Human IgG1 Expression in Silkworm Larval Hemolymph Using BmNPV Bacmids and its *N*-Linked Glycan Structure

Enoch Y. Park,[1,2*] Motoki Ishikiriyama,[1] Takuya Nishina,[1] Tatsuya Kato,[1] Hirokazu Yagi,[3] Koichi Kato,[3,4] and Hiroshi Ueda[5]

[1]*Laboratory of Biotechnology, Department of Applied Biological Chemistry, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka 422-8529, Japan*

[2]*Laboratory of Biotechnology, Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka 422-8529, Japan; telephone/fax: +81 54 238 4887; e-mail: acypark@ipc.shizuoka.ac.jp*

[3]*Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan*

[4]*Institute for Molecular Science and Okazaki Institute for Integrative Bioscience, National Institutes of Natural Science, 5-1 Higashiyama Myodaiji, Okazaki 444-8787, Japan*

[5]*Department of Chemistry and Biotechnology, School of Engineering, University of Tokyo, Bunkyo-ku, Tokyo 113-8656, Japan*

Abbreviations: Bacmid, baculovirus shuttle vector; BmNPV, *Bombyx mori* nucleopolyhedrovirus; BmNPV-*CP*$^-$, cysteine protease-deficient BmNPV; BmNPV-*CP*$^-$-*Chi*$^-$, cysteine protease- and chitinase-deficient BmNPV; BSA, bovine serum albumin; bx, bombyxin; d.p.i., days post-injection; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; V_H, heavy-chain antibody variable region fragment; V_L, light-chain antibody variable region fragment

*Corresponding author at: Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka 422-8529, Japan. Tel./fax: +81 54 238 4887. *E-mail address:* acypark@ipc.shizuoka.ac.jp (E.Y. Park).

Abstract:

A *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid expressing heavy and light chains of human 29IJ6 IgG was constructed and used to secrete recombinant antibody into silkworm larval hemolymph. Fifth instar silkworm larvae were reared and injected into the dorsum of the larvae with recombinant cysteine protease- and chitinase-deficient BmNPV (BmNPV-*CP*$^-$-*Chi*$^-$) bacmid/29IJ6 IgG and harvested after approximately 6 days. The total yield of recombinant 29IJ6 IgG was 36 μg/larvae, which is equivalent to 8 mg/kg of larvae. The recombinant antibody was purified to homogeneity using a HiTrap rProtein A FF column with a purification yield of 83.1%. The purified protein was identified by Western blot and ELISA experiments. The *N*-linked glycan structure of the purified protein was determined by the HPLC mapping method. The *N*-glycans of the 29IJ6 IgG glycoprotein produced in, and secreted by the silkworm larvae were composed exclusively of two kinds of paucimannose-type oligosaccharides, Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc.

Keywords: IgG; antibody; bacmid; silkworm; BmNPV

1. Introduction

Progress has been made in antibody engineering technology in the last decade, which has enabled the production of recombinant antibodies including chimeric, humanized and human antibodies. Different systems to produce recombinant antibodies have been developed and used, such as bacterial (Bird et al., 1988), yeast (Davis et al., 1991), insect (Bei et al., 1995; Hasemann and Cappa, 1990), mammalian (Jost et al., 1994), transgenic plant (Whitelam et al., 1994) and animal methodologies (Pollock et al., 1999). In order to obtain recombinant antibodies capable of full activity, expression in eukaryotic cells has been preferred over bacterial systems that often produce insoluble antibodies that remain inactive even after re-folding. Insect cells infected with recombinant baculovirus have been used for the high-level expression of antibodies (Hasemann and Cappa, 1990;Verma et al., 1998), because they are capable of a similar post-translational modification to that occurring in mammalian cells, and also because of the high expression levels achievable. Recombinant baculoviruses are used to infect insect cells, Sf9 and High Five cells, and recombinant antibodies are recovered from the infected cells. However, to improve the yield of recombinant antibodies, optimization of the reactor performance, reactor design and the development of appropriate media are still required. For example, the serial passage and preparation of large amounts of recombinant baculovirus for the infection of insect cells are major drawbacks when using baculovirus-insect cell expression systems.

An alternative baculovirus expression system involves the use of silkworms and enables the production of recombinant proteins in silkworm larvae or pupae. A *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid system has recently been developed (Motohashi et al., 2005). The BmNPV bacmid is a shuttle vector that can be replicated in *Escherichia coli*, cultured *B. mori* cells and silkworm larvae or pupae. This enables more rapid gene expression in silkworms compared with that in conventional baculovirus expression systems. Moreover, a cysteine protease-deficient BmNPV (BmNPV-*CP*$^-$) bacmid (Hiyoshi et al., 2007) and both cysteine protease- and chitinase-deficient BmNPV (BmNPV-*CP*$^-$-*Chi*$^-$) bacmids (Park et al., 2008) have been developed for the efficient production of gene products from silkworms. The protein expression of these bacmids is higher than that of the wild-type BmNPV bacmid due to the significant decrease in silkworm liquefaction and proteolytic degradation of the expressed proteins.

In this study we report the successful production of a functional human IgG in silkworm larvae. To evaluate the performance of our antibody expression system, a human anti-BSA IgG1 was chosen as a model antibody. As the source of variable region gene, the heavy (V_H) and light chain (V_L) genes of human single chain Fv 29IJ6 isolated from a synthetic library Tomlinson I+J were used (Aburatani et al., 2002; de Wildt et al., 2000). The genes for V_H/V_L were linked to the genes for heavy chain constant region of human IgG1 (Cγ1) and the human lambda light chain constant region Cλ, respectively, and expressed as 29IJ6 IgG. Moreover, p~~P~~urification of th~~e product~~is

29IJ6 IgG from silkworm larval hemolymph and identification of its *N*-linked glycan structures are also reported.

2. Materials and methods

2.1. Strain, plasmid and silkworm larvae

E. coli DH10Bac was purchased from Invitrogen (Carlsbad, CA, USA). Fifth-instar hybrid Kinsyu x Syowa silkworm larvae (Ehime Sansyu, Yahatahama, Japan) were used in this study. The larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27±1°C. Plasmids pUC18/VDJ-gamma1m (HG324) and pUC18/human IgCλ (HG302) were obtained from Human Science Research Resources Bank (Osaka, Japan).

2.2. PCR amplification of the genetic elements for IgG1 expression

Oligonucleotide primers used for the PCR amplification of heavy and light chain genes of the human antibody variable region (Fv) recognizing bovine serum albumin (BSA), and human ~~VDJ~~ Cγ1 ~~/ Ig~~ Cλ genes are listed in Table 1. Heavy (29IJ6VH) and light (29IJ6VL) chain genes of the human single chain Fv isolated from a synthetic library Tomlinson I+J were amplified from pIT2-29IJ6 (de Wildt et al., 2000; Aburatani et al., 2002) using primers 29IJ6VH-F and 29IJ6VH-R or 29IJ6VL-F and 29IJ6VL-R, respectively. The ~~S~~secretion form IgG1 constant region (Cλ1) cDNA was amplified from pUC18/VDJ-gamma 1m using Cgamma1-F and Cgamma1-R, ~~whilst~~ while the

human Ig Cλ sequence DNA from pUC18/human IgCλ was amplified using primers Clambda-F and Clambda -R. PCR was performed using the following program: 3 min at 95°C, 35 cycles at 95°C for 15 s, 55°C for 30 s and 68°C for 1 min, followed by a final extension at 68°C for 5 min. All of the amplified PCR fragments were purified using a GFX PCR purification kit (Biocompare Inc., San Francisco, CA) and stored at -20°C for subsequent cloning.

