Comparison of the *N*-linked glycosylation of human β1,3*N*-acetylglucosaminyltransferase 2 expressed in insect cells and silkworm larvae

Takashi Dojima[a], Takuya Nishina[b], Tatsuya Kato[b], Tsuyoshi Uno[c], Hirokazu Yagi[c], Koichi Kato[c,d,e,f] and Enoch Y. Park[a,b*]

[a]*Laboratory of Biotechnology, Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka 422-8529*
[b]*Laboratory of Biotechnology, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka 422-8529, Japan;*
[c]*Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan*
[d]*Institute for Molecular Science and Okazaki Institute for Integrative Bioscience, National Institutes of Natural Science, 5-1 Higashiyama Myodaiji, Okazaki 444-8787, Japan*
[e] *GLYENCE Co., Ltd., 2-22-8 Chikusa, Chikusa-ku, Nagoya 464-0858, Japan*
[f] *The Glycoscience Institute, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan*

[*] Corresponding author. Address: Laboratory of Biotechnology, Major in Bioscience, Graduate School of Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka 422-8529, Japan. Phone & Fax. +81 54 238 4887. *E−mail address:* acypark@ipc.shizuoka.ac.jp (E. Y. Park).

Abstract:

N-glycosylation of human β1,3*N*-acetylglucosaminyltransferase 2 (β3GnT2) is essential for its biological function. β3GnT2 fused to GFP_{uv} (GFP_{uv}-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, β1,3*N*-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 β1,3*N*-acetylglucosaminyltransferase 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.,

2004, Kato et al., 2005a) and silkworm larvae (Park et al., 2007). β3GnT is indispensable for synthesizing the lactose-*N*-triose II (GlcNAcβ1-3Galβ1-4Glc) that is converted into lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3GalNAcβ1-4Glc) and lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3GalNAcβ1-4Glc) (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 GFP_{uv}-β3GnT2 and analyzed by HPLC mapping. The purified GFP_{uv}-β3GnT2 could be used as a sample for *N*-glycan analysis on β3GnT2, because the GFP_{uv} 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 GFP_{uv}-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. mor*i actin promoter is widely used for gene expression of stably transformed insect cell line, which was constructed by inserting the GFP_{uv}-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 (His_6), the gfp_{uv} gene, and an enterokinase cleavage site, followed by the *β3GnT*2 gene from the N- to C-terminus.

2.2. Expression of GFP_{uv}-β3GnT2 in stable cell lines and silkworm larvae

Tn-pXme11CNX6 cells at a density of 5×10^5 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 GFP_{uv}-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^{o}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^{o}C. The supernatant samples were immediately frozen at -80^{o}C for GFP_{uv}-β3GnT2 purification. The β3GnT2 activity of hemolymph supernatant was lost after a few days of storage at 4^{o}C.

2.3. Purification of GFP_{uv}-β3GnT2

His-tagged GFP_{uv}-β3GnT2 was purified by affinity chromatography using a Ni^{2+} 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 GFP_{uv}-β 3GnT2 was dissolved in 20 mM phosphate buffer (pH 7.4), followed by dialysis overnight. The dialyzed sample was applied to the immobilized Ni^{2+} 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 GFP_{uv}-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^{o}C.

2.4. SDS-PAGE and lectin blot analysis

145 Protein samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis
146 (SDS-PAGE) using the Mini-PROTEAN II system (Bio-Rad, Hercules, USA). Bands
147 were detected using Coomasie Brilliant Blue (CBB). For the detection of fluorescent
148 GFP_{uv} fusion protein by SDS-PAGE, bands were detected by Molecular Image FX
149 (Bio-Rad).

150 The glycosylation status of β3GnT2 was assayed by lectin blot with Concanavalin A
151 (ConA, Sigma), which detects specifically linked Manα1-3(Manα1-6)Man. The
152 purified sample was run on SDS-PAGE under reducing conditions and transferred to a
153 polyvinylidine difluoride membrane. The membrane was incubated with
154 FITC-conjugated ConA at room temperature, followed by three washes with TBST
155 buffer (10 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.05% Tween 20) for 5 min each. The
156 bands were analyzed in a Fluor-S/MAX multi-image (Bio-Rad).

