Improved secretion of molecular chaperone-assisted human IgG in silkworm, and no alterations in theirN-linked glycan structures

Improved Secretion of Molecular Chaperone-assisted Human IgG in Silkworm, and No Alterations in their *N*-Linked Glycan Structures

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Abstract. Human 29IJ6 IgG was expressed in silkworm using a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid system. The mean amounts of 296IJ6 IgG produced in larval hemolymph and whole pupae were 30.1 μg/larva and 78.0 μg/pupa, respectively. The use of molecular chaperones including calreticulin (CRT), calnexin (CNX) and immunoglobulin heavy chain binding protein (BiP, GRP78) improved the production of 296IJ6 IgG secretion in the larvae fivefold. The total yield of recombinant 29IJ6 IgG was 239 μg/ml when coexpressed with CRT. However, the overexpression of molecular chaperones had negative effects on secretion. The *N*-linked glycans of secreted 296IJ6 IgG in silkworm hemolymph were dominated by paucimannose structures. Small amounts of GlcNAc residues linked to the Manα1,3 branch were detected. When molecular chaperones were coexpressed, the compositions of *N*-linked glycans in the IgG1 produced were unchanged compared with those produced without them. This suggests that *N*-glycosylation is controlled by a regulatory function in the Golgi apparatus even though the translation of 296IJ6 IgG was assisted by coexpression of molecular chaperones. Therefore, if the glycosylation pathways that coexpress *N*-acetylglucosaminyltransferase, galactosyltransferase and sialyltransferase could be improved, silkworm larvae might prove a useful system for producing human antibodies.

KEYWORDS: silkworm larvae, molecular chaperone, antibody, bacmid, *N*-glycan

Introduction

Currently licensed therapeutic antibodies are produced by mammalian cell culture using Chinese Hamster ovary (CHO) cells, or mouse NS0 or Sp2/0 plasma cell lines (*1*). The increasing demand for monoclonal antibodies (mAbs) with regards to amount and quality have led to the development of a variety of recombinant protein production systems, such as yeast, insect and mammalian cell lines, and transgenic plants and animals (*2*).

Silkworm larvae are useful and cost-effective hosts in terms of the amount of protein production that can be achieved. A high level of recombinant gene expression is made possible by the commonly used strong polyhedrin and p10 promoters, in which host cells have to translate and produce large amounts of heterologous protein within a short time. However, host protein synthesis can be shut down by baculovirus infections, which may lead to a limitation in the supply of secretory assistance factors (*3*, *4*). Improper secretory processing can be especially problematic by several days postinfection when the host cell's post-translational processing machinery has deteriorated (*5*). Because the cellular folding system is disrupted leading to an extensive aggregation and degradation of expressed protein, overall production is compromised. In fact, extensive aggregation was observed following IgG expression in insect cells driven by the strong polyhedrin promoter (*6*). To prevent these damaging events, insect cells and larvae require molecular chaperones to regulate and correct the folding, assembling and transition of proteins. Fortunately, this system can be applied for the coexpression of multiple proteins simultaneously.

During glycoprotein transportation from the endoplasmic reticulum (ER) to the Golgi apparatus (*7–10*), *N*-linked glycans are processed along the secretory pathway. The newly synthesized proteins in the ER each interact differently with molecular chaperones (*11*). Some of them interact at first with immunoglobulin heavy chain binding protein (BiP, GRP78) and then with calnexin (CNX) and calreticulin (CRT) (*12*). Otherwise, proteins in the ER bind to CNX and CRT but do not associate with BiP (*13*) or are sequentially associated with BiP and GRP94 (*14*). Furthermore, the formation of intramolecular disulfide bonds can be catalyzed by protein disulfide isomerase (PDI), by ERp57, or by both oxidoreductases acting together (*15*). The rules that govern such selection of differential chaperones are only poorly understood (*11*).

In this study, we investigated the *N*-linked glycosylation of IgG1 produced in silkworm larvae and pupae using a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid system. IgG1 was produced by coexpression with five molecular chaperones: CNX, CRT, ERp57, BiP and Hsp70. These molecular chaperones are susceptibility factor for IgG1 production in silkworm larvae. It is crucial to assure the consistency and reproducibility of protein synthesis, not only in terms of protein sequence and bioactivity, but also in terms of post-translational modifications (*16*). Each secreted IgG1 was purified and *N*-linked glycan structures were compared using high performance liquid chromatography (HPLC) mapping.

