Molecular chaperone-assisted production of human α-1,4-*N*-acetylglucosaminyltransferase in silkworm larvae using recombinant BmNPV bacmids

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Abstract: In this study, human α-1,4-*N*-acetylglucosaminyltransferase (α4GnT) fused with GFP_{uv} (GFP_{uv}-α4GnT) was expressed using both a transformed cell system and silkworm larvae. A Tn-pXgp-GFP_{uv}-α4GnT cell line, isolated after expression vector transfection, produced 106 mU/ml of α4GnT activity in suspension culture. When *Bombyx mori* nucleopolyhedrovirus containing a GFP_{uv}-α4GnT fusion gene (BmNPV-CP^-/GFP_{uv}-α4GnT) bacmid was injected into silkworm larvae, α4GnT activity in larval hemolymph was 352 mU/ml, 3.3-fold higher than that of the Tn-pXgp-GFP_{uv}-α4GnT cell line. With human calnexin (CNX) or human immunoglobulin heavy chain-binding protein (BiP, GRP78) coexpressed under the control of the ie-2 promoter, α4GnT activity in larval hemolymph increased by 1.4 - 2.0-fold. Moreover, when BmNPV-CP^-/GFP_{uv}-α4GnT bacmid injection was delayed for 3 hours after BmNPV-CP^-/CNX injection, the α4GnT activity increased significantly to 922 mU/ml, 8.7-fold higher than that of the Tn-pXgp-GFP_{uv}-α4GnT cell line. Molecular chaperone assisted-expression in silkworm larvae using the BmNPV bacmid is a promising tool for recombinant protein production. This system could lead to large-scale production of more complex recombinant proteins.

Keywords human chaperone · silkworm · bacmid · BmNPV · human α-1,4-*N*-acetylglucosaminyltransferase

Introduction

α-1,4-*N*-acetylglucosaminyltransferase (α4GnT) catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) to β-galactose (βGal) residues with α-1,4-linkages, forming the unique glycan GlcNAcα→4Galβ→R. The α4GnT enzyme appears to be expressed in gastric gland mucous cells and in Brunner's glands in gastroduodenal mucosa, since its product glycan is present in the mucin of gastric gland cells containing α1,4-GlcNAc-capped O-glycans [1-3]. α4GnT is also expressed in adenocarcinoma cells such as gastric, pancreatic and biliary tract cancers [4]. Quantitative analysis of α4GnT mRNA expressed in the peripheral blood detected pancreatic cancer cells expressing α1,4-GlcNAc-capped O-linked glycans, suggesting a possible use of α4GnT as a biomarker for pancreatic cancer [5, 6].

α4GnT cDNA was isolated by expression cloning from a gastric mucosa cDNA library [7]. α4GnT is a type II transmembrane protein with 3 amino acids of N-terminal domain, 22 amino acids of transmembrane/signal anchoring domain, a stem domain and a large catalytic domain, similar to other all glycosyltransferases. α4GnT also has four potential N-glycosylation sites. Except for this α4GnT, all *N*-acetylglucosaminyltransferases reported until now have transferred β-GlcNAc residues to their acceptors.

Glycosyltransferases mediate the specific transfer of a saccharide from a sugar nucleotide to an acceptor, and their rigid specificity is required for oligosaccharide synthesis [8]. However, preparing large amounts of glycosyltransferases and sugar

nucleotides is difficult. To resolve this problem, human α4GnT was produced in a baculovirus expression system using insect cells and larvae. Insect larvae, especially silkworm larvae, are used as living factories for the production of recombinant proteins [9-11]. Protein production using insect larvae is easy to manipulate and to scale-up. In this study, we expressed active human α4GnT in insect cells and silkworm larvae. Moreover, to improve production, α4GnT and human chaperones were coexpressed in the larvae. Coexpression of desired proteins and chaperones has been performed in the baculovirus expression system (BES) in various insect cells [12-14], but this is the first application in silkworm larvae.

In this report, a coexpression system of heterologous proteins and chaperones was established in silkworm larvae using a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid. Improved production of α4GnT could be achieved by delaying injection of recombinant bacmids. Molecular chaperone-assisted protein expression in silkworm larvae using recombinant BmNPV bacmid may allow the mass production of recombinant proteins for functional analysis and crystallization.

Materials and methods

Insect, cell line and cell culture

The Tn-5B1-4 cell line derived from *Tricoplusia ni* was purchased from Invitrogen (San Diego, CA). Tn-5B1-4 cells were cultivated in Express Five Serum-Free-Medium (Invitrogen) supplemented with 1% antibiotic-antimycotic (Invitrogen) and 18 mM

glutamate. *B. mori* fifth-instars (Ehime Sanshu, Ehime, Japan) were reared on an artificial feed, Silkmate 2 (NIHON NOSAN Co, Yokohama, Japan), in a 27°C incubator.

Construction of expression vectors and recombinant BmNPV bacmids

A 947 bp truncated fragment of human α4GnT gene was obtained from Quick-Clone human stomach cDNA (Clontech, Palo Alto, CA, USA) by PCR using α4GnT-F and α4GnT-R primers (Table 1). Amplified PCR fragments were digested with *Kpn*I and *Eco*RI, and inserted in the *Kpn*I and *Eco*RI sites of pBlueBacHis2-GFP_{uv} [15]. To add the signal peptide coding region of *B. mori* bombyxin (bx), each GFP_{uv}-α4GnT fusion fragment was amplified by PCR using bx-F and αGnT-R primers containing the signal peptide coding region. The DNA amplification cycle was 95°C for 3 min for one cycle, followed by 30 cycles of amplification by denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min. PCR products were inserted into pENTR/D-TOPO (Invitrogen), resulting in pENTR/GFP_{uv}-α4GnT. The GFP_{uv}-α4GnT fusion gene was inserted into pDEST8 (Invitrogen) using pENTR/GFP_{uv}-α4GnT by Gateway Cloning Technology (Invitrogen), resulting in pDEST/GFP_{uv}-α4GnT. Construction of the recombinant BmNPV bacmid BmNPV-CP^-/GFP_{uv}-α4GnT, was performed using the bacmid system of BmNPV with cysteine protease deleted (BmNPV-CP^-) to suppress the degradation of expressed recombinant proteins [16].