2.3. Construction of recombinant BmNPV bacmids

The recombinant BmNPV bacmid for IgG1 expression was constructed as follows (Fig. 1). *Xho* I- and *Nco* I-digested 29IJ6VL, and *Nco* I- and *Kpn* I-digested Cλ fragments were ligated into *Xho* I and *Kpn* I digested pFastBac Dual (Invitrogen) fragments using T4 ligase (pFastBac Dual/29IJ6VL-Cλ). *Bam* HI- and *Eco* RI- digested 29IJ6VH and *Eco* RI- and *Pst* I- digested γ1 fragments were ligated into *Bam* HI and *Pst* I digested pFastBac Dual/29IJ6VL-Cλ using T4 ligase (pFastBac Dual/29IJ6 IgG). Expression of the 29IJ6 IgG1 gene is controlled by a polyhedrin promoter in the pFastBac Dual/29IJ6 IgG, but that of the 29IJ6 lambda chain gene is controlled by a p10 promoter. To secrete expressed protein(s) into the silkworm larval hemolymph, the signal sequence from bombyxin (bx) was added to both the heavy and the light chain genes. In order to decrease the proteolytic degradation of expressed 29IJ6 IgG, a cysteine protease- and chitinase-deficient *Bombyx mori* multiple nucleopolyhedrovirus (BmNPV-*CP*$^-$-*Chi*$^-$) bacmid (Park et al., 2008) was used. The pFastBac Dual/29IJ6 IgG

was then transformed into each *E. coli* DH10BacBm-*CP*$^{-}$-*Chi*$^{-}$, cultivated in a LB (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 20 g of agar per liter) plate containing 50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracycline, 100 μg/ml 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-Gal) and 40 μg/ml isopropyl β-D-1-thiogalactopyranoside (IPTG). White colonies were selected, and positive clones harboring the BmNPV bacmid were used for expressing the IgG1~~, and the white colony was picked up as positive clones~~.

2.4. 29IJ6 IgG expression in harvested silkworm larvae and hemolymph

BmNPV bacmid DNA was injected directly into larvae on the first day of their fifth-instar. Four μg of both BmNPV bacmid and the helper plasmid pMON7124 DNA were suspended in 5 μl of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE)-C reagent (Invitrogen) and left to stand 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 mixture was then injected into the dorsum of the larvae using a syringe with a 26-gauge beveled needle. Expression of IgG in the silkworm larvae was confirmed at 6 days post-injection (d.p.i.).

Silkworm larvae at 6 d.p.i. during the fifth instar were bled by cutting the abdominal legs with scissors. Hemolymph was immediately mixed with 5 μl of 200 mM 1-phenyl-2-thiourea, and centrifuged at 9000 rpm for 10 min at 4^{o}C. The supernatant samples were immediately frozen at -80^{o}C for further analysis.

2.5. SDS-PAGE, Western blot, and lectin blot analyses

The protein content in the hemolymph was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. SDS-PAGE was performed with 10% polyacrylamide gel using the Mini-protein III System (Bio-Rad Laboratories, Hercules, CA, USA). The respective bands were detected using Coomasie Brilliant Blue (CBB). For the Western blot, the samples were boiled for 5 min before they were run on the SDS-PAGE gel. A~~n~~ mouse anti-human IgG (H+L) antibody (Jackson ImmunoResearch Lab., Inc. West Grove, PA, USA) was used as the primary antibody, and goat anti-mouse IgG-horseradish peroxidase (GE Healthcare, Piscataway, NJ, USA) (1:10,000) was used as the secondary antibody. The immunoblot bands were visualized using ECL plus Western blotting detection reagents (GE Healthcare), and analyzed using a Fluor-S/MAX multi-imager (Bio-Rad). Magic Mark XP Western Protein Standard (Invitrogen) was used as a protein molecular weight marker.

Proteins were transferred to the SDS-PAGE as described above. The gel was blocked by washing three times with TBST (10 mM TrisHCl, pH, 7.4, 150 mM NaCl, 0.05% Tween 20) for 10 min. It was then incubated with FITC-conjugated lectin for 1 h at room temperature, followed by three washes with TBST for 5 min. The lectins used were Concanavalin A (ConA), and agglutinins from *Lens culinaris* (LCA), *Arachis hypogaea* (PNA), wheat germ (WGA), *Aleuria aurantia* (AAL) and *Sambucus*

sieboldiana (SSA). The signal was analyzed using a Fluor-S/MAX multi-imager (Bio-Rad).

The protein concentration was measured using a Bradford protein assay kit (Bio-Rad), with BSA as a standard.

2.6. Quantification of 29IJ6 IgG and antigen binding

The expressed IgG in silkworm larvae was quantified using the Human IgG ELISA quantification kit (Bethyl Lab. Inc., Montgomery, TX, USA). One hundred μl/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 μl 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 wells, followed by incubation for 30 min at room temperature. The wells were washed three times with a 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-HRP conjugate diluted 50,000-100,000 times in sample/conjugate diluent (50 mM Tris-HCl, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0), was added to each well, incubated for 1 h and washed three times with 200 μl of washing buffer. 100 μl of substrate (0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (TMBZ) in 100 mM sodium acetate, pH 6.0, with 0.2% (v/v) of 30% hydrogen

peroxidase) was added to each well and left at room temperature for blue color development. The reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 solution. The color developed was measured at optical densities (ODs) of 450 and 655 nm. The value calculated by subtracting OD_{450} from OD_{655} was used as a measure of the amount of 29IJ6 IgG. Human reference serum (4 mg/ml) was used for the calibration of IgG as a standard.

The antigen binding assay was performed as follows. One hundred μl/well of 10 μg/ml BSA was used to coat a 96-well flexible assay plate for 1 h at room temperature. Two hundred μl of 20% skimmed milk in phosphate-buffered saline-Tween (PBST) buffer (137 mM NaCl, 10 mM sodium phosphate, 2.7 mM KCl and 0.1% Tween 20, pH 7.4) was added to the BSA-coated plate wells for blocking, followed by incubation for 1 h at room temperature. The wells were washed three times with PBST buffer. One hundred μl/well of diluted hemolymph (diluted 400-800 times) or purified 29IJ6 IgG (diluted 10-200 times) were then added to the BSA-coated plate wells, incubated for 1.5 h at room temperature and washed three times with 200 μl of PBST. As a second antibody, 100 μl of HRP conjugated goat anti-human IgG-Fc (Bethyl Lab. Inc., Montgomery, TX) was added to each well after being diluted 500-2000 times with 5% skimmed milk/PBS. The plate was incubated for 1 h at room temperature and washed three times with 200 μl of PBST. Detection was performed as described above. Data are presented as the means of triplicate samples.