Materials and Methods

Silkworm larvae, pupae and bacmids. *Bombyx mori* fifth-instar silkworm larvae and pupae (Fuyoutsukubane, Ehime Sansyu, Yahatahama, Japan) were used in this study. The larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) in an incubator at 25 °C.

The recombinant cysteine protease- and chitinase-deficient BmNPV containing the 29IJ6 IgG gene (rBmNPV-*CP*$^{-}$*Chi*$^{-}$/29IJ6 IgG) bacmid was used for IgG1 expression (*17*). The 29IJ6VH-Cγ and 29IJ6VL-Cλ genes are controlled by a polyhedrin promoter and a p10 promoter, respectively. To secret expressed protein into the silkworm larval hemolymph, the signal sequence from bombyxin (*bx*) was added to the *N*-termini of both heavy and light chain genes. The molecular chaperones CNX, CRT, ERp57, BiP and Hsp70 were expressed under the *ie*-2 promoter. Recombinant cysteine protease-deficient BmNPV (rBmNPV(*ie*)-*CP*$^{-}$) bacmids containing each chaperone were prepared as described (*18*).

29IJ6 IgG expression in silkworm larvae and pupae. The rBmNPV bacmid DNAs were injected directly into the first day of fifth-star larvae and pupae of silkworm. Fifty micrograms of rBmNPV bacmid was suspended in 5 μl of 1,2−dimyristyloxypropyl−3−dimethyl-hydroxy ethyl ammonium bromide (DMRIE)−C (Invitrogen, Carlsbad, CA, USA) as a transfection reagent and placed at room temperature for 45 min. The resulting mixture was diluted to a final volume of 50 μl with phosphate buffered saline (PBS) and 10 μl of the bacmid mixture was then

injected into the dorsum using a syringe with a 26-gauge beveled needle. To express proteins with chaperones, 22.5 μl aliquots containing 10 μg each of the rBmNPV-*CP*$^{-}$*Chi*$^{-}$/29IJ6 IgG and rBmNPV(*ie*)-*CP*$^{-}$ bacmids expressing the chaperone genes together with helper plasmids were injected.

The silkworm larvae and pupae were reared at 25 °C in a breeding incubator and harvested at 6 days post-injection (d.p.i.). The silkworm larval hemolymph was collected by cutting the caudal leg in a tube containing 5 μl of 200 mM 1-phenyl-2-thiourea and centrifuged at 9055 *g* for 10 min at 4 °C. The supernatants were immediately frozen at −80 °C for further analysis. The silkworm pupae were collected and frozen in liquid nitrogen, then ground in a mortar and added to 3.4 ml of homogenization buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.0) and sonicated on ice three times for 30 s each, with 30 s intervals, using a sonicator (VC130PB, Sonic & Materials Inc., Newton, CT, USA). They were then centrifuged at 5500 *g* for 10 min. The supernatant was filtered through 0.45 μm nitrocellulose membrane filters (Advantec, Tokyo, Japan) and used for further analysis. These experiments were performed on 10 larvae or pupae each time and were repeated five times at different time throughout one year. Data are reported as the mean and standard deviation.

SDS–PAGE and western blot analysis. The protein samples were analyzed using 12% SDS-polyacrylamide gel electrophoresis (SDS–PAGE) using the Mini-PROTEANE II system (Bio-Rad, Hercules, CA, USA). The respective bands were detected using Coomassie Brilliant Blue (CBB). For western blotting, the reduced

samples were boiled for 5 min before they were run on the SDS–PAGE gels. A mouse anti-human IgG (H+L) antibody (Jackson ImmunoResearch Lab., Inc. West Grove, PA, USA) was used as the primary antibody. The immunoblot bands were visualized using enhanced chemiluminescence (ECL) with western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA) and analyzed using a Fluor-S/MAX multi-image (Bio-Rad). Magic Mark XP Western Protein Standard (Invitrogen) was used as a molecular weight marker. The protein concentration was measured using the Bradford method with a protein assay kit (Bio-Rad).