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

158 *2.5. β3GnT2 activity assay*

159 The β3GnT2 activity assay was carried out in 50 mM Tris-HCl, pH 8.0, 15 mM
160 $MnCl_2$, 19 mM UDP-GlcNAc, 22 mM Galβ1-4GlcNAcβ-*p*NP, and 5 μl of the enzyme
161 solution (total volume 25 μl). The reaction started by the addition of the β3GnT2 sample
162 to the reaction mixture. For each sampling, 5 μl of the reaction mixture were added to
163 195 μl distilled water, and the mixture was boiled for 5 min. The resulting solution was
164 filtered with a 0.45-μm nitrocellulose filter (Millipore, Bedford, Massachusetts, USA)
165 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^{o}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 GFP_{uv}-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 GFP_{uv}-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 Ni^{2+} affinity column. Approximately 46% of the β3GnT2 activity bound to the column, whereas more than 50% containing protein was washed out. The absorbed GFP_{uv}-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 GFP_{uv}-β3GnT2 (75 kDa) and a contaminating protein (Fig. 1A).

The purity of the GFP_{uv}-β3GnT as a pool of *N*-glycoproteins was assayed by lectin blotting. A single GFP_{uv}-β3GnT2 band was detected by ConA (Fig. 1B), indicating no

207 contaminants of Man terminal residue on *N*-glycan in the purified sample. Therefore,
208 the purified pool was dialyzed against pure water overnight, followed by lyophilization
209 to analyze *N*-glycans on β3GnT2. One mg of β3GnT2 fusion protein was purified from
210 280 ml culture supernatant and the recovery yield was 8.8%. The specific β3GnT2
211 activity indicates a 52-fold enrichment from cell culture supernatant (Table 1).

212 *3.3. Purification of β3GnT2 expressed in silkworm larval hemolymph*

213 Twenty-five ml of hemolymph was collected and diluted five times with 20 mM
214 phosphate buffer (pH 7.4), followed by precipitation with 40-50% saturated ammonium
215 sulfate. The GFP_{uv}-β3GnT2-containing fraction was dialyzed against 20 mM phosphate
216 buffer (pH 7.4) and loaded on an immobilized Ni^{2+} affinity column. Elution was
217 performed by a stepwise gradient of imidazole concentrations from 100-500 mM.
218 Elution fractions that detected fluorescence were analyzed for β3GnT2 activity and
219 SDS-PAGE (Fig. 2A). *N*-linked glycoprotein contamination was assayed by lectin
220 blotting, and several bands were detected with ConA (Fig. 2B). Therefore, an additional
221 purification step of size exclusion chromatography was carried out using a Sephacryl
222 S-200 HR column. Fractions eluted with 20 mM phosphate buffer were assayed by
223 SDS-PAGE (Fig. 2C). Fractions with a single GFP_{uv}-β3GnT2 band were dialyzed
224 against pure water and lyophilized for *N*-glycan analysis. In these purification processes,
225 0.5 mg of β3GnT2 fusion protein was purified from 25 ml silkworm hemolymph and
226 the recovery yield was 5%. The specific β3GnT2 activity indicates 155-fold enrichment
227 from hemolymph (Table 2).

228 *3.4. Characterization of N-glycans*

229 The reducing ends of *N*-glycans released from purified GFP_{uv}-β3GnT2 by
230 glucoamidase A were reductively aminated with PA. The PA-oligosaccharides were
231 separated by ODS column and their profiles are shown in Fig. 3. Each separated fraction
232 (a through k) was then applied to an amide column. Structural assignment was
233 performed by HPLC mapping (Table 3).