2.7. Purification of expressed 29IJ6 IgG in silkworm larval hemolymph

A 3-4 ml sample of hemolymph from five larvae was diluted to 10 times its volume with a buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.0) and mixed with 40% saturated ammonium sulfate (pH 7.0) by vigorous stirring for 1.5 h. All subsequent procedures were performed at 4^{o}C. The preparation was centrifuged at 15000 rpm for 30 min, and the supernatant was mixed with 50% saturated ammonium sulfate (pH 7.0) for 1.5 h and then centrifuged again. Subsequently the resulting supernatant was mixed with 70% saturated ammonium sulfate using the similar procedure as above. Each precipitate was suspended with 20 mM sodium phosphate, pH 7.0, followed by dialysis against 20 mM sodium phosphate overnight. The dialyzed sample was applied at a flow rate of 0.5 ml/min to a HiTrap rProtein A FF 1 ml column (GE Healthcare) according to the manufacturer's protocol. The column was then subsequently eluted at 0.5-1 ml/min with elution buffer (0.1 M sodium citrate, pH 3.0). Six ml of the eluate from the column was dialyzed with 1 liter of distilled water overnight, and a 15 ml dialyzed sample was obtained. The sample was lyophilized for 72 h.

To compare the recovery yield of recombinant IgG, the ammonium sulfate purification step was omitted and a sample of hemolymph diluted 10 times was applied directly to a HiTrap rProtein A FF 1 ml column using the same protocol as described above.

2.8. Characterization of oligosaccharides by the HPLC mapping method

The experimental procedures used, including the chromatographic and mass spectrometric conditions, have been described previously (Nakagawa et al. 1995;

Takahashi et al. 1995; Yagi et al. 2005), but slight modifications were made in the preparation of the 2-aminopyridine derivatives of the N-glycans. The purified IgG (0.3 mg) was digested with PNGaseF (New England Biolabs, MA, USA) to release *N*-glycans. After removal of the peptide materials by SepPack reversed-phase cartridges (Waters, MA, USA), the reducing ends of the *N*-glycans were derivatized with 2-aminopyridine (Wako, Osaka, Japan). This mixture was applied to a DEAE column (Tosoh, Tokyo, Japan) or a TSK-gel Amide-80 column (Tosoh), and then each fraction separated on the amide column was applied to a Shim-pack HRC-ODS column (Shimadzu, Kyoto, Japan). The elution times of the individual peaks onto the amide-silica and ODS columns were normalized with respect to the degree of polymerization of 2-aminopyridine-derivatized (PA-) isomalto-oligosaccharide, and represented in units of glucose (GU). Thus, a given compound from these two columns provided a unique set of GU values, which corresponded to coordinates on the HPLC map. The PA-oligosaccharides were identified by comparison with the coordinates of around 500 reference PA-oligosaccharides in a home-made web application, GALAXY (Takahashi et al. 2003). The matrix assisted laser desorption/ionization time of the PA-oligosaccharides was assessed by flight mass spectrometry (MALDI-TOF-MS) and co-chromatography with reference to PA-oligosaccharides on the columns was used to confirm their identities.

3. Results

3.1. Expression of recombinant 29IJ6 IgG in silkworm larval hemolymph

BmNPV-*CP*$^-$-*Chi*$^-$/29IJ6 IgG with bx signal peptides was constructed for the secretion of 29IJ6 IgG into silkworm larval hemolymph which was sampled at 6 d.p.i. and subjected to Western analysis. The secretion of heavy and light chains in the hemolymph was confirmed (Fig. 2A, lane 2), and their predicted molecular weights were 52 and 26 kDa, respectively. The H_2L_2 antibody assembled was also observed in its non-reduced condition (Fig. 2A, lane 4), which suggests that heterodimerization does occur in the hemolymph. No band was observed from mock-injected silkworm larval hemolymph (Fig. 2A, lanes 1 and 3). The average amount of 29IJ6 IgG obtained was 46.1±10 μg/ml corresponding to 36.9±8.1 μg/silkworm larva (Fig. 2B).

3.2. Purification of the recombinant 29IJ6 IgG expressed in silkworm larval hemolymph

The hemolymph diluted 10 times and containing 188.9 μg of 29IJ6 IgG (0.81 μg/mg protein) was precipitated with 50% sodium sulfate and dialyzed against distilled water. In this fraction, 99.8 μg of 29IJ6 IgG (0.92 μg/mg protein) was obtained with a yield of 52.8%. This amount was subjected to a HiTrap rProtein A FF column, of which 25 μg (0.21 μg/mg protein) was found to be flow-through and 68.4 μg (118.8 μg/mg protein) was eluted with a yield of 36.2%. The eluted sample was subjected to an antigen binding assay, and no differences between samples were found (data not shown).

To improve the purification yield, the sample diluted 10 times was filtered through a 4.5 μm Millipore filter and subjected directly to the HiTrap rProtein A FF column.

Protein A column chromatography removed most of the larval protein as demonstrated by the Coomassie-stained SDS-PAGE gel run under reducing conditions (Fig. 3A, lane 6). This simple purification resulted in the production of 350 μg of recombinant IgG with an increase in purity from 1.3 μg per mg total protein in the crude larval hemolymph (Table 2). The estimated total recovery was 83.1% (6.6 mg/kg larva).

The functional activity of recombinant 29IJ6 IgG was demonstrated by ELISA using the purified 29IJ6 IgG and larval hemolymph, which were compared for their antigen binding with anti-human IgG-Fc-HRP antibody. Typical profiles of both samples are shown in Fig. 3B. The working range of the 29IJ6 IgG was from 1 to 100 ng. The calculated 50% saturation values were between 7 and 10 ng for both samples, suggesting that no significant inactivation of recombinant IgG occurred during the purification step.

3.3. Lectin binding assay and identification of the N-linked glycan structure of the recombinant 29IJ6 IgG expressed in silkworm larval hemolymph

The reactivity of recombinant 29IJ6 IgG with various lectins was examined (Fig. 4). The ConA lectin reacted with recombinant 29IJ6 IgG, indicating the presence of a non-reducing terminal α-mannosyl group (Fig. 4). In addition, the reaction with LCA lectin suggested the presence of an α 1-6 fucose residue attached at the reducing terminus. Similarly, reactions with AAL, PNA and WGA lectins indicated the presence of α-Fuc1-6GlcNAC, Galβ1-3GlcNAc disaccharide of *O*-glycans, and GlcNAc,

respectively. Conversely the SSA lectin, known to recognize Siaα2-6Gal, did not react with recombinant 29IJ6 IgG, suggesting that sialic acid was absent. The lectin binding properties are summarized in Table 3.