Quantification of 29IJ6 IgG. The expressed IgGS were quantified using the Human IgG enzyme linked immunosorbent assay (ELISA) quantification kit (Bethyl Lab. Inc., Montgomery, TX, USA). One hundred microliters per well of 10 μg/ml goat anti-human IgG affinity-purified solution was used as a coating antibody in a coating buffer containing 0.05 M carbonate-bicarbonate (pH 9.6). This was used to coat a 96-well flexible assay plate for 1 h at room temperature. Two hundred microliters of blocking solution (10% skimmed milk in 50 mM Tris-HCl 0.14 M NaCl, pH 8.0) was added to the antibody-coated plate well, followed by incubation for 30 min at room temperature. The wells were washed three times with washing solution (50 mM Tris-HCl, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and the diluted expressed protein was then added to the antibody-coated plate wells, incubated for 1 or 2 h at room temperature and washed three times with 200 μl of washing buffer. As a second antibody, 100 μl of goat anti-human IgG-horseradish peroxidase (HRP) conjugate

151 diluted 50,000–100,000 times in sample/conjugate diluent (50 mM Tris-HCl, 0.14 M
152 NaCl, 1% BSA, 0.05% Tween 20, pH 8.0), was added to each well, incubated for 1 h
153 and washed three times with 200 μl of washing buffer. One hundred microliters of
154 substrate (0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine, TMBZ, in 100 mM sodium acetate,
155 pH 6.0, with 0.2% v/v of 30% hydrogen peroxidase) was added to each well and left at
156 room temperature for blue color development. The reaction was stopped by the addition
157 of 50 μl of 2 M H_2SO_4 solution. The color developed was measured at optical densities
158 (ODs) of 450 and 655 nm. The value calculated by subtracting OD_{450} from OD_{650} was
159 used as a measure of the amount of 29IJ6 IgG. Human reference serum (4 mg/ml) was
160 used for the calibration of IgG as a standard.

161 **Purification of expressed 29IJ6 IgG from silkworm larval hemolymph and**
162 **pupae.** Samples of hemolymph from larvae and supernatants from pupal homogenates
163 were filtered with 0.45 μm nitrocellulose membrane filter and diluted fivefold with
164 buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.0). The diluted sample was
165 applied to a HiTrap rProtein A FF column (5.0 ml bed volume, GE Healthcare)
166 according to the manufacture's protocol. The column was then subsequently eluted at
167 0.5-1.0 ml/min with elution buffer (0.1 M sodium citrate, pH 3.0). Chromatography
168 was carried out using an AKTA 10S unit controlled by UNICORN software version
169 5.11 (GE Healthcare). Fifteen milliliters of the eluate from the column was dialyzed
170 with 1 liter of pure water overnight and a dialyzed sample was lyophilized for 72 h.

171 **Structural analysis of *N*-linked glycans.** All experimental procedures used,

172 including the chromatographic conditions and glycosidase treatments, have been
173 described previously (*19–21*). The purified IgG1 was further proteolyzed with
174 chemotrypsin and trypsin mixture and then digested with glycoamidase A to release
175 *N*-linked glycans. After removal of the peptide materials, the reducing ends of the
176 *N*-linked glycans were derivatized with 2-aminopyridine (PA) (Wako Pure Chemical
177 Ind., Ltd., Osaka, Japan). The mixture of PA-derivatives of the *N*-linked glycans was
178 individually separated and identified sequentially on a TSK-gel Amide-80 HPLC
179 column (Tosoh, Tokyo, Japan) and a Shim-pack HRC-ODS column (Shimadzu, Kyoto,
180 Japan). The identification of *N*-linked glycan structures were based on their elution
181 positions on two kinds of columns in comparison with PA-glycans in the GALAXY
182 database (*22*). The *N*-glycans were confirmed by co-chromatography and
183 matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy
184 (MALDI-TOF-MS) (*23*).
185

Results

Comparison of 29IJ6 IgG expression levels in the larval hemolymph and pupae of silkworm, and *N*-linked glycan structures. The 29IJ6 IgG was expressed in the larval hemolymph and pupae of silkworm injected with 10 μg/larva of rBmNPV bacmid and reared for 6 days. The expressed 296IJ6 IgG levels in silkworm larvae and pupae were quantified using ELISA. The mean amounts of 296IJ6 IgG in larval hemolymph and whole pupae were 30.1 μg/larva and 78.0 μg/pupa, respectively.