234 The purified β3GnT2 sample from silkworm larvae contained
235 GlcNAcβ1,2Manα1,3(GlcNAcβ1,4)(Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc (16.2%)
236 and
237 Galβ1,4GlcNAcβ1,2Manα1,3(GlcNAcβ1,4)(Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc
238 (21.3%), which were not observed in samples from Tn-pXme11CNX6 cells (Table 3).
239 These data indicate that activities of β1,2*N*-acetylglucosaminyltransferase I (GlcNAcT
240 I), β1,4*N*-galactosyltransferase (GalT) and β1,4*N*-acetylglucosaminyltransferase III
241 (GlcNAcT III) might exist in silkworm larvae but not in Tn-pXme11CNX6 cells.

242 The *N*-glycan structure is summarized in Table 4. The different glycoforms were
243 classified as high-mannose (with more than three mannose residues), paucimannose
244 (containing two core GlcNAc residues and three or fewer mannose residues), and a
245 terminal glycan other than mannose, such as GlcNAc or galactose. The percentage of
246 high-mannose structures produced in Tn-pXme11CNX6 and silkworm larvae were
247 19.4% and 9.3%, respectively. The Tn-pXme11CNX6 sample contained larger amounts
248 of three to seven mannose residues without fucosylation (23.6%), compared to samples

249 from silkworm larvae (0.9%). Most of the paucimannosidic glycans were different in
250 addition with or without fucose residues. The *N*-glycans expressed in Tn-pXme11CNX6
251 were α(1,3) and α(1,6) fucosylated (36.0%). In silkworm larvae, the *N*-glycan structure
252 was α(1,6) fucosylated (29.4%). The fucosylated *N*-glycans produced by
253 Tn-pXme11CNX6 and silkworm larvae comprised 58.2% and 29.4%, of total *N*-glycans
254 respectively.

255

256 **4. Discussion**

257 The baculovirus-insect cell expression system is widely used for recombinant
258 protein production in insect cells. However, this system has limited commercial use
259 because of a difference in glycosylation between insect cells and mammalian cells.
260 *N*-glycans from insect cell lines are not usually processed to terminally sialylated
261 complex-type structures but are instead modified to paucimannosidic or oligomannose
262 structures (Tomiya et al., 2004). This is because the terminal residue of
263 *N*-acetylglucosamine can be removed by a membrane bound
264 β1,2*N*-acetylglucosaminidase (GlcNAcase) during intracellular trafficking in insect
265 cells (Altmann et al., 1995). However, there have been reports of terminal Gal residues
266 on recombinant glycoprotein produced by insect cells (Oganah et al., 1996, Hsu et al.,
267 1997, Joshi et al., 2000).

268 In this study, we characterized *N*-glycans of a β3GnT2 fusion protein expressed by a
269 non-virus expression system in stably transformed *T. 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 protreins 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

291 with *B. mori* larvae, the secreted alkaline phosphatase contained significant quantities of
292 fucosylated oligosaccharides containing dimannose and linear trimannose, with virtually
293 no terminal α1,3-linked mannose. On recombinant interleukin-3, the predominant
294 oligosaccharide was Manα1,6Manβ1,4GlcNAc1,4(Fucα1,6)GlcNAc (Hogeland et al.,
295 1994). When BmNPV bacmids were used, recombinant IgG contained oligosaccharides
296 that were also predominantly Manα1,6Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc (77.5%)
297 (Park et al., 2009). These previous studies suggest that *B. mori* larvae cells may express
298 high levels of hydrolyzing enzyme that can catalyze a terminal α1,3-linked mannose
299 residue. In this study, a Man_2GlcNAc(Fuc)GlcNAc structure was detected on 29.4% of
300 β3GnT2 produced by silkworms.