Molecular chaperones were expressed under either the polyhedrin or the ie-2

105 promoter. The BmNPV-*CP*$^-$ bacmid containing each chaperone under the polyhedrin
106 promoter was constructed by the same method as BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT. Genes
107 of calnexin (CNX), calreticulin (CRT), ERp57, immunoglobulin heavy chain binding
108 protein (BiP, GRP78) and heat shock protein 70 (Hsp70) were amplified by PCR using
109 primers shown in Table I and Quick-Clone human stomach cDNA (Clontech) as a
110 template. The amplification cycle was 95°C for 3 min for one cycle, followed by 30
111 cycles of amplification by denaturation at 95°C for 30 s, annealing at 50°C for 30 s and
112 extension at 72°C for 1.5 or 2 min.

113 The ie-2 promoter from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus
114 works at the early stage of infection [17] and its promoter activity is lower than that of
115 the polyhedrin promoter. To construct BmNPV-*CP*$^-$ bacmids containing each chaperone
116 under the ie-2 promoter (BmNPV(ie)-*CP*$^-$ bacmids), the polyhedrin promoter region of
117 the pDEST8 vector containing each chaperone gene was exchanged with the ie-2
118 promoter region from pIB vector (Invitrogen). Then, using each resulting plasmid as a
119 destination vector, five kinds of BmNPV(ie)-*CP*$^-$ bacmids containing each chaperone
120 under the ie-2 promoter were constructed by the same method as
121 BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT.

122 Insect cell expression vectors were constructed by inserting the GFP$_{uv}$-α4GnT
123 gene with a bx signal peptide coding sequence, or the signal peptide coding sequence
124 of glycoprotein 64 (gp) from *Autographa californica* multiple nucleopolyhedrovirus
125 (AcMNPV) into the pXinsect-DEST38 vector (Invitrogen) using Gateway Cloning

Technology, to obtain pXbx/GFP_{uv}-α4GnT and pXgp/GFP_{uv}-α4GnT.

Transfection, isolation and cultivation of stable cell lines

Tn-5B1-4 cells were transfected with either pXbx/GFP_{uv}-α4GnT or pXgp/GFP_{uv}-α4GnT and pBmA:neo (Invitrogen) using Cellfectin (Invitrogen) as previously described [15]. Transfected cells were subcultured in Express Five medium containing 700 μg/ml Geneticin (Invitrogen) for 4-5 weeks to obtain polyclonal stably transformed cell lines. Colonies of these cell lines were picked and cultured separately to isolate monoclonal cell lines. To produce GFP_{uv}-α4GnT fusion protein, isolated cells were cultivated in suspension cultures in 100-ml flasks with a working volume of 20 ml, in Express Five medium. Agitation speed and temperature were controlled at 100 rpm and 27°C, respectively.

Bacmid injection

Forty-five microliters of recombinant bacmid (18 μg) solution containing helper plasmid was suspended with 5 μl of DMRIE-C reagent (Invitrogen) and incubated at room temperature for 45 min. Forty microliters of bacmid-DMRIE-C mixture was injected into the dorsal of the larvae using a syringe with a 26-gauge beveled needle. To coexpress with chaperones, 22.5 μl, containing 9 μg of the BmNPV-*CP*$^-$/GFP_{uv}-α4GnT bacmid and 9 μg of the BmNPV-*CP*$^-$ bacmid containing the chaperone genes with helper plasmid was mixed, and then injected by the same protocol as single bacmid injection. To improve α4GnT expression, time-delayed

injection of the two bacmids was performed. The BmNPV-*CP*$^-$ bacmid containing each chaperone gene was injected 3, 6, 12, 18, and 24 h earlier than BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid injection. Number of silkworm larvae for this study was 10 larvae each experiment and average activity was taken as data.

Analysis of α4GnT activity

The α4GnT activity was measured by mixing 50 μl of enzyme solution and 50 μl substrate solution (50 mM Tris-HCl (pH 8.0), 20 mM $MnCl_2$, 10 mM uridine 5'-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), 10 mM *p*-nitrophenyl-galactose (Galβ-*p*NP), 2 mM ATP) and incubating at 37°C. At each sampling time, 10 μl of incubated reaction mixture was added to 190 μl of distilled water, followed by boiling for 5 min. After passing through a 0.45 μm nitrocellulose filter (Millipore, Bedford, Massachusetts, USA), filtrates were analyzed by HPLC. Mightysil RP-18 (H) GP 150–4.6 (KANTO CHEMICAL Co Inc., Tokyo, Japan) was used as column. Reaction products were eluted with 10% methanol and detected at an absorbance of 300 nm. HPLC was performed at 40°C with a flow rate of 0.8 ml per min. One unit of enzyme activity was defined as the amount of enzyme capable of catalyzing the transfer of 1 μmol of GlcNAc per minute.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), fluorescent image analysis and Western blot

To detect the expression of recombinant protein, samples were subjected to SDS-PAGE

170 on 10 or 12% polyacrylamide gels using the Mini-protean II system (Bio-Rad). For
171 fluorescent image analysis, samples were suspended in sample buffer (Aoki et al.,
172 1996) without boiling and then directly subjected to SDS-PAGE. After SDS-PAGE,
173 fluorescent bands for the GFP_{uv}-α4GnT fusion protein were detected with a Molecular
174 Imager FX (Bio-Rad).
175 For Western blots, samples were subjected to SDS-PAGE, followed by transfer to
176 polyvinylidene fluoride membranes using a Mini Trans-Blot Electrophoretic Transfer
177 Cell (Bio-Rad). After blocking in 5% skim milk in Tris-buffered saline containing
178 0.1% Tween 20, the membrane was incubated in 1:1,000 each antibody (anti-HisG
179 antibody, anti-human calnexin, anti-human Bip) for 1 hour. The membrane was washed,
180 and incubated in 1:20,000 anti-rabbit or anti-mouse IgG antibody labeled with
181 horseradish peroxidase for 1 hour. Detection of CNX and CRT was performed using
182 ECL Plus Western blotting reagent (GE Healthcare UK Ltd., Buckinghamshire HP7
183 9NA, England). Specific bands were detected using a Fluor-S/MAX multi-imager
184 (Bio-Rad).
185
186 **Results**
187
188 Expression of human α4GnT in stable insect cell lines
189 Human α4GnT was expressed in a transformed insect cell system using Tn-5B1-4 cells.
190 Five cell lines of Tn-pXbx/GFP_{uv}-α4GnT was isolated after transformation with
191 pXbx/GFP_{uv}-α4GnT, while six cell lines of Tn-pXgp/GFP_{uv}-α4GnT were isolated after