The *N*-glycan structure from silkworm larval hemolymph contained $Man_2GlcNAc(Fuc)GlcNAc$ (47.4%), $Man_3GlcNAc(Fuc)GlcNAc$ (28.5%) and a small amount of $GlcNAcMan_3GlcNAc(Fuc)GlcNAc$ (3.4%; Tables 1 and 2). In contrast, the *N*-linked glycan structure of 296IJ6 IgG in the whole pupae contained paucimannosidic glycans (52.1%) as in the hemolymph and 14.6% of a terminal GlcNAc residue. High mannose structures were expressed in the whole pupae (33.3%) as shown in Table 2. This suggested that proteins expressed from the whole pupae resulted from insufficient processing of *N*-linked glycans. It also suggested that the pupae had higher *N*-acetylglucosaminyltransferase activity than the larvae. Furthermore α1,6-fucose residues were attached to the 296IJ6 IgG expressed in silkworm larvae and pupae: this allergenic residue has not been detected before.

Human chaperone-assisted recombinant 29IJ6 IgG expression. Five kinds of molecular chaperones were used for coexpression to test for any enhancement of 29IJ6 IgG secretion. Coexpression of human chaperone with 29IJ6 IgG was performed by

injection of 10 μg of each of the rBmNPV bacmids into silkworm larvae. In all cases, secretion of heavy chain (HC) and light chain (LC) proteins in the hemolymph were identified by western blot analysis under reducing conditions (Fig. 1A). The fully assembled IgG (H_2L_2) was also detected as well as intermediate immunoglobulin oligomer assembles (H_2L and H_2) under non-reducing conditions (Fig. 1B).

When CRT, CNX and BiP were coexpressed with 296IJ6 IgG in silkworm larvae, the amounts of secreted 296IJ6 IgG in larval hemolymph were increased significantly to 229, 220 and 119 μg/ml, respectively (Fig. 2). These levels were approximately 5-fold higher than those produced without coexpression of molecular chaperones (43.0 μg/ml). Coexpression levels of secreted 296IJ6 IgG with ERp57 or Hsp70 were 175 and 117 μg/ml, respectively, and these were 3–4-fold higher than controls. These results show that CRT, CNX and BiP had significant effects on 296IJ6 IgG folding and assembly in post-translational modification. The combined effects of CRT with the other molecular chaperones were also investigated. Levels of 296IJ6 IgG in the larval hemolymph were decreased notably in comparison with those seen using only CRT (Fig. 3).

Effect of calreticulin on recombinant 29IJ6 IgG expression. Coexpression of several proteins in silkworm larvae was easily achieved using the bacmid system and the expression level was assumed to be controlled by the amount of injected bacmid DNA. The expressions of 296IJ6 IgG and the molecular chaperone were controlled by the polyhedrin promoter and *ie*-2, respectively. There are great differences in

transcriptional levels between the polyhedrin and the *ie*-2 promoters. The expression phase is also different in that the *ie*-2 promoter works at an early stage of infection but the polyhedrin promoter works at a later stage (*24*). When the injection level of the CRT bacmid was increased from 5 to 100 μg/ml, the secreted IgG1 level decreased significantly from 239 to 16 μg/ml in the larval hemolymph (Fig. 4). This indicates that excessive CRT expression inhibited the secretion of IgG1. Intracellular trafficking of 296IJ6 IgG in silkworm larvae might require not only CRT but also other proteins for interaction, such as ERp57.

_N_-linked glycans in human chaperone-assisted recombinant 29IJ6 IgG.