301 In general, the galactosylated glycans are not a substrate for GlcNAcase, while
302 paucimannosidic forms are not a substrate for GalT. A balance of competitive rates
303 between both enzymes should be a key factor for obtaining complex glycans from insect
304 cells (Altmann et al., 1999). Additionally, we can find the Gal terminal residue on
305 Manα(1,3) branches with bisecting GlcNAc residues in silkworm larvae. However,
306 there were no biantennary complex-type structures that contained *N*-glycans of GlcNAc
307 residues on the Manα(1,6) branches, despite the availability of an accepter substrate
308 GlcNAc for GlcNAcT II in silkworm larvae. In *N*-glycosylation biosynthesis, GlcNAcT
309 III can also play a regulatory role, as addition of the bisecting GlcNAc eliminates the
310 potential for α-mannosidase II, GlcNAcT II and core FucT in mammalian cells (Pristal
311 et al., 1997). Therefore, GlcNAcase activity also might interfere with the β3GnT2
312 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 endoplasimic reticulum and the Golgi complex of *T. ni* and silkworm is similar to in mammalian cell lines. $Man_9GlcNAc_2$ is processed by α-mannosidase I to generate the $Man_5GlcNAc_2$ structure. In the general pathway, GlcNAc is added to the α1,3-Man branch of $Man_5GlcNAc_2$ by GlcNAcT I, after which two Man residues are removed by α-mannosidase II to $GlcNAcMan_3GlcNAc_2$. However, alternative pathways may exist in stably transformed *T. ni* cells and silkworm larvae. *N*-glycan intermediates with terminal GlcNAc residues linked to the Manα(1,3) branch without bisecting GlcNAc residues such as $GlcNAcMan_5GlcNAc_2$, $GlcNAcMan_3GlcNAc_2$, and $GlcNAcMan_3GlcNAc(Fuc)GlcNAc$, 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*-acetylglucosamine residues. Significant levels of non-fucosylated $Man_3GlcNAc_2$ and $Man_2GlcNAc_2$ exist in stably transformed *T. ni* cells and silkworm larvae, respectively. Recently, α-mannosidase III was isolated from Sf9 insect cells by Kawar et al (2001), which catalyzes $Man_5GlcNAc_2$ to $Man_2GlcNAc_2$ without the prior addition of a terminal GlcNAc residue. In addition, a similar enzyme that could hydrolyze $Man_5GlcNAc_2$-PA to $Man_{2-4}GlcNAc_2$-PA was found in various types of mouse cells and tissues (Chui et al., 1997). However, a fucosyltransferase (FucT) that requires the presence of GlcNAcβ(1,2) on the Manα(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

335 $Man_5GlcNAc_2$ to $Man_3GlcNAc_2$ might be predominant for non-fucosylated
336 paucimannosidic structures in silkworm but have low activity in stably transformed *T.*
337 *ni* cells. Alternatively, fucosylated paucimannosidic structures might be processed
338 through the general pathway, based on the model that FucT requires the presence of
339 GlcNAcβ(1,2) on the Manα(1,3) branch (Staudacher et al., 1998). The α-1,3
340 fucosylated trimannosidic structure is processed by this general pathway in stably
341 transformed *T. ni* cells.

342 The presence of a bisecting structure such as
343 Galβ1,4GlcNAcβ1,2Manα1,3(GlcNAcβ1,4)(Manα1,6)Manβ1,4GlcNAcβ1,4GlcNAc is
344 not a typical structure for lepidopteran insect *N*-glycosylation. In this study, the Gal
345 terminal residue linked to the *N*-glycan was always in non−fucosylation by bisecting
346 GlcNAc residue in silkworm larvae. This is favorable for therapeutic antibody IgG
347 production, because antibody-dependent cellular cytotoxicity is enhanced by
348 defucosylation of complex type *N*-glycans (Shinkawa et al., 2003). Therefore, silkworm
349 larvae may be a useful host for producing human glycoproteins by improvement of
350 glycosylation pathways that include GlcNAcT II and sialyltransferase.

351 **Acknowledgements**

352 This work was supported by the Program of Basic Research Activities for Innovative
353 Biosciences (PROBRAIN), Japan.

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454
455

Figure legends

Fig. 1. Confirmation of GFP_{uv}-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, purifiedGFP_{uv}-β3GnT2 by Ni^{2+} affinity chromatography detected using CBB; lane D, lectin blotting of purified GFP_{uv}-β3GnT2 by Ni^{2+} affinity chromatography detected by ConA.