The structures of the *N*-linked glycans were determined by comparing their positions with those of known standard oligosaccharides on a two-dimensional map. Identification of a sample PA-oligosaccharide was confirmed by co-chromatography with a known PA-oligosaccharide on the ODS and amide-silica columns. The detailed chromatogram and structure of the oligosaccharides are shown in Fig. 5. The recombinant 29IJ6 IgG antibody had a non-reducing terminal α-mannosyl group lacking sialic acid, whilst fucose was connected to GlcNAc near Asn. Non-reducing terminal α-mannosyl sugars were calculated to account for 90.2% of the total sugar content and included 12.7% antennary sugars. PNA lectin showed the possible attachment of Galβ1-4GlcNAc, which may be Galβ1-4GlcNAcβ1-2Manα1-. This unidentified oligosaccharide might be included with others (9.8%) in Fig. 5B.

4. Discussion

In this study, a model human IgG1 was expressed in silkworm larval hemolymph. The amount of IgG secreted from the larvae was 46 μg/ml, which is higher than that obtained using insect cells where the expression level of secreted human HAV16 antibody using High Five insect cells was reported at around 6-18 μg/ml (Liang et al., 2001). In another study, multiple–protease-deficient strains of the methylotrophic yeast *Ogataea minuta* secreted 10 μg/ml of antibody, where the partial degradation of

antibody was suppressed (Kuroda et al., 2007). CHO-DG44 cells from mammals have been reported to produce 35 μg/ml of monoclonal antibody against botulinum neurotoxin serotype A (Mowry et al., 2004). Although the specificity varied between antibodies, comparison with these other experiments demonstrates that silkworm larvae are a potential host capable of expressing a comparable or superior level of human IgG. However, a larger amount of antibody can be produced using transgenic animals. For example, 4 g/l and 14 g/l of hBR96-2, a humanized IgG1, were recorded in the milk of transgenic mice and goats, respectively (Pollock et al., 1999). However, these authors also reported that the other female founder produced only low levels (0.1 g/l) of antibody, which suggests that significant fluctuations in antibody production occur in transgenic animals.

To further improve the expression level in insect cells, coexpression of a molecular chaperone is effective. Hsu and Betenbaugh (1997) reported that the coexpression of chaperone BiP enhanced the level of soluble intracellular and secreted IgG obtained from *Trichoplusia ni* insect cells by 90%. Cytosolic hsp70 chaperones, which are known to associate and prevent aggregation of polypeptides in vitro, form a specific hsp70-immunoglubulin complex in vivo and increase intracellular immunoglobulin solubility (Ailor and Betenbaugh, 1998). In silkworm larvae, coexpression of CRT or CNX can increase IgG expression to levels five times greater than that without the chaperone (EY Park et al., in preparation). This indicates that the coexpression of chaperones in silkworm larvae also improves the level of IgG expression.

The *N*-glycans on 29IJ6 IgG produced in the silkworm larvae were composed exclusively of two kinds of paucimannose-type oligosaccharides, Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc. These patterns contained fucose α1,6-linked to the innermost GlcNAc residue. Misaki et al. (2003) reported the presence of Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (6.4%) and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (1.1%) in *N*-linked glycan structures of mouse interferon-β produced by *Bombyx mori* larvae. Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc may be derived from $GlcNAcMan_5GlcNAc_2$ through mannosidase-mediated excision of α-linked mannosyl residues, followed by GlcNAcase-mediated deletion of a β-1,2-linked terminal GlcNAc residue (Watanabe et al., 2002). Likewise mannosidase-mediated excision of α-linked mannosyl residues from Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc may lead to Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc. Shinkawa et al. (2003) reported that the most important carbohydrate structure in terms of the enhancement of antibody-dependent cellular cytotoxicity (ADCC) is the fucose attached to the innermost GlcNAc of the biantennary complex oligosaccharides. Comparing IgG1s with low and high fucose contents, the former had a higher level of ADCC before separation. A terminal galactose residue was detected during the lectin analysis, indicating the presence of the core Galβ1-3GlcNAc disaccharide of *O*-glycans.

The *N*-linked glycan structures of human 291J6 IgG produced from silkworm larvae were similar to that of *Trichoplusia ni* TN-5B1-4 cells, but the intracellular IgG

contained 50% higher mannose-type *N*-glycan with lower levels of complex, hybrid and paucimannosidic type structures (Hsu et al., 1997). To improve the IgG quality produced from silkworm larvae, sialylation of the *N*-glycans of the recombinant protein is required. Watanabe et al. (2002) observed that GlcNAcase-dependent depletion of *N*-acetylglucosamine residues from intermediate *N*-glycans is critical for the assembly of paucimannosidic *N*-glycans in insect cells and, more importantly, that insect cells, under specific conditions, retain the ability to construct sialylated *N*-glycans like those in mammalian cells. In future, transgenic silkworm provided with both defucosylation and sialylation activities will offer the most enhanced alternative IgG expression system, which will extend the use of silkworm larvae in recombinant protein therapeutics.

Acknowledgements

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

References

Aburatani, T., Ueda, H., Nagamune, T., 2002. Importance of a CDR H3 basal residue in VH/VL interaction of human antibodies. J. Biochem. 132, 775-782.

Ailor, E., Betenbaugh, M.J., 1998. Overexpression of a cytosolic chaperone to improve solubility and secretion of a recombinant IgG protein in insect cells. Biotechnol. Bioeng. 58, 196-203.

Bei, R., Schlom, J., Kashmiri, S.V., 1995. Baculovirus expression of a functional single-chain immunoglobulin and its IL-2 fusion protein. J. Immunol. Methods 186, 245-255.

Bird, R.E., Hardman, K.D., Jacobson, J.W., 1988. Single-chain antigen-binding proteins. Science 242, 423-426.

Davis, G.T., Bedzyk, W.D., Voss, E.W., Jacobs, T.W., 1991. Single chain antibody (SCA) encoding genes: One-step construction and expression in eukaryotic cells. BioTechnology 9, 165-169.

Hasemann, C.A., Cappa, J.D., 1990. High-level production of a functional immunoglobulin heterodimer in a baculovirus expression system. Proc. Natl. Acad. Sci. 87, 3942-3946.

Hiyoshi, M., Kageshima, A., Kato, T., Park, E.Y., 2007. Construction of cysteine protease deficient *Bombyx mori* nuclear polyhedrovirus bacmid and its application on improved expression of fusion protein. J. Virus Method 144, 91-97.

Hsu, T.-A., Betenbaugh, M.J., 1997. Coexpression of molecular chaperone BiP improves immunoglobulin solubility and IgG secretion from *Trichoplusia ni* insect cells. Biotechnol. Prog. 13, 96-104.

Hsu, T.-A., Takahashi, N., Tsukamoto, Y., Kato, K., Shimada, I., Masuda, K., Whiteley, E.M., Fan, J.-Q., Lee, Y.C., Betenbaugh, M.J., 1997. Differential *N*-Glycan patterns of secreted and intracellular IgG produced in *Trichoplusia ni* cells. J. Biol. Chem. 272, 9062-9070.