192 transformation with pXgp/GFP_{uv}-α4GnT. The Tn-pXbx/GFP_{uv}-α4GnT 2 cell line
193 was cultivated in a culture flask, and α4GnT activity was found to be 3.5 mU/ml after
194 6 days of culture. When Tn-pXgp/GFP_{uv}-α4GnT cell lines were cultured separately to
195 confluence in 6-well plates, the extracellular α4GnT activity in the
196 Tn-pXgp/GFP_{uv}-α4GnT 5 cell line was 16.4 mU/ml, which was 4.6-fold higher than
197 the Tn-pXbx/GFP_{uv}-α4GnT cell line (Table 2). Tn-pXgp/GFP_{uv}-α4GnT cell lines
198 number 3 and 5 were picked and cultivated in a culture flask. Extracellular α4GnT
199 activity in Tn-pXgp/GFP_{uv}-α4GnT cell line 5 reached 106 mU/ml, which was 30-fold
200 higher than the Tn-pXbx/GFP_{uv}-α4GnT 2 cell line (Fig. 1A). Extracellular α4GnT
201 activity in Tn-pXgp/GFP_{uv}-α4GnT cell line 3 was 31 mU/ml. The maximum cell
202 concentration of each of the Tn-pXgp/GFP_{uv}-α4GnT cell lines was above 4.5×10^6
203 cells/ml after 5 days of culture (Fig. 1B), indicating that α4GnT productivity of
204 Tn-pXgp/GFP_{uv}-α4GnT in cell line 5 was higher than that cell line 3. A previous report
205 found that a GFP_{uv}-β3GnT2 fusion protein was not secreted by the gp signal peptide in
206 Bm5 cells isolated from *B. mori* ovaries [10]. This indicates that the bx signal peptide
207 may not be functional in Tn-5B1-4 cells, but Bm5 cells, because bx signal peptide is
208 from bombyxin of silkworm. GFP_{uv}-α4GnT could be detected by Western blot at
209 approximately 75 kDa, in the culture supernatant of Tn-pXgp/GFP_{uv}-α4GnT cell line 5
210 (Fig. 1C).
211
212 Expression of human α4GnT in silkworm larvae
213 In a previous study, we confirmed that the bx signal peptide allowed recombinant

proteins to be secreted into culture supernatants of Bm5 cells, and into the hemolymph of silkworm larvae, but the gp signal peptide did not [10]. Therefore, the bx signal peptide was adopted for silkworm larvae expression. The BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid, which contained the GFP$_{uv}$-α4GnT gene fused to the bx signal sequence, was injected into silkworm larvae. After breeding for 6-7 days, hemolymph was collected and expression of GFP$_{uv}$-α4GnT was confirmed on SDS-PAGE gels, judging by GFP$_{uv}$ fluorescence. A distinct band was detected below 75 kDa (Fig. 2). The molecular weight discrepancy between Fig. 1C and Fig. 2 is because Fig 2 used non-denatured samples to detect GFP$_{uv}$ fluorescence. Non-denatured GFP$_{uv}$-α4GnT fusion proteins sometimes have a low molecular weight on SDS-PAGE [18]. No GFP$_{uv}$ fluorescence was detected in mock-injected silkworm larval hemolymph, indicating that the band in Fig. 2 is the GFP$_{uv}$-α4GnT fusion protein. α4GnT activity in hemolymph was 352 mU/ml, which was approximately 3.5-fold higher than the Tn-pXgp/GFP$_{uv}$-α4GnT cell line 5. When twice as much BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid DNA was injected, the α4GnT activity was the same as for standard bacmid volumes. GFP$_{uv}$ fluorescence was also observed in fat body fractions, where α4GnT activity was also detected, indicating that active GFP$_{uv}$-α4GnT was secreted into hemolymph efficiently, and simultaneously accumulated in fat bodies (data not shown).

Human molecular chaperone-assisted expression of GFP$_{uv}$-α4GnT in silkworm larval hemolymph

To improve GFP$_{uv}$-α4GnT secretion into larval hemolymph, GFP$_{uv}$-α4GnT was

236 coexpressed with human chaperone. Five kinds of human chaperones were
237 co-expressed with GFP_{uv}-α4GnT in silkworm larvae.
238 For co-expression of GFP_{uv}-α4GnT with chaperones, the polyhedrin and ie-2
239 promoters used. When the CNX or Bip chaperones were expressed with the ie-2
240 promoter, coexpressed α4GnT activity in hemolymph was approximately 1.2 fold
241 higher than the mock bacmid injection (Fig. 3). When CNX or Bip was coexpressed
242 under the control of the polyhedrin promoter, α4GnT activity in larval hemolymph was
243 approximately 1.4 or 2.0-fold higher, respectively, than without chaperone
244 coexpression (Fig. 4). Chaperone-assisted expression of α4GnT in the silkworm larvae
245 fat body also increased compared to silkworm larvae fat bodies with the
246 BmNPV-*CP*$^-$/GFP_{uv}-α4GnT bacmid, but without chaperone (data not shown).
247 Western blots confirmed the expression of CNX and Bip in silkworm larvae.
248 Expression of CNX and Bip under control of the ie-2 promoter was not observed in the
249 microsome fraction of fat body (data not shown). CNX and Bip expression under the
250 control of the polyhedrin promoter was not observed in larval hemolymph, but was
251 observed in the microsome fraction of the fat body (Fig. 5). The difference in
252 chaperone expression between the ie-2 and polyhedrin promoters was caused by
253 differences in promoter activity. Expression of CNX or Bip under the control of ie-2
254 promoter was so low that CNX and Bip were not detected by Western blot, although
255 α4GnT activity was enhanced by coexpression. Degradation of CNX was observed in
256 the fat body, but that of Bip was not. Degradation of CNX was also observed in