Fig. 2. Confirmation of GFP_{uv}-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 GFP_{uv}-β3GnT2 detected using CBB; lane D, eluent of Ni^{2+} affinity chromatography detected using CBB; lane E, lectin blotting of Ni^{2+} affinity chromatography eluent detected by ConA; lane F, purified GFP_{uv}-β3GnT2 by size exclusion chromatography from eluent of Ni^{2+} 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,

477 respectively. *N*-glycans enclosed by dotted-lines are not detected by the HPLC

478 mapping.

479

Table 1

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

Sample	Volume (ml)	Total β3GnT (mU)	Total protein (mg)	Specific activity (mU/mg)	Recovery (%)
Culture supernatant	280	3,080	756	4.07	100
Flowthrough	555	1,665	821	2.03	54.0
Wash	75	465	73.2	6.35	15.1
Elution pool	16	272	1.28	212.5	8.83

Table 2

Purification of recombinant β3GnT2 from silkworm larval hemolymph.

Step	Volume (ml)	Total β3GnT (mU)	Total protein (mg)	Specific activity (mU/mg)	Purification (-fold)	Recovery (%)
Hemolymph	25	800	1550	0.52	1	100
50 % $(NH_4)_2SO_4$ precipitate	25	275	113	2.43	4.67	34.4
Ni^{2+} affinity chromatography	0.75	39.8	0.81	49.1	94.4	5.0
Size exclusion chromatography	7.0	40	0.479	80.8	155.4	5.0

Table 3
Proposed structures of *N*-glycan forms from recombinant β3GnT2 expressed in stably transformed *T. ni* cell and silkworm.

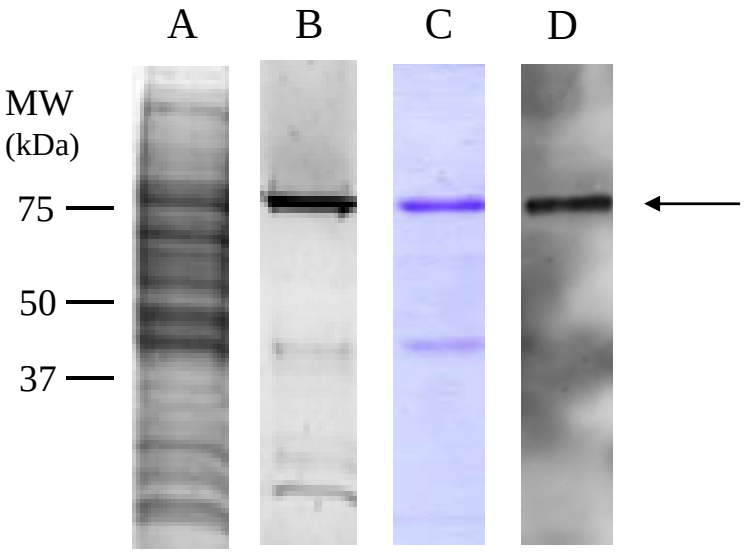
Peak	GU (ODS)	GU (Amide)	Molecular mass[a]	Structure	Relative Quantity (%)[b] Silk worm	*T. ni* cell
a	5.1	9.7	1962	Manα1-2Manα1-6 Manα1-2Manα1-3 Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	4.5	6.8
b	5.4	9.0	1800	Manα1-2Manα1-6 Manα1-3 Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	3.9	-
c1[c]	5.9	7.1	1475	Manα1-6 Manα1-3 Manα1-6 Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	-	5.2
c2[c]	5.9	8.0	1637	Manα1-6 Manα1-3 Manα1-6 Manα1-2Manα1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	-	1.5
d	7.1	6.1	1313	Manα1-6 Manα1-3 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	0.9	5.9
e	7.3	4.3	988	Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	-	11.0
f	7.4	3.3	827	Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	14.0	4.0
g+(g')	8.7 (5.8)	5.6	1282	Manα1-6 Fucα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc Fucα1-3	-	36.0
h+(h')	10.0(7.4)	4.7	1135	Manα1-6 Fucα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc	-	22.2
i+(i')	10.1(7.7)	3.5	973	Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	29.4	-
j	10.4	5.1	1395	Manα1-6 GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2 Manα1-3	16.2	-
k	10.7	5.8	1555	Manα1-6 GlcNAcβ1-4 Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1-2 Manα1-3	21.3	-
others					12.5	11.5

