

Silkworm expression system as a platform technology in life science

Tatsuya Kato · Mizuho Kajikawa · Katsumi Maenaka · Enoch Y. Park

Received: 24 July 2009 / Revised: 17 September 2009 / Accepted: 17 September 2009 / Published online: 15 October 2009
© The Author(s) 2009. This article is published with open access at Springerlink.com

Abstract Many recombinant proteins have been successfully produced in silkworm larvae or pupae and used for academic and industrial purposes. Several recombinant proteins produced by silkworms have already been commercialized. However, construction of a recombinant baculovirus containing a gene of interest requires tedious and troublesome steps and takes a long time (3–6 months). The recent development of a bacmid, *Escherichia coli* and *Bombyx mori* shuttle vector, has eliminated the conventional tedious procedures required to identify and isolate recombinant viruses. Several technical improvements, including a cysteine protease or chitinase deletion bacmid and chaperone-assisted expression and coexpression, have led to significantly increased protein yields and reduced costs for large-scale production. Terminal *N*-acetyl glucosamine and galactose residues were found in the *N*-glycan structures produced by silkworms, which are different from those generated by insect cells. Genomic elucidation of silkworm has opened a new chapter in utilization of silkworm. Transgenic silkworm technology provides a stable production of recombinant protein. Baculovirus surface display expression is one of the low-cost approaches toward silkworm larvae-derived recombinant subunit vaccines. The expression of pharmaceutically relevant proteins, including cell/viral surface proteins,

membrane proteins, and guanine nucleotide-binding protein (G protein) coupled receptors, using silkworm larvae or cocoons has become very attractive. Silkworm biotechnology is an innovative and easy approach to achieve high protein expression levels and is a very promising platform technology in the field of life science. Like the “Silkroad,” we expect that the “Bioroad” from Asia to Europe will be established by the silkworm expression system.

Keywords Silkworm · *Bombyx mori* nucleopolyhedrovirus (BmNPV) · Bacmid · Protein expression · Baculovirus display

Introduction

Various systems to produce recombinant proteins are available. Bacteria, especially *Escherichia coli*, are usually chosen first, due to the abundance of tools available for gene manipulations and the simple process for protein production. However, bacteria are severely limited because of their poor capacity to modify expressed proteins cotranslationally and posttranslationally, in spite of recent improvements of strains. Yeasts have some advantages over bacteria. Recombinant proteins expressed in yeast can be glycosylated, but certain modifications are not possible, especially in the case of complex form of *N*-glycosylation.

Insect cell systems are now popular and used widely to produce proteins from higher eukaryotes because insect cells have a similar pattern and capacity of cotranslational and posttranslational modifications as mammalian cells, including glycosylation, phosphorylation, and protein processing. The insect cell systems include the baculovirus expression system and the stably transformed cell system. In the baculovirus expression system, not only cultured

T. Kato · E. Y. Park (✉)

Faculty of Agriculture, Integrated Bioscience Section,
Graduate School of Science and Technology, Shizuoka University,
836 Ohya, Suruga-ku,
Shizuoka 422-8529, Japan
e-mail: acypark@ipc.shizuoka.ac.jp

M. Kajikawa · K. Maenaka

Medical Institute of Bioregulation, Kyushu University,
3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan

cells but also insect larvae and pupae can be used for protein production.

The silkworm, *Bombyx mori*, has been used for silk production for centuries and recently also for protein production, as a bioreactor. Since Maeda et al. (1985) reported the production of human α -interferon in silkworm larvae, using recombinant *B. mori* nucleopolyhedrovirus (BmNPV), the production of many proteins has been achieved. TORAY Ind. Inc. (Tokyo, Japan) used silkworms to produce two recombinant proteins for veterinary use. Intercat, a drug principally composed of feline interferon, for feline calicivirus infections (commonly known as “cat flu”) was introduced in February 1994 as the world’s first antiviral drug for animal use. Interdog, a drug principally composed of canine interferon- γ for canine atopic dermatitis, was launched in December 2005. Nowadays, recombinant proteins produced from silkworms are being investigated for commercial purposes.