Jost, C.R., Kurucz, I., Jacobus, C.M., Titus, J.A., George, A.J.T., Segal, D.M., 1994. Mammalian expression and secretion of functional single-chain Fv molecules. J. Biol. Chem. 269, 26267-26272.

Kuroda, K., Kitagawa, Y., Kobayashi, K., Tsumura, H., Komeda, T., Mori, E., Motoki, K., Kataoka, S., Chiba, Y., Jigami, Y., 2007. Antibody expression in protease-deficient strains of the methylotrophic yeast *Ogataea minuta*. FEMS Yeast 7, 1307-1316.

Liang, M., Dübel, S., Li, D., Queitsch, I., Li, W., Bautz, E.K.F., 2001. Baculovirus expression cassette vectors for rapid production of complete human IgG from phage display selected antibody fragments. J. Immunol. Methods 247, 117-130.

Misaki, R., Nagaya, H., Fujiyama, K., Yanagihara, I., Honda, T., Seki, T., 2003. *N*-linked glucan structures of mouse interferon-β produced by *Bombyx mori* larvae. Biochem. Biophys. Res. Commun. 311, 979-986.

Motohashi, T., Shimojima, T., Fukagawa, T., Maenaka, K., Park, E.Y., 2005. Efficient large-scale protein production of larvae and pupae of silkworm by *Bombyx mori* nuclear polyhedrosis virus bacmid system. Biochem. Biophys. Res. Commun. 326, 564-569.

Mowry, M.C., Meagher, M., Smith, L., Marks, J., Subramanian, A., 2004. Production and purification of a chimeric monoclonal antibody against botulinum neurotoxin serotype A. Protein. Expr. Purif. 37, 399-408.

Nakagawa, H., Kawamura, Y., Kato, K., Shimada, I., Arata, Y., Takahashi. N., 1995. Identification of neutral and sialyl *N*-linked oligosaccharide structures from human

serum glycoproteins using three kinds of high-performance liquid chromatography. Anal. Biochem. 226, 130-138.

Park, E.Y., Abe, T., Kato, T., 2008. Improved expression of fusion protein using a cysteine protease and chitinase deficient *Bombyx mori* multiple nucleopolyhedrovirus bacmid in silkworm larvae. Biotechnol. Appl. Biochem. 49, 135-140.

Pollock, D.P., Kutzko, J.P., Birck-Wilson, E., Williams, J.L., Echelard, Y., Meade, H.M., 1999. Transgenic milk as a method for the production of recombinant antibodies. J. Immunol. Methods 231, 147-157.

Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., Shintara, K., 2003. The absence of fucose but not the presence of galactose or bisecting *N*-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J. Biol. Chem. 31, 3466-3473.

Takahashi, N., Kato, K., 2003. GALAXY (Glycoanalysis by the three axes of MS and chromatography): a Web application that assists structural analyses of *N*-glycans. Trends Glycosci. Glycotech. 15, 235-251.

Takahashi, N., Nakagawa, H., Fujikawa, K., Kawamura, Y., Tomiya, N., 1995. Three-dimensional elution mapping of pyridylaminated *N*-linked neutral and sialyl oligosaccharides. Anal. Biochem. 226, 139-146.

Verma, R., Boleti, E., George, A.J., 1998. Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. J. Immunol. Methods 216, 165-181.

Watanabe, S., Kokuho, T., Takahashi, H., Takahashi, M., Kubota, T., Inumaru, S., 2002. Sialylation of *N*-glycans on the recombinant proteins expressed by a baculovirus-insect cell system under β-*N*-acetylglucosaminidase inhibition. J. Biol. Chem. 277, 5090–5093.

Whitelam, G.C., Cockburn, W., Owen, M.R., 1994. Antibody production in transgenic plants. Biochem. Soc. Trans. 22, 940-944.

de Wildt, R.M., Mundy, C.R., Gorick, B.D., Tomlinson, I.M., 2000. Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat. Biotechnol. 18, 989-994.

Yagi, H., Takahashi, N., Yamaguchi, Y., Kimura, N., Uchimura, K., Kannagi, R., Kato, K., 2005. Development of structural analysis of sulfated *N*-glycans by multidimensional high performance liquid chromatography mapping methods. Glycobiol. 15, 1051-1060.

Figure legends

Fig. 1. Schematic diagram of recombinant BmNPV-*CP*$^{-}$-*Chi*$^{-}$ bacmid/29IJ6 IgG construction. ~~The heavy chain gene was cloned under a polyhedron promoter; light chain, under p10 promoter.~~ The human variable region genes for BSA were amplified from pIT2-29IJ6 by PCR. Also, the secretion form human γ1 and Cλ were from pUC18/VDJ-gamma 1m and pUC18/human IgCλ, respectively. rVL and Cλ fragments were digested by restriction enzymes, and ligated into *Xho* I and *Kpn* I-digested pFastBac Dual fragment (pFastBac Dual/29IJ6VL-Cλ). Similarly, the VH and γ1 fragments were restriction digested, and ligated into *Bam* HI and *Pst* I-digested pFastBac Dual/29IJ6VL-Cλ (pFastBac Dual/29IJ6 IgG). Expression of the 29IJ6 IgG1 and 29IJ6 lambda in the pFastBac Dual/29IJ6 IgG is controlled by a polyhedrin promoter and a p10 promoter, respectively. To secrete expressed protein(s) into the silkworm larval hemolymph, the signal sequence from bombyxin (bx) was added to both the heavy and the light chain genes. The pFastBac Dual/29IJ6 IgG was then transformed into each *E. coli* DH10Bac harboring cysteine protease- and chitinase-deficient *Bombyx mori* multiple nucleopolyhedrovirus (BmNPV-*CP*$^{-}$-*Chi*$^{-}$), cultivated and positive colony was screened.

書式変更: フォント : Times New Roman

書式変更: フォント : Times New Roman

書式変更: フォント : Times New Roman

–

Fig. 2. **A,** Detection of 29IJ6 IgG. Supernatant (3 μl) was subjected to Western analysis in the reduced condition (lanes 1 and 2) and in the non-reduced condition (lanes 3 and 4). Peroxidase conjugated goat anti-human IgG (H+L) was used for the detection.

書式変更: インデント : 最初の行 : 7.5 mm

Lanes 1 and 3 denote mock-injected silkworm larval hemolymph. M denotes the molecular weight marker. Arrows indicate the molecular weight of heavy and light chains, respectively. **B,** 29IJ6 IgG quantification using ELISA of larval hemolymph of BmNPV-*CP*$^{-}$-*Chi*$^{-}$ bacmid/29IJ6 IgG-injected silkworm (closed circles) and larval hemolymph of mock-injected silkworm (open circles).

Fig. 3. **A,** Coomassie brilliant blue stained reduced SDS-PAGE gel of larval hemolymph of BmNPV-*CP*$^{-}$-*Chi*$^{-}$ bacmid/29IJ6 IgG-injected silkworm and purified recombinant 29IJ6 IgG; lanes: M, molecular marker; 1, larval hemolymph; 2, flow-through after immobilized protein A column chromatography; 3, washed fraction of protein A column chromatography; 4-8, elute from protein A column chromatography. **B,** Antigen binding assay of purified recombinant IgG (open circles) and crude larval hemolymph (closed circles).

