GlcNAc residue. In addition, a similar enzyme that could hydrolyze Man$_5$GlcNAc$_2$-PA to Man$_2$-4GlcNAc$_2$-PA was found in various types of mouse cells and tissues (Chui et al., 1997). However, a fucosyltransferase (FucT) that requires the presence of GlcNAc$\beta$(1,2) on the Man$\alpha$(1,3) branch for its action, has not been cloned from any lepidopteran insects (Tomiya et al., 2004, Staudacher et al., 1998). This suggests that an alternative pathway from...
Man$_3$GlcNAc$_2$ to Man$_3$GlcNAc$_2$ might be predominant for non-fucosylated paucimannosidic structures in silkworm but have low activity in stably transformed $T.$ *ni* cells. Alternatively, fucosylated paucimannosidic structures might be processed through the general pathway, based on the model that FucT requires the presence of GlcNAc$\beta$(1,2) on the Man$\alpha$(1,3) branch (Staudacher et al., 1998). The $\alpha$-1,3 fucosylated trimannosidic structure is processed by this general pathway in stably transformed $T.$ *ni* cells.

The presence of a bisecting structure such as Gal$\beta$1,4GlcNAc$\beta$1,2Man$\alpha$1,3(GlcNAc$\beta$1,4)(Man$\alpha$1,6)Man$\beta$1,4GlcNAc$\beta$1,4GlcNAc is not a typical structure for lepidopteran insect $N$-glycosylation. In this study, the Gal terminal residue linked to the $N$-glycan was always in non–fucosylation by bisecting GlcNAc residue in silkworm larvae. This is favorable for therapeutic antibody IgG production, because antibody-dependent cellular cytotoxicity is enhanced by defucosylation of complex type $N$-glycans (Shinkawa et al., 2003). Therefore, silkworm larvae may be a useful host for producing human glycoproteins by improvement of glycosylation pathways that include GlcNAcT II and sialyltransferase.

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Figure legends

Fig. 1. Confirmation of GFPuv-β3GnT2 produced by Tn-pXme11CNX6 cell line. Lane A, cell culture supernatant detected using CBB; lane B, cell culture supernatant detected by Molecular Image FX; lane C, purified GFPuv-β3GnT2 by Ni²⁺ affinity chromatography detected using CBB; lane D, lectin blotting of purified GFPuv-β3GnT2 by Ni²⁺ affinity chromatography detected by ConA.

Fig. 2. Confirmation of GFPuv-β3GnT2 produced in silkworm larval hemolymph using rBmNPV bacmid. Lane A, silkworm larval hemolymph detected using CBB; lane B, silkworm larval hemolymph detected by Molecular Image FX; lane C, 50% ammonium sulfate precipitate of GFPuv-β3GnT2 detected using CBB; lane D, eluent of Ni²⁺ affinity chromatography detected using CBB; lane E, lectin blotting of Ni²⁺ affinity chromatography eluent detected by ConA; lane F, purified GFPuv-β3GnT2 by size exclusion chromatography from eluent of Ni²⁺ affinity chromatography detected using CBB.

Fig. 3. N-glycosylation profiles of recombinant β3GnT2 expressed in T. ni cell line and silkworm larvae on an ODS column. The purified protein was digested with glycoamidase A to release N-glycans. The reducing ends of the N-glycans were derivatized with 2-aminopyridine. The epidemic by-products of the pyridylamination reaction are indicated with prime, e.g. h’ and i’.

Fig. 4. Proposed N-glycan processing pathway in the T. ni cell line and silkworm larvae. Open and closed arrows indicate pathway of silkworm larvae and T. ni cell line,
respectively. $N$-glycans enclosed by dotted-lines are not detected by the HPLC mapping.
Table 1

Purification of recombinant β3GnT2 from Tn−pXme11CNX6 by Ni^{2+} affinity chromatography.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>Total β3GnT (mU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (mU/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>280</td>
<td>3,080</td>
<td>756</td>
<td>4.07</td>
<td>100</td>
</tr>
<tr>
<td>Flowthrough</td>
<td>555</td>
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<td>821</td>
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</tr>
<tr>
<td>Wash</td>
<td>75</td>
<td>465</td>
<td>73.2</td>
<td>6.35</td>
<td>15.1</td>
</tr>
<tr>
<td>Elution pool</td>
<td>16</td>
<td>272</td>
<td>1.28</td>
<td>212.5</td>
<td>8.83</td>
</tr>
</tbody>
</table>
Table 2