257 Tn-5B-4 cells transformed with CNX expression plasmid [13].

258 The ie-2 promoter works at the early stage of infection and the polyhedrin
259 promoter works at the very late stage of infection [17]. To express molecular
260 chaperone more efficiently before the expression of GFP_{uv}-α4GnT,
261 BmNPV(ie)-*CP*$^-$/CNX or BmNPV(ie)-*CP*$^-$/Bip bacmids were injected into silkworm
262 larvae before injection of the BmNPV-CP^-/GFP_{uv}-α4GnT bacmid. Bacmid-injected
263 silkworm larvae were bred for 6 days and larval hemolymph was harvested (Fig. 6A).
264 When BmNPV-*CP*$^-$/GFP_{uv}-α4GnT bacmid injection was delayed for 3 hours after
265 BmNPV(ie)-*CP*$^-$/CNX bacmid injection, α4GnT activity in larval hemolymph was
266 approximately 1.9-fold higher (922 mU/ml) than in larval hemolymph without
267 chaperone (479 mU/ml) (Fig. 6B). α4GnT activity was retained in a 24-hour injection
268 delay, but was degraded without chaperone expression.

269 When the BmNPV-*CP*$^-$/GFP_{uv}-α4GnT bacmid was injected 6 hours after
270 BmNPV(ie)-*CP*$^-$/Bip bacmid injection, α4GnT activity in hemolymph was
271 approximately 1.5-fold higher (603 mU/ml) than without chaperone (391 mU/ml) (Fig.
272 6C). α4GnT activity was also retained after 24-hour injection delay. The
273 BmNPV-*CP*$^-$/CNX bacmid, which contains the CNX gene controlled by the polyhedrin
274 promoter, was also injected into silkworm larvae before injection of the
275 BmNPV-*CP*$^-$/GFP_{uv}-α4GnT bacmid, after which bacmid-injected silkworm larvae
276 were bred for 6 days and larval hemolymph was harvested. Expression of
277 GFP_{uv}-α4GnT in hemolymph was enhanced slightly, but α4GnT activity was not

278 observed after 9, 12, 18, and 24 hour delays in BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid
279 injection (data not shown)
280
281 **Discussion**
282 In this study, α4GnT was expressed in both Tn-5 B1-4 cells and silkworm larvae as a
283 GFP$_{uv}$ fusion protein. The α4GnT activity in the hemolymph of
284 BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid-injected silkworm larvae was 352 mU/ml, which
285 was 3.3-fold higher than that of the highest Tn-pXgp/GFP$_{uv}$-α4GnT cell line (Table 3).
286 The α4GnT activity in larval hemolymph was improved by coexpression with
287 molecular chaperones under the control of the polyhedrin or ie-2 promoters. In
288 particular, ie-2 promoter-driven coexpression of CNX or Bip increased activity in
289 hemolymph by 1.1-1.2 fold, even though chaperone expression was too low to be
290 detected by Western blot. Moreover, a 3-hour delay in injection of the
291 BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid after injection of BmNPV(ie)-*CP*$^-$/CNX bacmid
292 increased α4GnT activity to 921 mU/ml in larval hemolymph, which was
293 approximately 8.7-fold higher than that of the Tn-pXgp/GFP$_{uv}$-α4GnT cell line. This
294 suggested that even a small amount of molecular chaperone, expressed in the
295 Endoplasmic reticulum (ER) at the early stage of infection, could enhance the folding
296 of GFP$_{uv}$-α4GnT, and that chaperone expression can be effective for recombinant
297 expression in silkworm larvae. Moreover, α4GnT activity was retained after chaperone
298 coinjection with the BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid (Fig. 6), suggesting that CNX
299 and Bip might protect GFP$_{uv}$-α4GnT from degradation in the fat body during the late

300 stage of baculovirus infection.

301 CNX and CRT are lectin-like chaperones and bind to the $Glc_1Man_9GlcNAc_2$ chain
302 of *N*-linked oligosaccharides in glycoproteins, and help glycoproteins fold correctly
303 with the help of ERp57, a thiol-disulfide oxido-reductase involved in formation,
304 reduction and isomerization of disulfide bonds [19, 20]. α4GnT has four potential
305 *N*-glycosylation sites and eight cysteine residues [7]. An increase of activity in
306 hemolymph was observed only when CNX or ERp57, and not CRT were coexpressed,
307 suggesting that GFP_{uv}-α4GnT might bind to CNX and ERp57 in ER in fat body cells,
308 enhancing the folding of GFP_{uv}-α4GnT, and increasing α4GnT activity in larval
309 hemolymph. Recently, it was reported that substrate specificity of ERp57 is determined
310 by interaction with CNX and CRT [21]. If GFP_{uv}-α4GnT is coexpressed with CNX and
311 ERp57, its folding might be even more efficiently enhanced than with coexpresssion
312 with a single chaperone. The expression level of the cocaine-sensitive serotonin
313 transporter decreased after coexpression with CNX and ERp57, however, compared to
314 coexpression with only CNX [22]. Bip belongs to the HspA family and is also an ER
315 folding assistant [23]. Bip enhanced the level of soluble intracellular and secreted IgG
316 in Tn-5B1-4 cell cultures [24], and nicotine receptor subunits are associated with three
317 chaperones (CNX, ERp57 and Bip) [25]. The activity of α4GnT in larval hemolymph
318 increased with coexpression of CNX, ERp57 and Bip, suggesting that α4GnT might
319 associate with these three chaperones in the ER.

320 Expression of CNX or Bip under the control of the ie-2 promoter was detected in

the microsome fraction by Western blot, and α4GnT activity in larval hemolymph increased with coexpression of CNX or Bip. Enhanced secretion of α4GnT activity in hemolymph was observed only with early injection of CNX or Bip gene-containing bacmids, suggesting that molecular chaperone expression under the control of the ie-2 promoter caused accumulation in the ER that might enhance correct folding and stability of GFP_{uv}-α4GnT fusion proteins.