Fig. 4. Lectin blotting assay of recombinant 29IJ6 IgG. Protein was transferred to SDS-PAGE and was incubated with FITC-conjugated lectin.

書式変更: インデント : 最初の行 : 7.5 mm, 行間 : 固定値 30 pt

Fig. 5. **A,** *N*-Glycosylation profile of recombinant 29IJ6 IgG on an amide column. The purified protein was digested with PNGaseF to release *N*-glycans. The reducing ends of the *N*-glycans were derivatized with 2-aminopyridine, and were subjected to a TSK-gel Amide-80 column. Subsequently, each fraction was applied to a Shim-pack HRC-ODS column (Shimadzu, Kyoto, Japan). The elution times of the individual peaks onto the amide-silica and ODS columns were normalized with respect to the degree of

書式変更: インデント : 最初の行 : 1.77 字

polymerization of 2-aminopyridine-derivatized (PA-) isomalto-oligosaccharide and represented in units of glucose (GU). The identification and confirmation of PA-oligosaccharides are described in detail in the Materials and Methods. **B,** ~~The~~ The proposed structure of PA-oligosaccharides obtained from recombinant 29IJ6 IgG expressed in silkworm larval hemolymph. ~~proposed structure of PA-oligosaccharides obtained from recombinant 29IJ6 IgG expressed in silkworm larval hemolymph.~~

書式変更: インデント : 最初の行 : 1.77 字, 行間 : 固定値 30 pt

Table 1

Primers used for the cloning of 29IJ6 IgG

Name	Oligonucleotide sequences	Cloning sites
bx signal-29IJ6VL-F	5’-CCGCTCGAGCGGATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAAT GTGGGTGTCAACACAAGACATCCAGATGACCCAGTCT-3’	*Xho* I
29IJ6VL-R	5’-CATGCCATGGCATGCCGTTTGATTTCCACCTT-3’	*Nco* I
bx signal-29IJ6VH- F	5’-CGCGGATCCGCGATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAAT GTGGGTGTCAACACAAGAGGTGCAGCTGTTGGAGTC-3’	*Bam* HI
29IJ6VH-R	5’-CCGGAATTCCGGGCTCGAGACGGTGACCA-3’	*Eco* RI
Clambda- F	5’-CATGCCATGGCCCAAGGCCAACCCCACGGT-3’	*Nco* I
Clambda-R	5’-CAGGGTACCCTATGAACATTCTGTAGGGGCCACT-3’	*Kpn* I
Cgamma1- F	5’-CAGGAATTCTCCACCAAGGGCCCATCGGTCT-3’	*Eco* RI
Cgamma1-R	5’-CAACTGCAGTCATTTACCCGGAGACAGGGAG-3’	*Pst* I

Underlining denotes restriction site of primer.

Table 2

Purification of recombinant 29IJ6 IgG from silkworm larval hemolymph

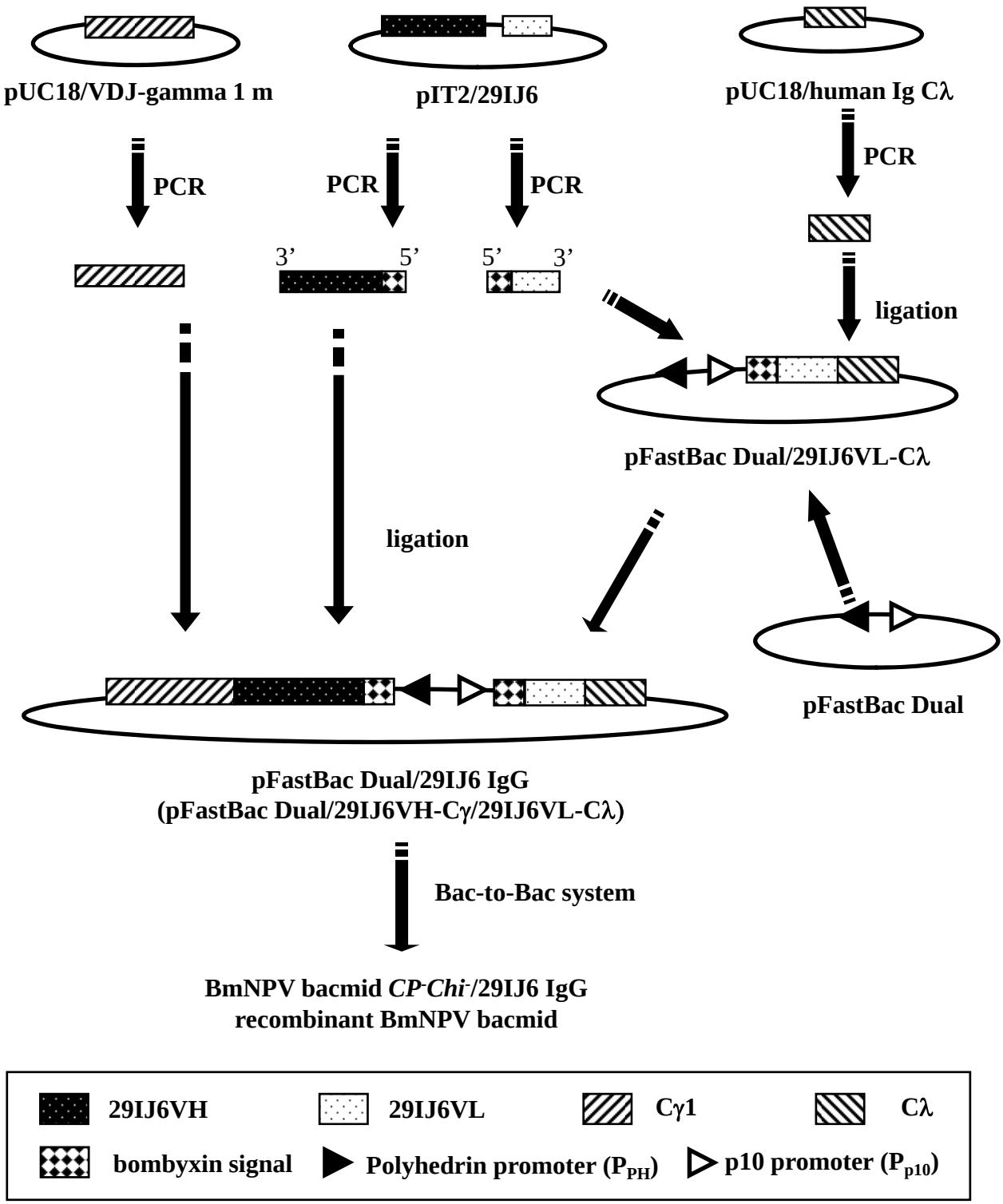
	Volume (ml)	29IJ6 IgG concentration (μg/ml)	Total 29IJ6 IgG (μg)	Protein concentration (mg/ml)	Total protein (mg)	Specific 29IJ6 IgG content (μg/mg)	Recovery (%)
Hemolymph	28	6.4	178.3	5.0	139.0	1.3	100
Flow-through	28	0.0	0.0	4.3	121.0	0.0	0
Wash	6	0.0	0.0	0.5	3.0	0.0	0
Elution	3.9	38.0	148.2	0.1	0.4	350.0	83