This review provides an overview of the production of recombinant proteins using silkworm larvae and pupae and the improvement of silkworm expression systems by the development of the BmNPV bacmid.

Silkworm genome sequence

Silkworms are the larvae of the silk moth, *B. mori*. *B. mori* was domesticated from its wild ancestor, *Bombyx mandarina*, and has been used for silk production for about 5,000 years. Silkworm silk in the natural form consists of silk fibroin, the core filaments, and silk sericin, a coating protein surrounding fibroin. Sericin is removed in commercial silk, and this silk has been used in textile production for centuries.

B. mori is an important model organism of Lepidoptera in the fields of genetics, physiology, and biochemistry. Two whole-genome shotgun (WGS) sequencing projects for *B. mori* were reported in 2004 (Mita et al. 2004; Xia et al. 2004), and its expressed sequence tag database was constructed (Mita et al. 2003). In 2008, two data sets from WGS projects were merged and assembled with new data (International Silkworm Genome Consortium 2008). The silkworm has 28 chromosomes and an estimated genome size of around 500 Mb. A large number of transposable elements (TEs) exist in the *B. mori* genome, accounting for 43.5% of the whole. TEs occupy from 2.7% to 25% in the whole genome in 12 *Drosophila* species and 47% in *Aedes aegypti* (Mita et al. 2004). The gene-finder algorithm BGF (BGI GeneFinder) predicted that approximately 18,510 genes are encoded in the silkworm genome. This gene number in the silkworm genome is higher than that in *Drosophila melanogaster* (13,379 genes), which belongs to Diptera (*Drosophila* 12 Genome Consortium 2007; Nene

et al. 2007; International Silkworm Genome Consortium 2008). Moreover, orthology relationship of genes from *B. mori*, *D. melanogaster*, *A. aegypti*, and *Caenorhabditis elegans* by phylogenetic methods showed 13,450 gene families. Approximately 11.45% and 25.9% of the gene families are specific to insects and vertebrates, respectively, and 3,223 genes are silkworm specific (The International Silkworm Genome Consortium 2008). A genome-wide microarray analysis of fifth-instar larvae of the silkworm identified tissue- and developmental-stage-specific genes (Xia et al. 2007). Tissue-specific genes and genes differentially expressed in different tissues were identified. Moreover, to construct the secondary protein database of the silkworm, proteomic identification of protein-coding genes was performed by tandem mass spectrometry, using silkworm pupae as the sample (Zhang et al. 2009).

Baculovirus expression system in silkworm larvae or pupae

Silkworm larvae have been used as a bioreactor for recombinant protein production for decades. Maeda et al. (1985) first reported the production of human interferon alpha (IFN- α) in the hemolymph of silkworm larvae using BmNPV, containing the gene encoding human α -interferon driven by the polyhedrin promoter. Recombinant proteins expressed in silkworm larvae are shown in Table 1. Many proteins from eukaryotes were expressed in silkworm larvae and purified. In general, the expression level of recombinant proteins in silkworm larvae is higher than that in cultures cells from insect and animals. The mouse interleukin-3 activity in the hemolymph of silkworm larvae was about 20-fold and 10,000-fold higher than those in the culture supernatants of BmN cells and COS7 cells, respectively (Miyajima et al. 1987). The hemolymph of infected silkworms showed human butyrylcholinesterase activities 23- and 280-fold higher than those in BmN cells and Chinese hamster ovary (CHO) cells, respectively (Wei et al. 2000). One milligram of purified human macrophage colony-stimulating factor (CSF) was obtained from the hemolymph of ten silkworm larvae, and 160 μ g of human growth factor was purified from 1 ml of hemolymph (Kadono-Okuda et al. 1995; Qiu et al. 1994).