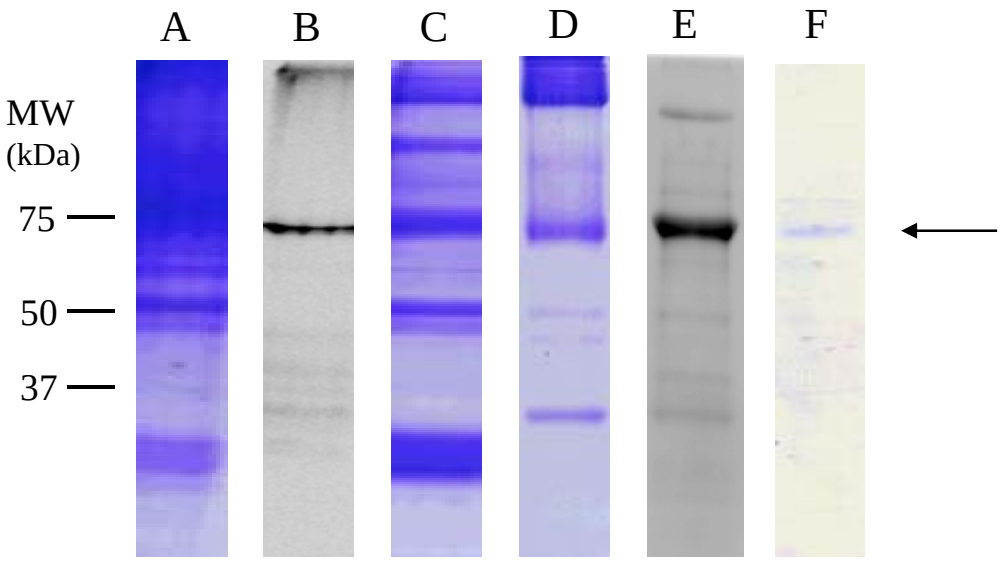
[a]Average mass calculated from *m/z* values of $[M+H]^+$ and /or $[M+Na]^+$ ions for PA-oligosaccharides.
[b]Molar percent of each glycan was calculated on the basis of peak areas in Fig. 3.
[c]Molar percent of each glycan was calculated on the basis of peak areas in the elution profile on the amide column.