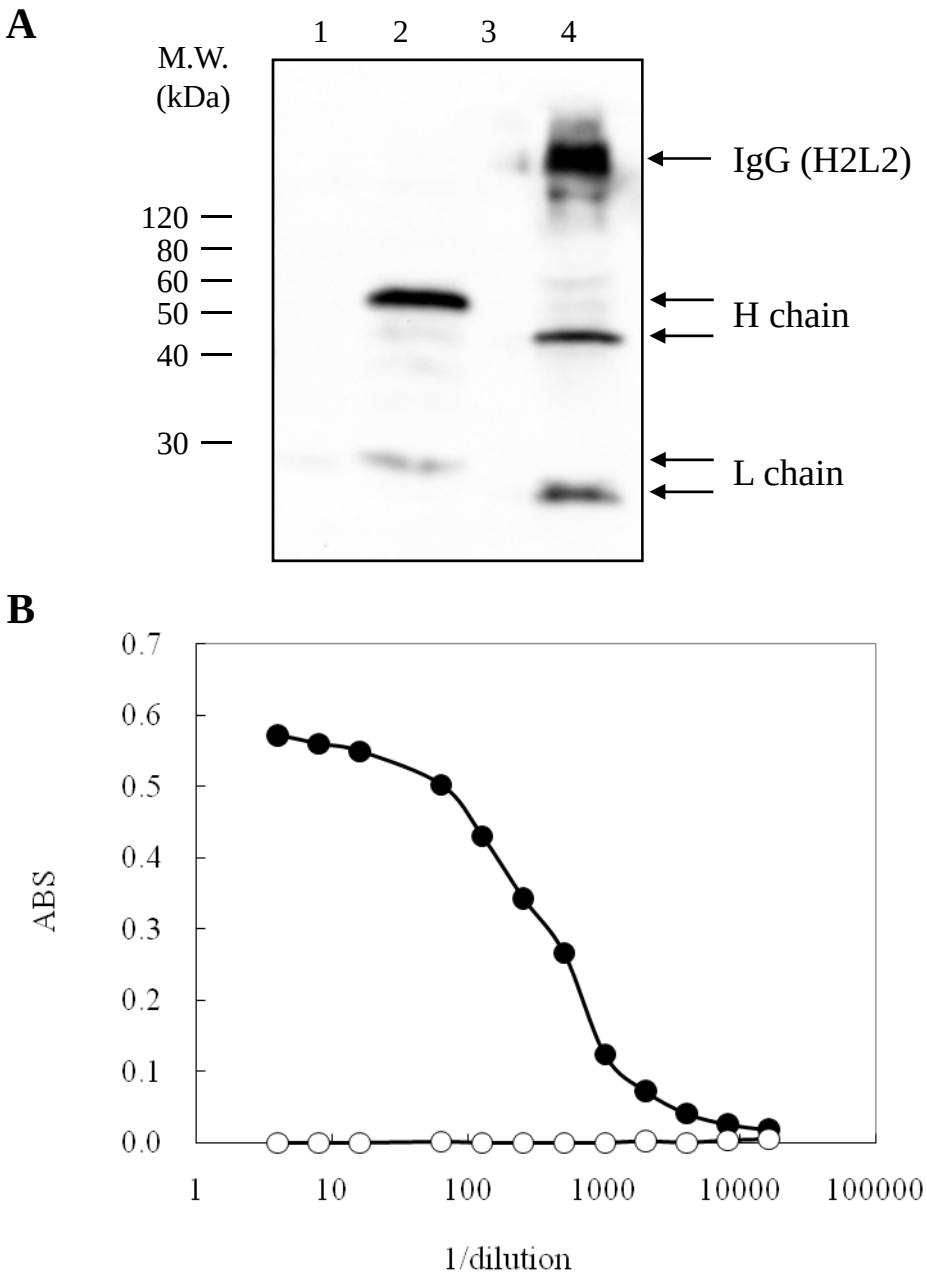
Table 3

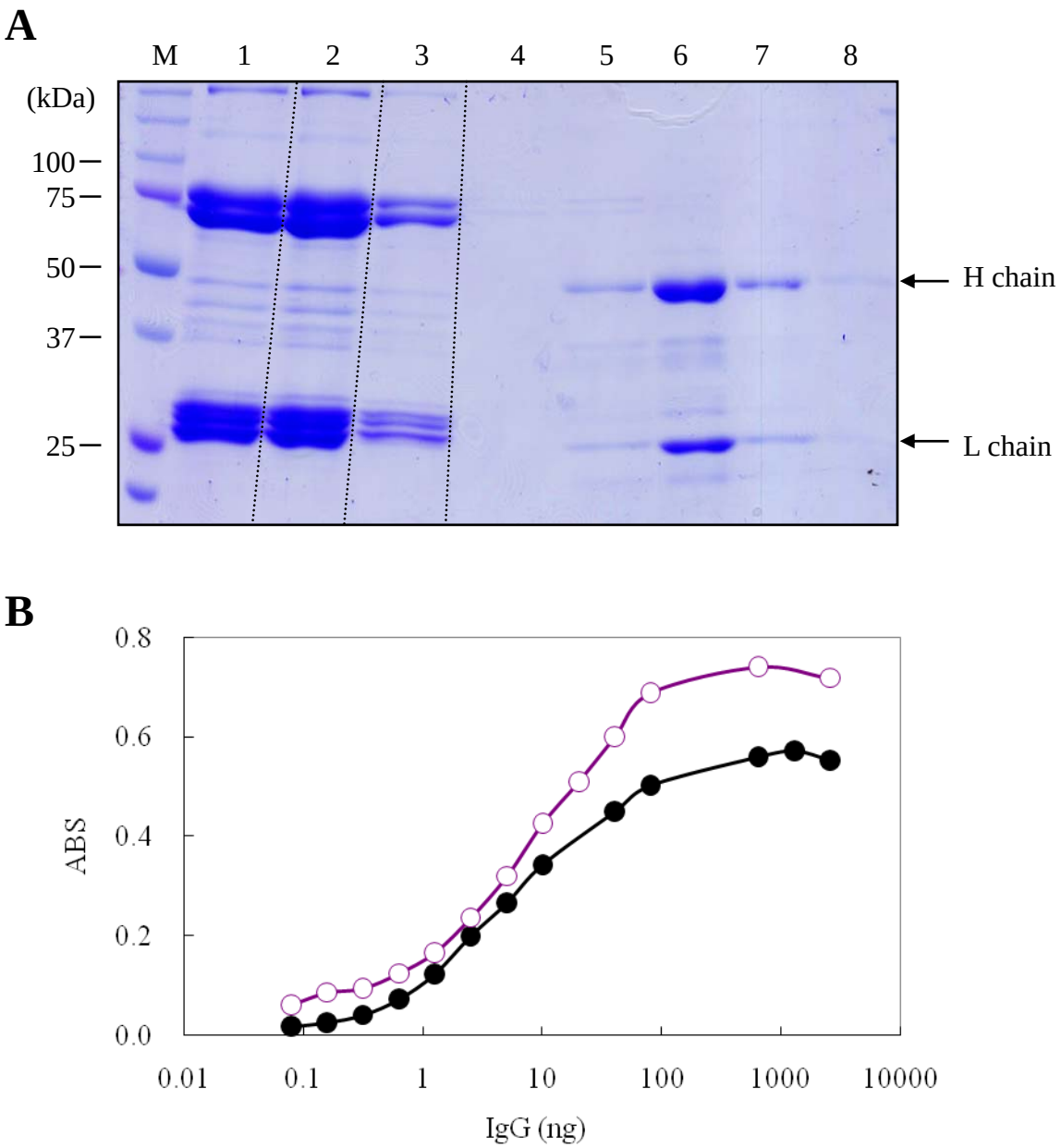
Lectin binding properties of 29IJ6 IgG

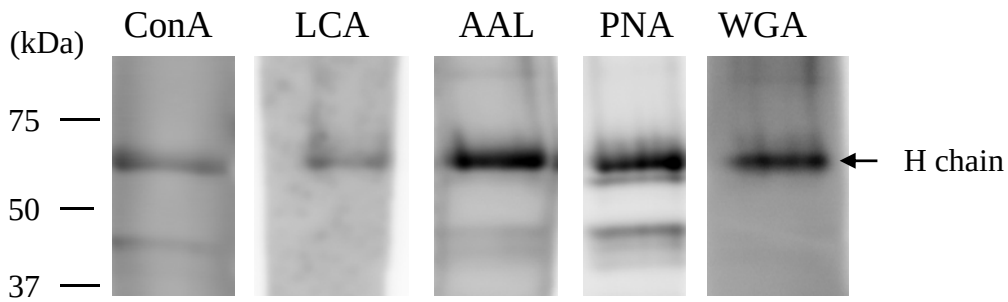
Lectin	Results	Specificity and linkage
Canavalia ensiformis (ConA)	+	Manα1-6(Manα1-3)Man
Lens culinaris (LCA)	+	Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4 (Fucα1-6)GlcNAc
Aleuria aurantia (AAL)	+	Fucα1-6GlcNAc
Arachis hypogaea (PNA)	+	Galβ1-3GlcNAc
Triticum vulgaris (WGA)	+	GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc or Sia
Sambucus sieboldiana (SSA)	-	Siaα2-6Gal

Man, mannose; Fuc; fucose; Gal, galactose; GlcNAc, *N*-acetyl glucosamine; Sia, sialic acid

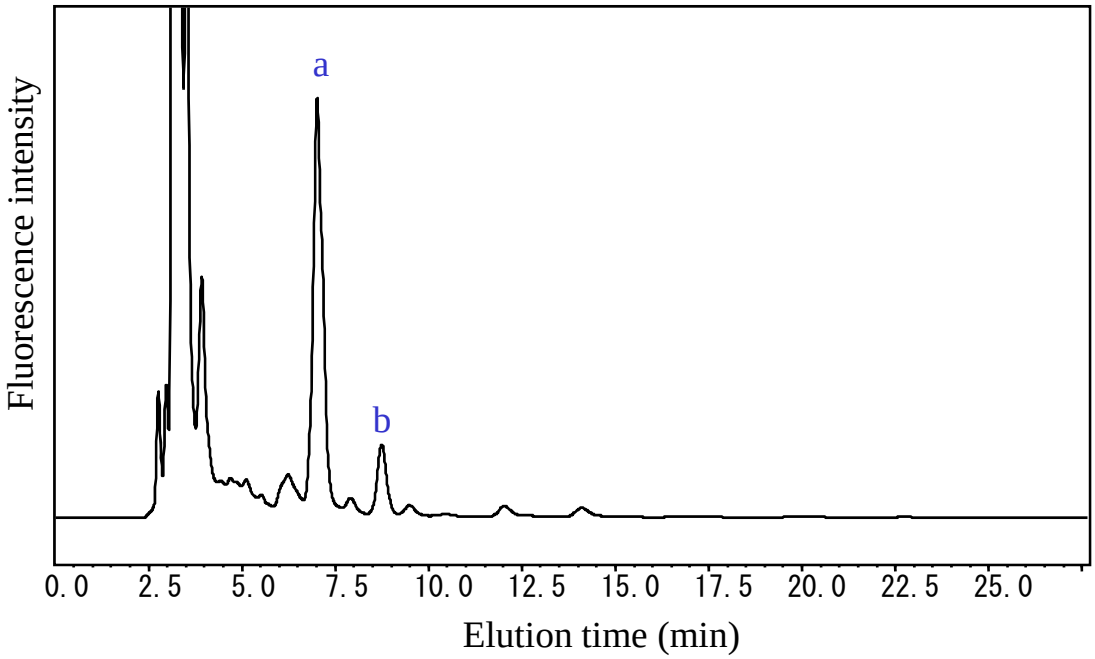

pUC18/VDJ-gamma 1 m
pIT2/29IJ6
pUC18/human Ig Cλ
PCR
PCR
PCR
PCR
3'
5'
5'
3'
ligation
pFastBac Dual/29IJ6VL-Cλ