Coexpression of recombinant proteins with molecular chaperones has been performed in various expression systems, including bacteria, yeast, insect cells and mammalian cells. This study showed that molecular chaperone-assisted expression is applicable to the silkworm expression system. Coexpression of several proteins in silkworm is easily achieved using the bacmid system, and expression levels can be controlled by regulating the amount of injected bacmid DNA. A time-delayed injection of the recombinant protein coding bacmid relative to the molecular chaperone-encoding bacmid is a powerful tool for recombinant protein production. This system could lead to the large-scale production of more complex proteins.

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424

425 **Figure legends**

426 **Fig. 1** (A) time course of extracellular α4GnT activity; (B) cell number and viability
427 of Tn-pXgp/GFP_{uv}-α4GnT cell line 3 or Tn-pXgp/GFP_{uv}-α4GnT cell line 5 in a
428 suspension culture; (C) detection of GFP_{uv}-α4GnT in culture supernatants of
429 Tn-pXgp/GFP_{uv}-α4GnT 5 cells by Western blot. Each cell line was cultivated for 7
430 days by suspension cultivation, with sampling each day. Symbols: (A) circle,
431 Tn-pXgp/GFP_{uv}-α4GnT 3 cells; square, Tn-pXgp/GFP_{uv}-α4GnT 5 cells; (B) circle,
432 Tn-pXgp/GFP_{uv}-α4GnT 3 cells; square, Tn-pXgp/GFP_{uv}-α4GnT 5 cells. Closed and
433 open symbols indicate cell number and viability, respectively. (C)**:** lane 1, protein
434 marker; lane 2, Tn-pXgp/GFP_{uv}-α4GnT 5 cells. Arrow indicates GFP_{uv}-α4GnT fusion
435 protein.

436 **Fig. 2** Expression of GFP_{uv}-α4GnT in hemolymph of silkworm larvae injected with
437 BmNPV-*CP*$^-$/GFP_{uv}-α4GnT bacmid. GFP_{uv}-α4GnT fusion protein was detected by
438 GFP_{uv} fluorescence on SDS-PAGE gel. Lane 1: protein marker, Lane 2:
439 BmNPV-*CP*$^-$/GFP_{uv}-α4GnT, Lane 3: mock. Arrow indicates GFP_{uv}-α4GnT fusion
440 protein.

441 **Fig. 3** α4GnT activity in hemolymph of silkworm larvae injected with
442 BmNPV-*CP*$^-$/GFP_{uv}-α4GnT and chaperone genes in BmNPV(ie)-*CP*$^-$ bacmids, for
443 coexpression of GFP_{uv}-α4GnT and each chaperone. BmNPV-*CP*$^-$/GFP_{uv}-α4GnT and
444 chaperone genes in BmNPV(ie)-*CP*$^-$ bacmids were mixed at a 1:1 ratio and injected

445 into ten silkworm larvae. After 6 days, hemolymph was recovered from 10 silkworm
446 larvae and α4GnT activity was measured. This experiment was repeated 3 times within
447 6 months. Error bars indicate standard error of the mean of three experiments (SEM, n
448 = 3).

449 **Fig. 4** α4GnT activity in hemolymph of silkworm larvae injected with
450 BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT and chaperone genes CNX or Bip in BmNPV-*CP*$^-$ and
451 BmNPV(ie)-*CP*$^-$ bacmids, for coexpression of GFP$_{uv}$-α4GnT and chaperones.
452 Experimental conditions were the same as in Fig. 3, except for number of used
453 silkworm larva. In this experiment only ten silkworm larvae were used, and error bars
454 indicate standard error of the mean of 10 silkworm larvae. Open bar, grey bar and
455 closed bar indicate mock bacmid injection, CNX bacmid injection and Bip bacmid
456 injection, respectively.

457 **Fig. 5** (A) CNX or (B) Bip expression under the polyhedrin promoter in the
458 microsome fraction of silkworm larvae fat bodies during coexpression with
459 GFP$_{uv}$-α4GnT fusion protein. Fat bodies of silkworm larvae co-expressing
460 GFP$_{uv}$-α4GnT with CNX or Bip under the polyhedrin promoter were recovered and
461 sonicated in Tris buffer. The microsome fraction was collected by ultracentrifugation
462 for Western blotting. Lane 1: protein marker, lane 2: microsome fraction under the
463 control of the polyhedrin promoter, lane 3: microsome fraction in mock.

464 **Fig. 6** Coexpression of BmNPV-*CP*$^-$/GFP$_{uv}$-α4GnT bacmid, injected into silkworm

465 larvae at 3, 6, 9, 12, 18 or 24 hours after injection of a chaperone gene (CNX or Bip) in
466 a BmNPV-*CP*$^{-}$ bacmid. A: Scheme of coexpression experiment. B: α4GnT activity in
467 hemolymph of seven or ten silkworm larvae injected with BmNPV(ie)-*CP*$^{-}$/CNX. C:
468 α4GnT activity in the hemolymph of 7 or 10 silkworm larvae injected with
469 BmNPV(ie)-*CP*$^{-}$/Bip. Open bars and closed bars indicate mock bacmid injection and
470 chaperone bacmid injection, respectively. This experiment was repeated 3 times within
471 6 months. Error bars indicate standard error of the mean of three experiments (SEM, n
472 = 3).