N-linked glycan structure was analyzed for the purified secreted 296IJ6 IgG coexpressing molecular chaperones. The compositions of *N*-linked glycan were Manα1,3(Manα1,6)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc, Manα1,6Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc and GlcNAcβ1,2Manα1,3(Manα1,6)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc (Table 1). In all cases, there were few differences in *N*-linked glycan structure regardless of the molecular chaperone used (Table 2). Manα1,6Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc (a in Table 1) and Manα1,3(Manα1,6)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc (b in Table 1) were predominant in the silkworm larvae hemolymph (Table 2). In addition, small amounts of GlcNAc residue linked to the Manα1,3 branch were detected. The *N*-linked glycan profile is affected by manufacturing conditions such as the culture medium used, the efficiency of protein expression and the physiological status of the

host cell (*25, 26*). However, we found that these molecular chaperones did not affect *N*-glycosylation in this expression system.

Discussion

Rahman and Gopinathan (*27*) described the infection process of BmNPV. *B. mori* has an "open circulatory system" so the fat bodies and other tissues lie submerged in hemolymph within the larvae. Several proteins synthesized in the fat bodies consequently get leached out into the hemolymph without actually depending on the secretory process. This will be enhanced following viral infection because BmNPV multiplication is maximal in the fat bodies. After virus infection the fat body cells are lysed in significant numbers, leading to the leaching of the proteins into hemolymph. This was also shown when using BmNPV bacmids containing a viral protease: more than 90% of total protein was detected in the hemolymph of silkworm larvae (*28*). However, the secreted proteins appear to be especially susceptible to intracellular aggregation because of the complexity of the secretion pathway. It has been reported that secreted levels of expressed protein can be 10 to 100 times lower than intracellular levels (*29*). Most newly synthesized proteins are aggregated and degraded or misfolded because of the inadequacy of post-translational modifications. However, when a cysteine protease-deficient BmNPV (BmNPV-CP^{-}) bacmid was transfected into silkworm larvae, only 30% of the protein was secreted into the hemolymph and the rest remained in the fat body (*30*). This resulted from delayed fat body cell lysis because of deletion of the viral protease.

Post-translational modification is effected by molecular chaperones in the ER for folding and assembly, followed by glycan processing in the Golgi apparatus. Protein folding and assembly within cells is a complex process with stringent quality control mechanisms (*31*), which is largely regulated by enzymes and an array of molecular chaperones. This is why we used the BmNPV-*CP*-*Chi*$^{-}$ bacmid for investigating the *N*-linked glycan structures of human IgG.

Previously, we have demonstrated efficient heterologous protein production in insect cells and larvae (*32*–*34*) and a human antibody has been expressed in silkworm larvae (*17*). Human IgG antibodies are composed of two identical heavy chain polypeptides (MW ~25 kDa) and two identical light chain polypeptides (~50 kDa) that oligomerize to form a heterotetramer (~150 kDa) (*35*). The limiting process of mAb production is likely to be the phase of folding and assembly in the ER, mediated by the sequential interactions of HC and LC polypeptide (*36*).

Here we demonstrated that an *N*-linked glycan in IgG1 protein could be produced by silkworm larval hemolymph and pupae. Levels expressed in the pupae were higher than in the larval hemolymph. However, it is difficult to collect pupal hemolymph because it is thick and sticky. Therefore, whole pupae were used for the analysis of IgG1 expression levels and the composition of the *N*-linked glycans. In whole pupae, the trimannosyl core structure made up 30% of the *N*-linked glycans (Table 2). Additionally, 14.6% of terminal GlcNAc residues were found linked to the Manα1,3 branch. This indicates that the pupal activity of a specific *N*-acetylglucosaminidase was higher than in larvae. The other residues, accounting for 33.3%, were predominantly

292 high mannose-containing structures. In contrast, the *N*-linked glycans of larvae were
293 predominantly of a paucimannose type, with small amounts of GlcNAc residues linked
294 to the Manα1,3 branch.