ligation
pFastBac Dual
pFastBac Dual/29IJ6 IgG
(pFastBac Dual/29IJ6VH-Cγ/29IJ6VL-Cλ)
Bac-to-Bac system
BmNPV bacmid CP-Chi-/29IJ6 IgG
recombinant BmNPV bacmid
29IJ6VH
29IJ6VL
Cγ1
Cλ
bombyxin signal
Polyhedrin promoter (PPH)
p10 promoter (Pp10)


A
1
2
3
4
M.W.
(kDa)
120
80
60
50
40
30
IgG (H2L2)
H chain
L chain
B
ABS
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
10
100
1000
10000
100000
1/dilution


A
M
1
2
3
4
5
6
7
8
(kDa)
100
75
50
37
25
H chain
L chain
B
0.8
0.6
0.4
0.2
0.0
ABS
0.01
0.1
1
10
100
1000
10000
IgG (ng)


(kDa)
ConA
LCA
AAL
PNA
WGA
75
50
37
H chain

A



B

peak	GU(ODS) GU(Amide)	MS value $[M+H]^+$	Structure	Relative quantity (mol %)
a	10.3 4.6	974.5	Manα1、 6 Fucα1 6 Manβ1-4GlcNAcβ1-4GlcNAc	77.5
b	10.3 5.1	1136.7	Manα1、 6 3 Manα1′ Fucα1 6 Manβ1-4GlcNAcβ1-4GlcNAc	12.7
others				9.8