Purification of recombinant β3GnT2 from silkworm larval hemolymph.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total β3GnT (mU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (mU/mg)</th>
<th>Purification (-fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolymph</td>
<td>25</td>
<td>800</td>
<td>1550</td>
<td>0.52</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>50 % (NH4)2SO4 precipitate</td>
<td>25</td>
<td>275</td>
<td>113</td>
<td>2.43</td>
<td>4.67</td>
<td>34.4</td>
</tr>
<tr>
<td>Ni²⁺ affinity chromatography</td>
<td>0.75</td>
<td>39.8</td>
<td>0.81</td>
<td>49.1</td>
<td>94.4</td>
<td>5.0</td>
</tr>
<tr>
<td>Size exclusion chromatography</td>
<td>7.0</td>
<td>40</td>
<td>0.479</td>
<td>80.8</td>
<td>155.4</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Table 3
Proposed structures of N-glycan forms from recombinant β3GnT2 expressed in stably transformed T. ni cell and silkworm.

<table>
<thead>
<tr>
<th>Peak</th>
<th>GU (ODS)</th>
<th>GU (Amide)</th>
<th>Molecular mass a</th>
<th>Structure</th>
<th>Relative Quantity (%) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Silk worm</td>
<td>T. ni cell</td>
</tr>
<tr>
<td>a</td>
<td>5.1</td>
<td>9.7</td>
<td>1962</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>9.0</td>
<td>1800</td>
<td>Manα1-2Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>5.9</td>
<td>7.1</td>
<td>1475</td>
<td>Manα1-2Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>- 5.2</td>
</tr>
<tr>
<td></td>
<td>5.9</td>
<td>8.0</td>
<td>1637</td>
<td>Manα1-2Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>- 1.5</td>
</tr>
<tr>
<td>d</td>
<td>7.1</td>
<td>6.1</td>
<td>1313</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>4.3</td>
<td>988</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>- 11.0</td>
</tr>
<tr>
<td>f</td>
<td>7.4</td>
<td>3.3</td>
<td>827</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>5.6</td>
<td>1282</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>- 36.0</td>
</tr>
<tr>
<td>h</td>
<td>10.0</td>
<td>4.7</td>
<td>1135</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>- 22.2</td>
</tr>
<tr>
<td>i</td>
<td>10.1</td>
<td>3.5</td>
<td>973</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>29.4</td>
</tr>
<tr>
<td>j</td>
<td>10.4</td>
<td>5.1</td>
<td>1395</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>16.2</td>
</tr>
<tr>
<td>k</td>
<td>10.7</td>
<td>5.8</td>
<td>1555</td>
<td>Manα1-2Manα1-4GlcNAcβ1-4GlcNAc</td>
<td>21.3</td>
</tr>
<tr>
<td>others</td>
<td></td>
<td></td>
<td>12.5</td>
<td></td>
<td>11.5</td>
</tr>
</tbody>
</table>

*aAverage mass calculated from m/z values of [M+H]+ and /or [M+Na]+ ions for PA-oligosaccharides.

*bMolar percent of each glycan was calculated on the basis of peak areas in Fig. 3.

*cMolar percent of each glycan was calculated on the basis of peak areas in the elution profile on the amide column.
Table 4

Glycosylation of β3GnT2 by Tn−pXme11CNX6 and silkworm larvae.

<table>
<thead>
<tr>
<th>Glycan type (%)</th>
<th>Tn−pXme11CNX6</th>
<th>Silkworm larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>High mannosidic N−glycan</td>
<td>19.4</td>
<td>9.3</td>
</tr>
<tr>
<td>Paucimannosidic N−glycan</td>
<td>73.2</td>
<td>43.4</td>
</tr>
<tr>
<td>GlcNAc residue on Manα(1,3) branch</td>
<td>ND</td>
<td>16.2</td>
</tr>
<tr>
<td>Gal residue on Manα(1,3) branch</td>
<td>ND</td>
<td>21.3</td>
</tr>
<tr>
<td>Fucosylated N−glycans</td>
<td>58.2</td>
<td>29.4</td>
</tr>
</tbody>
</table>
Fig. 1, Dojima et al.
Fig. 2, Dojima et al
Fig. 3, Dojima et al
Fig. 4 Dojima et al.