295 Immunoglobulin assembly occurs during movement through the ER lumen and is
296 followed by trafficking of the native mAb through the Golgi apparatus followed by
297 secretion (*37*). The glycoproteins of newly synthesized proteins act as signals for
298 enhanced folding and quality control and/or the degradation of misfolded proteins (*38*).
299 Therefore, the secretion and *N*-glycosylation for IgG1 in silkworm larvae were
300 investigated using molecular chaperone-assisted translation. Abundant expression of
301 HC and LC, controlled by polyhedrin and p10 promoters, respectively, was achieved in
302 the silkworm larvae. Using the molecular chaperones CRT, CNX and BiP, increased
303 IgG1 secretion levels up to five times higher than without the assistance of chaperones.
304 The total yield of recombinant 29IJ6 IgG was 160.3 mg/larva following CRT
305 coexpression. Thus, these chaperones had significant effects on IgG1 folding and
306 assembly during post-translational modification. There may be two distinct chaperone
307 systems in the silkworm ER, one comprising BiP, PDI and GrP94; the other ERp57,
308 CNX and CRT (*11*). Therefore, coexpression of both chaperones might help in
309 improving IgG1 production in silkworm. However, coexpression using combinations of
310 CRT with other chaperones had little or no effect on IgG1 secretion in this model.
311 Additionally, the amount of IgG1 secretion was decreased by the injection of high
312 levels of CRT bacmids. CRT and CNX are clearly involved in retaining influenza
313 hemagglutinin fragments in the ER (*39*). For human thyrotropin receptor (TSHR),

belonging to the G protein coupled receptor family, overexpressed CRT or CNX protected TSHR from being degraded immediately after synthesis, but this protection had disappeared completely after 5 h (*40*). In addition, when BiP was overexpressed the formation of correctly folded human thyroperoxidase (hTPO) decreased 2.5-fold after 5 h. The increased binding rate of BiP did not enhance the folding of the protein but rather led to its degradation and thus confirmed that BiP had negative effects on the folding of hTPO (*41*).

Most mammalian recombinant glycoproteins produced by insect cell or larval expression systems are produced with mannose-terminated glycans or other less complete structures, compared with the native protein produced in the original tissues (*42*). Although the *N*-linked glycans of the IgG1 produced in the present study were dominated by paucimannose structures, small amounts of GlcNAc residues linked to the Manα1,3 branch were detected. As a nascent glycoprotein passes through the ER and Golgi apparatus, enzymes trim and add different sugars to this *N*-linked glycan. Therefore, we tested for any *N*-glycosylation differences in the IgG1 produced using molecular chaperone enhancement. This tested whether the trimming and terminal glycosylation capacity in Golgi apparatus was sufficient for increased IgG1 production, such as an increase in high-mannose structures and disappearance of terminal GlcNAc residues. However, the compositions of the *N*-linked glycans were no different from those produced without using molecular chaperones. This suggests that *N*-glycosylation was being controlled by a regulatory function in the Golgi apparatus even though the throughput of 296IJ6 IgG was increased following the coexpression of molecular

chaperones. These modification steps can vary in mammalian and insect hosts leading to differences in the structures of *N*-linked glycans (*5*).

In the case of β1,3*N*-acetylglucosaminyltransferase 2 expressed in silkworm larvae, *N*-linked glycans with a terminal Gal and bisecting GlcNAc residues were detected (*43*). However, for IgG1 expression, paucimannosidic *N*-linked structures were predominant in the present study. Antibodies are *N*-glycosylated on the C_H2 domain of the Fc fragment at the Asn297 residue. *N*-glycosylation of the Fc domain of recombinant IgG produced in CHO and NS0 cells is mainly characterized by core-fucosylated complex type structures with low levels of galactosylation and sialylation (*1*). Increased terminal sialylation can increase the serum half-life of many proteins (*44*). However, Fc sialylation has both positive and negative effects on antibody functions (*45–47*). Increased sialylation of Fc glycans results in decreased Antibody-dependent cellular cytotoxicity (ADCC) activity of IgGs as terminal sialylation negatively affects antibody binding to the FcγRIIIa receptor (*46, 47*). In addition, increased sialylation can result in decreased binding to certain immobilized or cell surface expressed antigens (*43*). On the other hand, the effect of high mannose type Fc glycans on the serum half-life of IgGs can vary according to the antibody concerned, with the mechanism not yet resolved (*45, 48, 49*). Therefore, if the glycosylation pathways could be improved by the coexpression of *N*-acetylglucosaminyltransferase, galactosyltransferase and sialyltransferase, silkworm larvae would prove a useful host system for producing human antibodies.