Table 1 Primers used for the construction of BmNPV bacmids

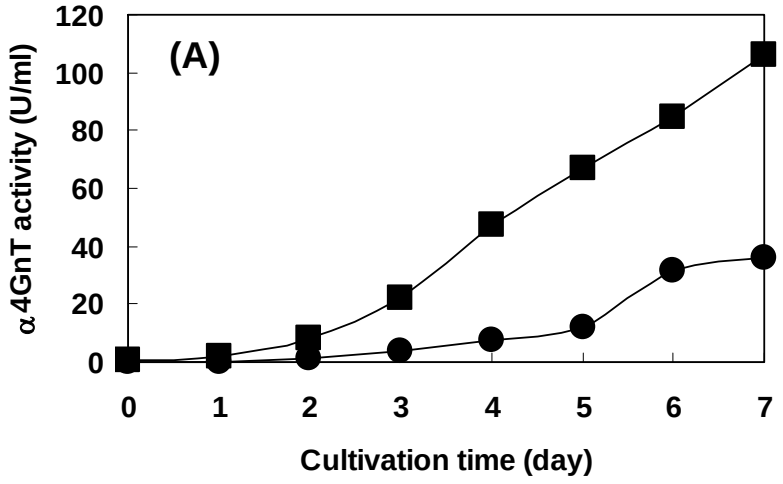
Name	5' - 3'
α4GnT-F	CATGGTACCAAGTCCAGCTGCCTCTTCTGTTTGCCTTCT
α4GnT-R	CCGGAATTCTTATTTGTTACCTGGACCCAGCTCCCCAG
bx-F	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAAT-GTGGGTGTCAACACAACCGCGGGGTTCTCATCATC
gp-F	CACCATGCCCATGTTAAGCGCTATTGTTTTATATGTCCTTTTGGCGGC-GGCGGCGCATTCTGCCTTTGCGCCCCCGCGGGGTTCTCATCATC
BiP-F	CACCATGAAGCTCTCCCTGGTGGCCGCGATGCT
BiP-R	CCAGTATTTACAATATTACAGCACTAGCAGATCAGTGTC
CRT-F	CACCATGCTGCTATCCGTGCCGCTGCT
CRT-R	TCTCTACAGCTCGTCCTTGGCCTG
CNX-F	CACCATGGAAGGGAAGTGGTTGCTGTG
CNX-R	GTTTCACTCTCTTCGTGGCTTTCTGTTTC
ERp57-F	CACCATGCGCCTCCGCCGCCTAGCGCTGTT
ERp57-R	GCTTTAGAGATCCTCCTGTGCCTTC
HSP70-F	CACCATGGCCAAAGCCGCGGCGAT
HSP70-R	CAGCAATCTTGGAAAGGCCCCTAATCTACCTC

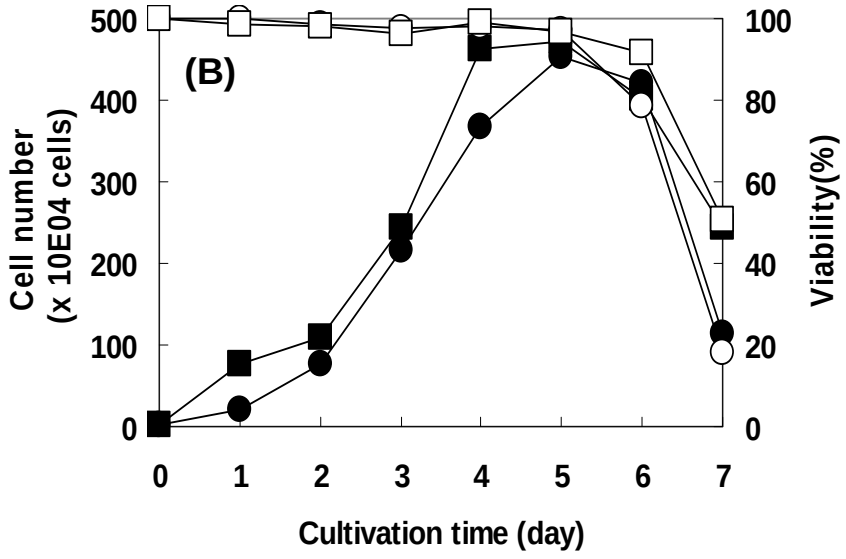
Table 2 Extracellular α4GnT activity in cell line adhesive cultures at 6 days

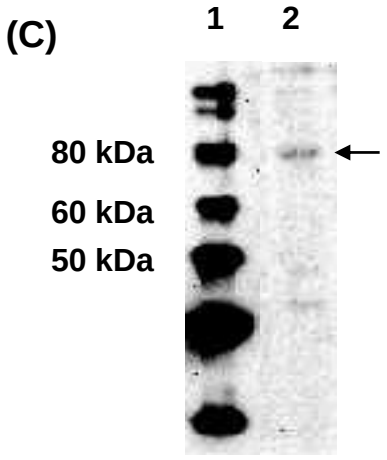
Tn-pXgp-GFP_{uv}-α4GnT cell line	Activity (mU/ml)
1	0.1
2	0.7
3	3.4
4	2.5
5	16.4
6	0.5

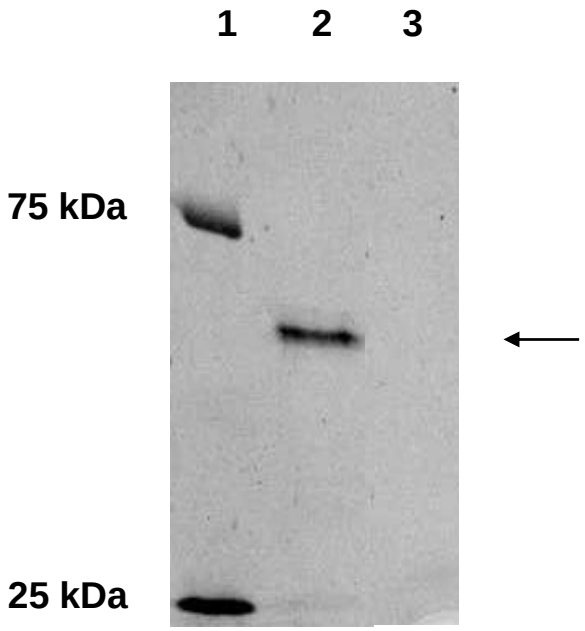
Table 3 Summary of α4GnT activity in cell culture and silkworm larvae

	Activity (mU/ml)	Fold over cell line
Culture supernatant of Tn-pXgp/GFP_{uv}-α4GnT cell line 5	106	1.0
Silkworm larval hemolymph	352	3.3
Silkworm larval hemolymph		
with CNX coexpression	921	8.7
with Bip coexpression	602	5.7


(A)
α4GnT activity (U/ml)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Cultivation time (day)


(B)
Cell number
(x 10E04 cells)
500
400
300
200
100
0
Viability(%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Cultivation time (day)