The degradation of the expressed proteins is often observed in silkworm larvae. This is probably caused by a baculovirus cysteine protease, v-cath, in the baculovirus-insect cell expression system (Kadono-Okuda et al. 1995). To overcome this problem, BmNPV lacking the cysteine protease gene (BmNPV-CP⁻) was constructed by cotransfection with BmNPV genome DNA and pBmFCPdLZ containing the cysteine protease gene partially substituted with the β -galactosidase gene fused with the hsp70

Table 1 Expression of recombinant proteins in silkworm larvae and pupae

Proteins	Used viruses or bacmids	Expression level	References
Intracellular protein			
Firefly luciferase	BmNPV	13 mg per larva	Palhan et al. (1995)
Secretory proteins			
Human interferon- α	BmNPV	50 mg in hemolymph	Maeda et al. (1985)
Human macrophage colony-stimulating factor	BmNPV	1 mg/10 larvae (after purification)	Qiu et al. (1994)
Human growth factor	BmNPV	160 μ g/ml hemolymph (after purification)	Kadono-Okuda et al. (1995)
Rat interleukin-5	Cysteine protease depleted BmNPV	1 mg/ml hemolymph 51 mg/4 larvae (after purification)	Ishihara et al. (1999)
Human butyrylcholinesterase	BmNPV	35 μ g/ml hemolymph	Wei et al. (2000)
Bovine interleukin-21	HyNPV	50 μ g/ml hemolymph	Muneta et al. (2004)
Bovine interferon-t	Cysteine protease depleted BmNPV	4.6 mg/100 larvae (after purification)	Nagaya et al. (2004)
Porcine lactoferrin	HyNPV	20.5 mg/100 pupae (after purification)	Wang et al. (2005)
Human granulocyte macrophage colony-stimulating factor	BmNPV	100 μ g per pupa	Chen et al. (2006)
GFP _{uv} - β 3GnT2 fusion protein	BmNPV bacmid	91 μ g/ml hemolymph	Park et al. (2007)
EGFP-spider dragline silk fusion protein	BmNPV bacmid	6 mg/a larva	Zhang et al. (2008)
Cholera toxin B	BmNPV	54.4 μ g/ml hemolymph	Gong et al. (2005)
Human stem cell factor	BmNPV	3 μ g/ml hemolymph	Han et al. (2004)
anti-BSA scFV	Cysteine protease and chitinase depleted BmNPV	188 μ g/ml hemolymph	Ishikiriyama et al. (2009)
Human anti-BSA IgG1	Cysteine protease and chitinase depleted BmNPV	36 μ g per larva	Park et al. (2009)
Human α 2,6-sialyltransferase	Cysteine protease and chitinase depleted BmNPV	2.2 mg/11 larvae (after purification)	Ogata et al. (2009b)
Transmembrane proteins			
Human (pro)renin receptor	Cysteine protease depleted BmNPV	31 μ g per larva (after purification)	Du et al. (2008)
Human prorenin-(pro)renin receptor complex	Cysteine protease depleted BmNPV	70 μ g/15 larvae (after purification)	Du et al. (2009b)

promoter. Efficient production of undegraded firefly luciferase was achieved (Suzuki et al. 1997). Using this BmNPV-*CP*⁻ strain, 4.6 mg of bovine interferon- τ was purified from the hemolymph of 100 silkworm larvae (Nagaya et al. 2004). This expression level is at least tenfold higher than those of other expression systems. Moreover, insect-derived cellulose was expressed using BmNPV in which the cysteine protease and chitinase genes (BmNPV-*CP*⁻-*Chi*⁻) were deleted, to suppress larvae liquefaction caused by baculovirus infection (Lee et al. 2006).