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501 **Figure Legends**

502 **Figure 1.** Western blot analysis of secreted 29IJ6 IgG in silkworm larval hemolymph
503 under reducing (A) and nonreducing conditions (B). Lane 1, IgG expression
504 without molecular chaperone assistance; lanes 2–6, CNX-, BiP-, ERp57-, CRT-
505 and Hsp70-assisted expression of IgG. H_2L_2, H_2L and H_2 in (B) denote the
506 complete IgG, heavy chain dimer–light chain and heavy chain dimer, respectively.

507 **Figure 2.** Molecular chaperone-assisted secretion of IgG in silkworm larval
508 hemolymph. Control indicates IgG expression without chaperone-coexpression.
509 Error bars denote standard deviation.

510 **Figure 3.** Multiple molecular chaperones-assisted secretion of IgG in silkworm larval
511 hemolymph. Control indicates IgG expression without chaperone coexpression.
512 Error bars denote standard deviations.

513 **Figure 4.** Effects of the injected amounts of rBmNPV(*ie*)-*CP*$^{-}$/CRT bacmid on IgG1
514 secretion in silkworm larval hemolymph. Error bars denote standard deviations.

Table 1 Structures of *N*-linked glycans on IgG expressed in silkworm

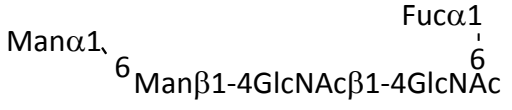
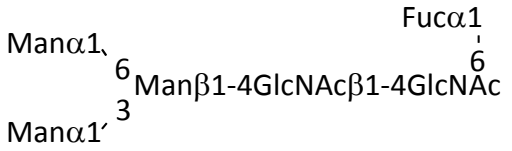
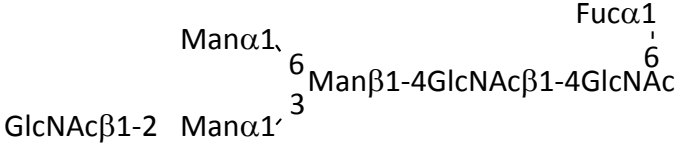
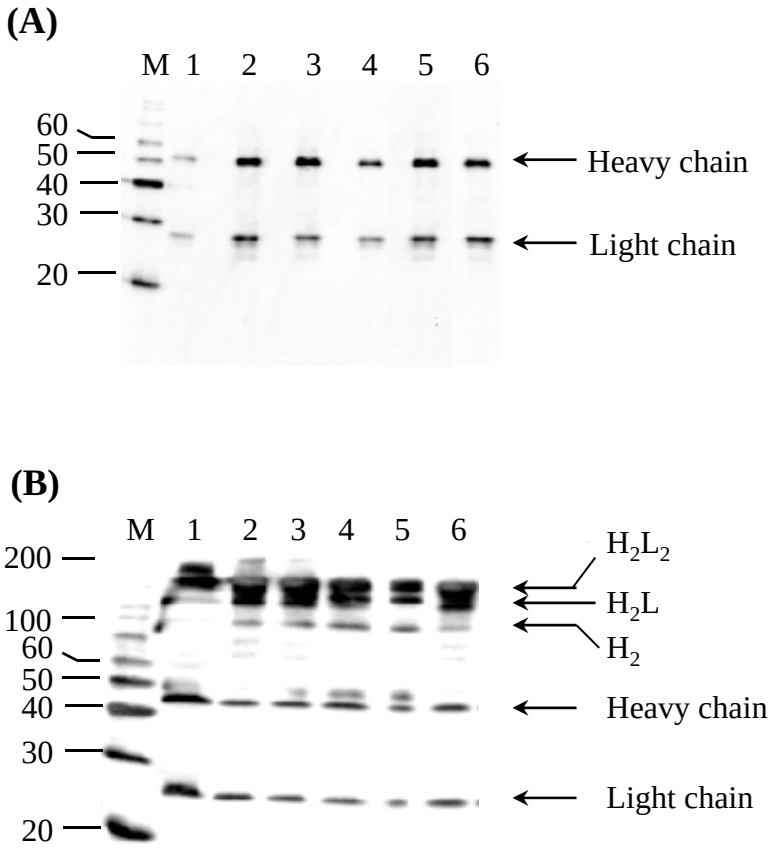
	GU (ODS)	GU (Amide)	MS value $[M+H]^+$	Structure
a	10.4	3.5	973	
b	10.3	4.8	1135	
c	10.3	5.2	1338	