(C)
1
2
80 kDa
60 kDa
50 kDa


1
2
3
75 kDa
25 kDa

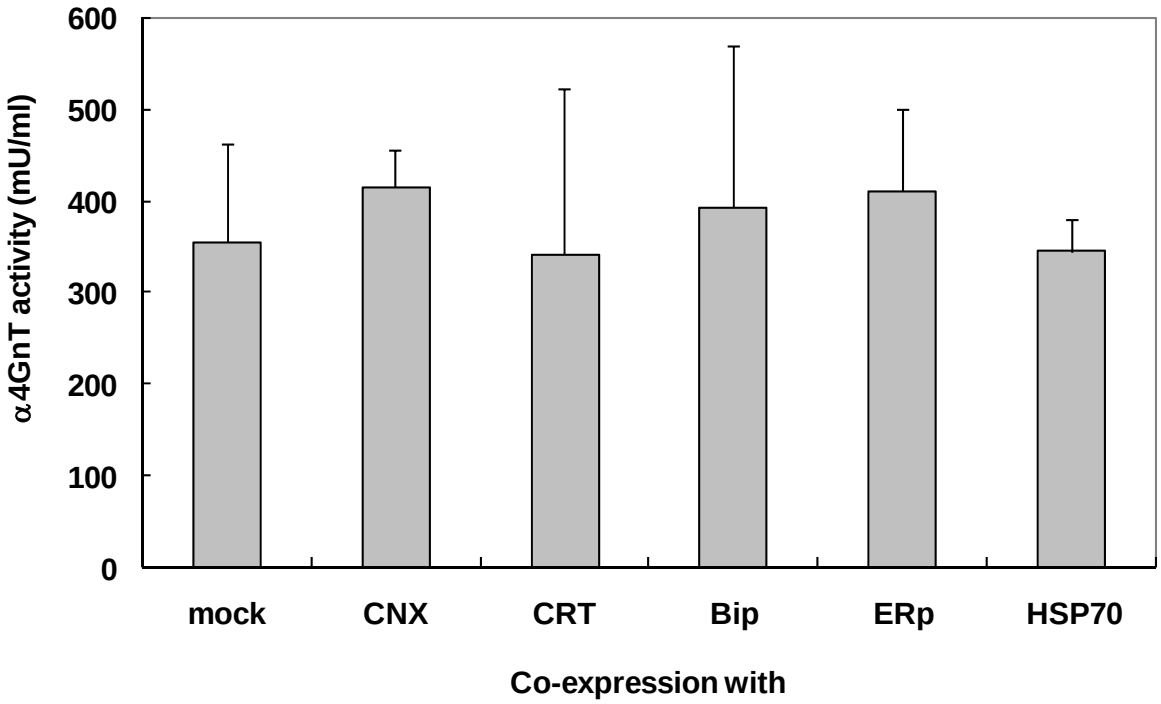
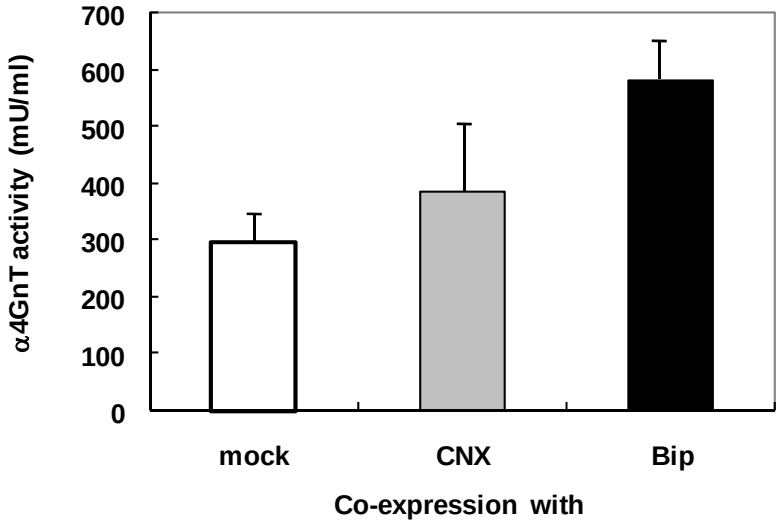
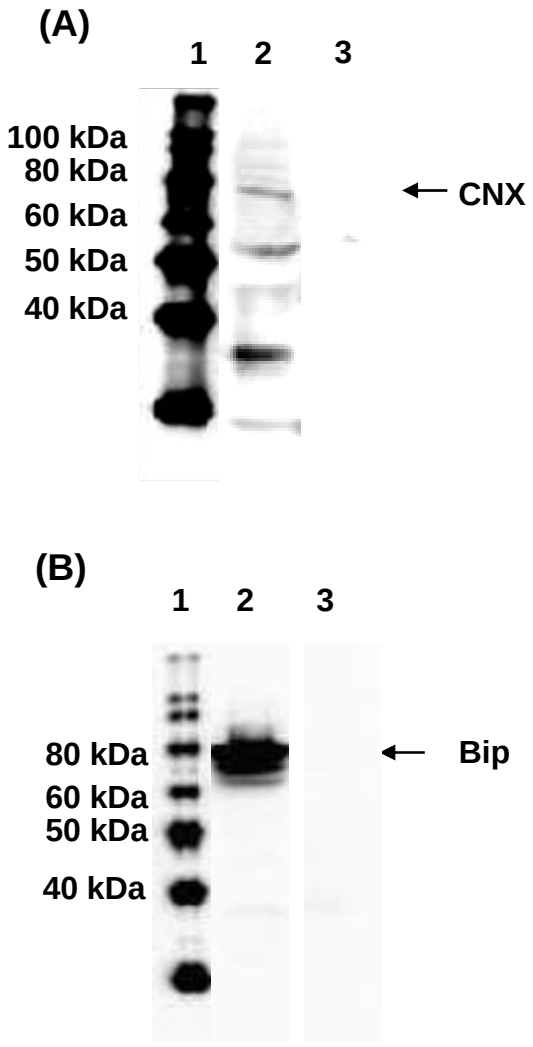
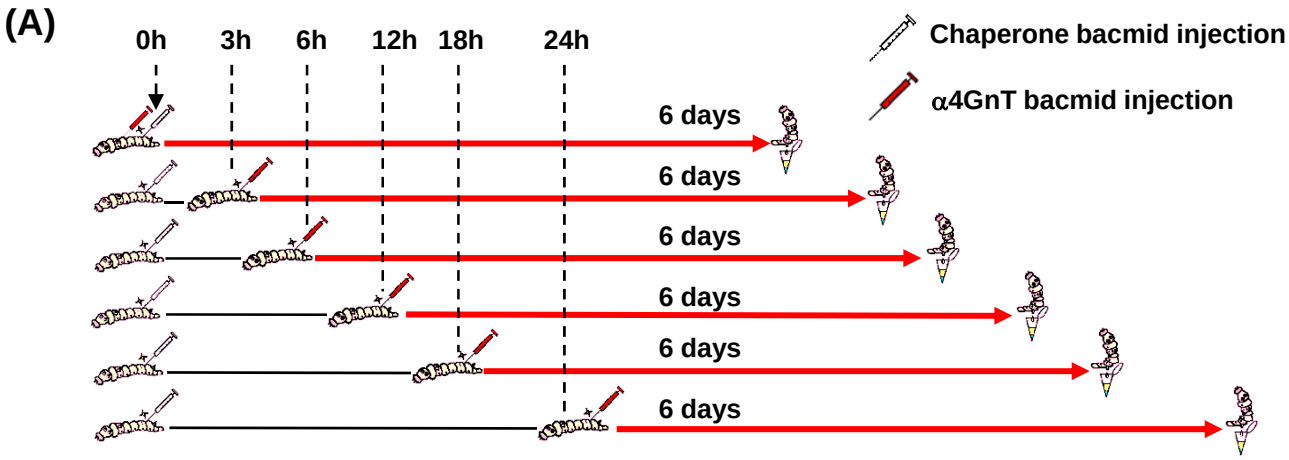
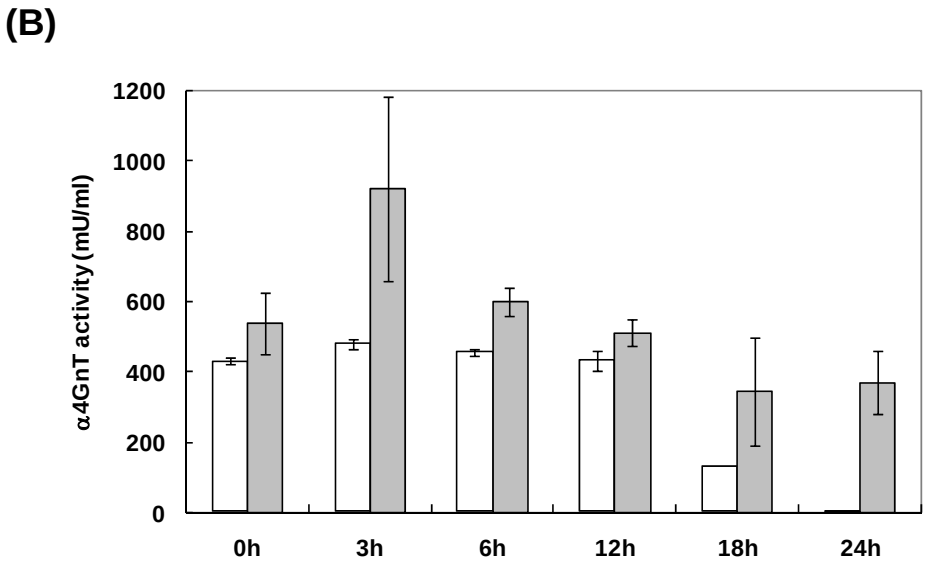