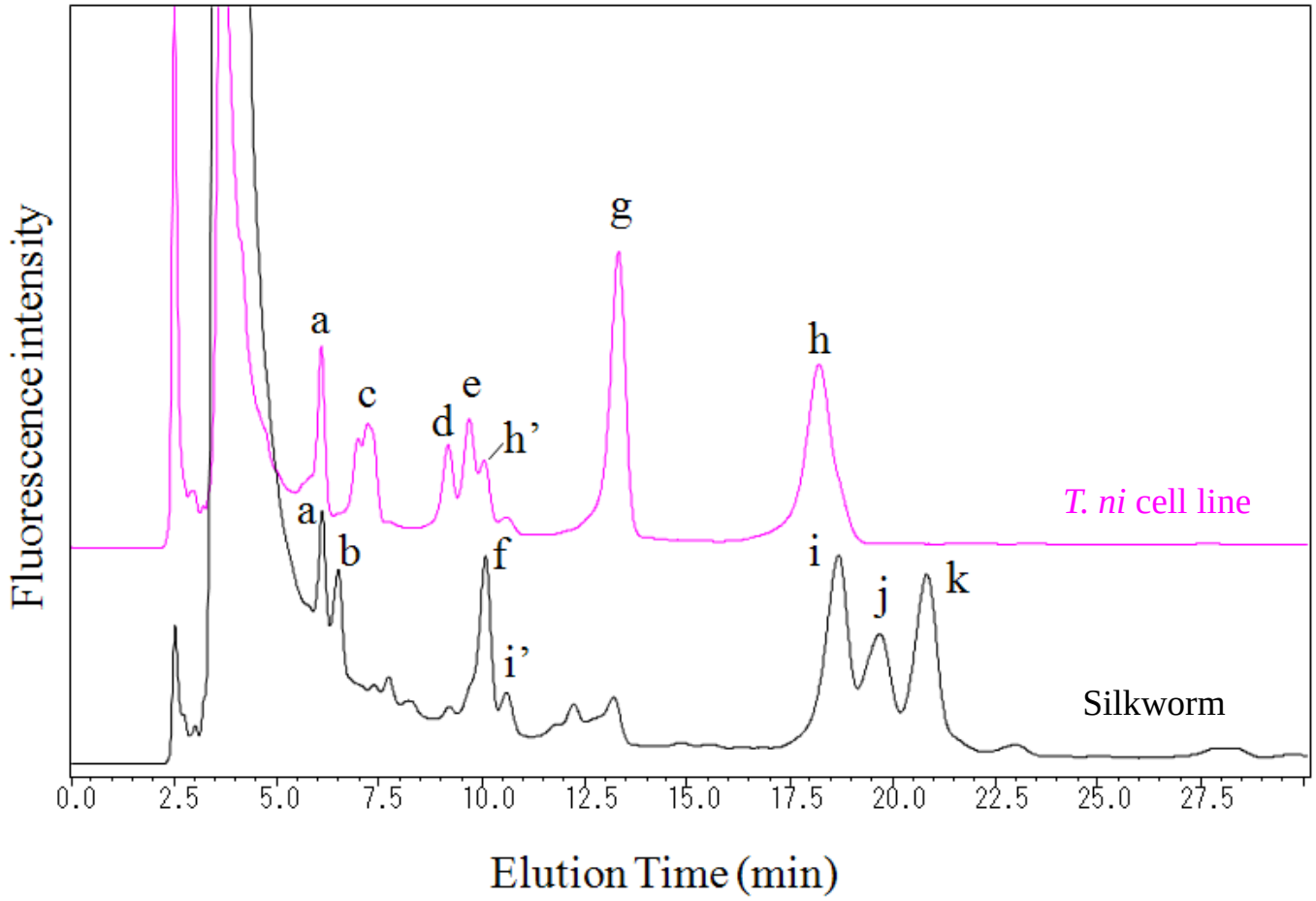
Table 4

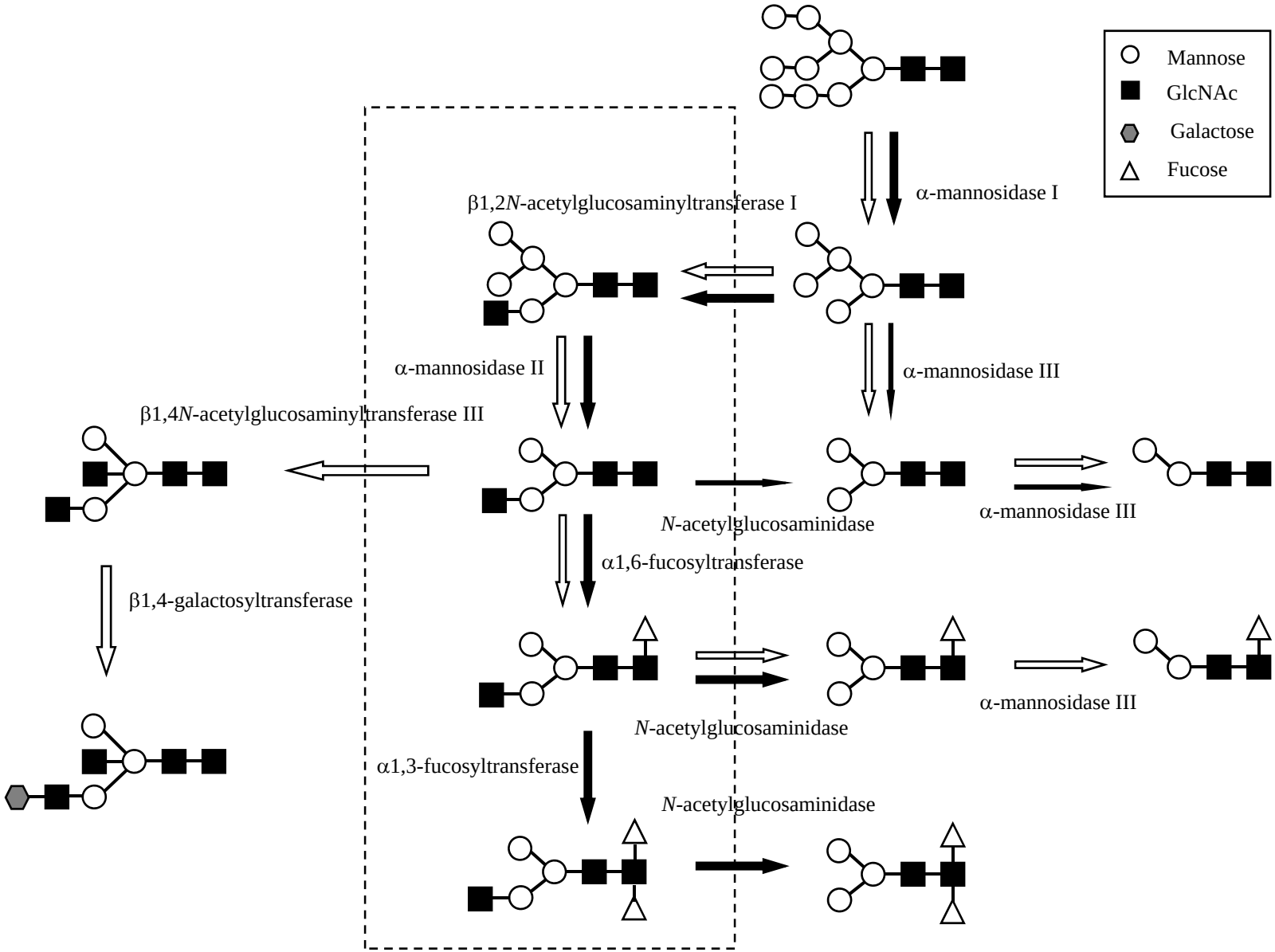
Glycosylation of β3GnT2 by Tn−pXme11CNX6 and silkworm larvae.

Glycan type (%)	Tn−pXme11CNX6	Silkworm larvae
High mannosidic *N*−glycan	19.4	9.3
Paucimannosidic *N*−glycan	73.2	43.4
GlcNAc residue on Manα(1,3) branch	ND	16.2
Gal residue on Manα(1,3) branch	ND	21.3
Fucosylated *N*−glycans	58.2	29.4


A
B
C
D
MW
(kDa)
75
50
37


A
B
C
D
E
F
MW
(kDa)
75
50
37


Fluorescence intensity
a
c
d
e
h'
g
h
T. ni cell line
a
b
f
i'
i
j
k
Silkworm
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
Elution Time (min)


Mannose
GlcNAc
Galactose
Fucose
α-mannosidase I
β1,2N-acetylglucosaminyltransferase I
α-mannosidase II
α-mannosidase III
β1,4N-acetylglucosaminyltransferase III
N-acetylglucosaminidase
α-mannosidase III
α1,6-fucosyltransferase
β1,4-galactosyltransferase
N-acetylglucosaminidase
α-mannosidase III
α1,3-fucosyltransferase
N-acetylglucosaminidase