Table 2 Relative quantity of *N*-linked glycan composition on IgG expression in silkworm

Structure	Larvae						Pupae
	–	CNX	CRT	BiP	ERp57	Hsp70	–
a	47.4	49.1	46.1	45.7	44.4	46.0	18.6
b	28.5	31.7	33.2	31.3	32.8	32.5	33.5
c	3.4	3.7	3.8	3.7	4.1	4.0	14.6
others	20.7	15.5	16.9	19.3	18.7	17.5	33.3


(A)
M 1 2 3 4 5 6
60
50
40
30
20
Heavy chain
Light chain
(B)
M 1 2 3 4 5 6
200
100
60
50
40
30
20
H_2L_2
H_2L
H_2
Heavy chain
Light chain

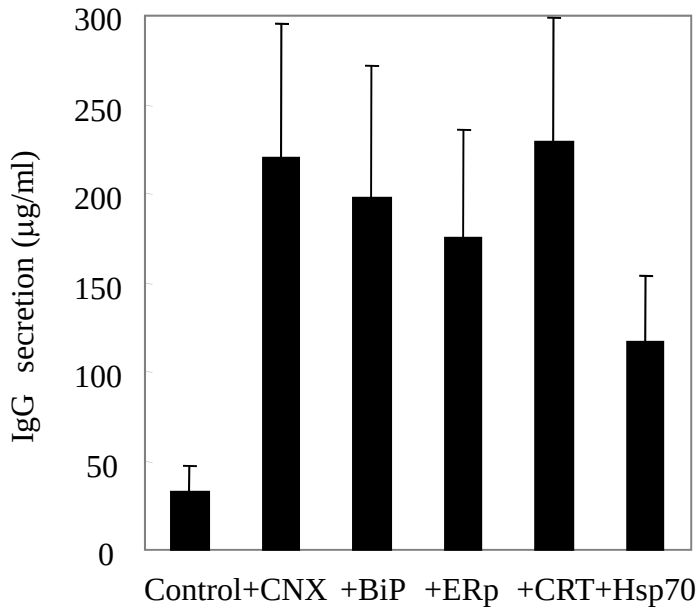

IgG secretion (μg/ml)
300
250
200
150
100
50
0
Control
+CNX
+BiP
+ERp
+CRT+Hsp70

Fig. 3, Dojima et al.

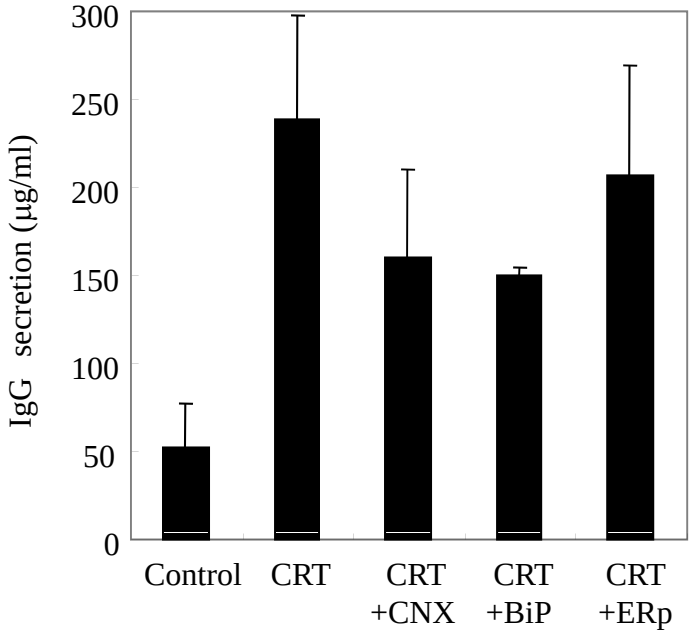


Fig. 4, Dojima et al.