600
500
400
300
200
100
0
α4GnT activity (mU/ml)
mock
CNX
CRT
Bip
ERp
HSP70
Co-expression with

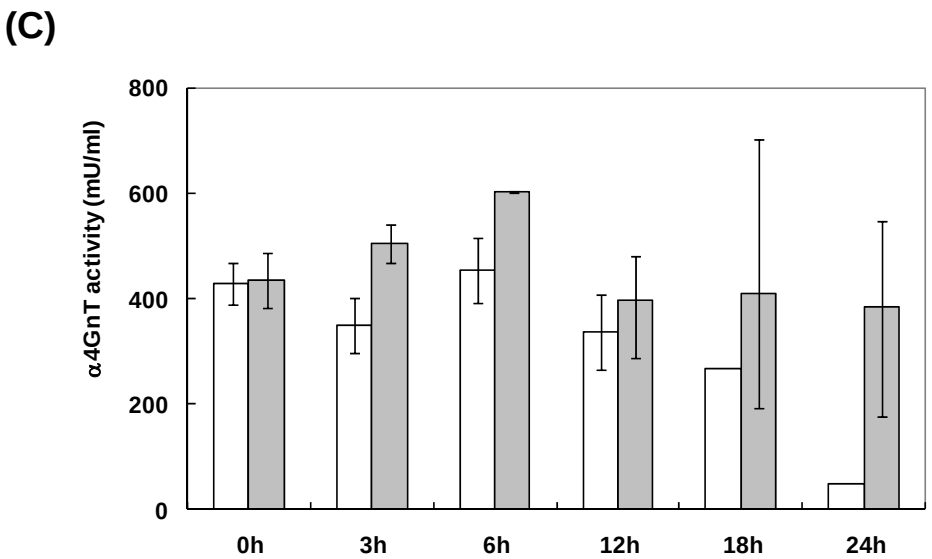
Fig. 4 Nakajima et al.




(A)
1
2
3
100 kDa
80 kDa
60 kDa
50 kDa
40 kDa
CNX
(B)
1
2
3
80 kDa
60 kDa
50 kDa
40 kDa
Bip


(A)
0h
3h
6h
12h
18h
24h
Chaperone bacmid injection
α4GnT bacmid injection
6 days
6 days
6 days
6 days
6 days
6 days


(B)
α4GnT activity (mU/ml)
1200
1000
800
600
400
200
0
0h
3h
6h
12h
18h
24h


(C)
α4GnT activity (mU/ml)
800
600
400
200
0
0h
3h
6h
12h
18h
24h