Several rounds of virus amplification steps are needed for BmNPV because the virus titer from *B. mori* cells is lower than that of Sf-9 cells, and it is more difficult to obtain a high titer of BmNPV than *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which is generally used in the baculovirus expression system. To amplify BmNPV rapidly, a hybrid virus of AcMNPV and BmNPV was

isolated and applied to the expression of recombinant proteins in silkworm larvae (Mori et al. 1992). A BmScH DNA fragment, the 572-bp *SacI*-*HindIII* fragment of the DNA helicase gene from BmNPV, determines the host specificity of BmNPV, and AcMNPV with the recombinant BmScH DNA fragment from BmNPV can infect BmN cells (Maeda et al. 1993). Two adjacent nucleotides (A and T) are the minimal essential sequence necessary to expand the host range of AcMNPV (Kamita and Maeda 1997). Muneta et al. (2004) reported that 500 μ g of bovine interleukin-21 (IL-21) was purified from 30 ml of hemolymph using a hybrid baculovirus. Silkworm pupae have also been used as a bioreactor for recombinant protein production. Pupae can survive at 4°C for a long time and do not require mulberry leaves or an artificial diet during their growth. Human granulocyte macrophage colony-stimulating factor (hGM-CSF) was produced in pupae and purified from a homogenate (Chen et al. 2006). The hGM-CSF expressed in pupae

had a molecular mass of 29 kDa, which is greater than that produced in the hemolymph of silkworm larvae. Moreover, the hGM-CSF expressed in pupae was glycosylated without its signal peptide. These results suggest that some comodification and postmodifications are different between silkworm larvae and pupae.

BmNPV bacmid development and its applications

To construct a recombinant baculovirus containing a gene of interest, cultured cells must be transfected with the baculovirus and the transfer vector. Moreover, this system requires the isolation and amplification of the recombinant baculovirus. It takes a long time (3–6 months) to prepare the high-titer recombinant baculovirus solution, and these steps are very tedious and troublesome. A novel system for the efficient production of recombinant AcMNPV was reported, which is based on site-specific transposition in *Escherichia coli* (Luckow et al. 1993). Luckow and colleagues constructed a recombinant baculovirus vector (bacmid) that can replicate in *E. coli* as a large plasmid. This system is known as the Bac-to-Bac baculovirus expression system, and various kits based on this Bac-to-Bac system have been distributed from Invitrogen Corp. This system reduces the time required to isolate and purify a recombinant baculovirus and allows the simultaneous construction of multiple recombinant baculoviruses.

Motohashi et al. (2005) constructed a BmNPV bacmid and established the Bac-to-Bac system using BmNPV. GFP_{uv} was expressed with only the injection of BmNPV bacmid DNA into silkworm larvae and pupae. This bacmid system provided rapid expression of recombinant proteins, since it did not require the preparation of a baculovirus solution by transfection, as compared to the baculovirus expression system using cultured cells (Fig. 1). Furthermore, this BmNPV bacmid system dramatically reduced the time needed for recombinant protein production by silkworm expression.

Improved protein expression by a modified BmNPV bacmid

Several proteins were produced using this BmNPV bacmid in silkworm larvae (Yue et al. 2006; Zhang et al. 2008). However, the degradation of recombinant proteins by baculoviral cysteine protease also occurred (Park et al. 2007). Therefore, the BmNPV-CP⁻ bacmid was constructed, and the GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase2 (β3GnT2) fusion protein (GGT2) was expressed successfully using this bacmid (Hiyoshi et al. 2007). The protease activity in the hemolymph of silkworm larvae injected with

the BmNPV-CP⁻ bacmid was reduced by 85%, as compared to that in the hemolymph of BmNPV-bacmid-injected silkworm larvae. GGT2 degradation was also suppressed by using the BmNPV-CP⁻ bacmid, and the β3GnT activity was improved by 30%. Moreover, the β3GnT activity of the hemolymph was improved 2.8-fold by using the BmNPV-CP⁻-Chi⁻ bacmid over that obtained with the unmodified BmNPV bacmid (Park et al. 2008). Liquefaction of larvae was delayed by using the BmNPV-CP⁻ and BmNPV-CP⁻-Chi⁻ bacmids (Fig. 2). The expression level of human anti-bovine-serum-albumin (BSA) single-chain Fv (scFv) fragment in hemolymph was 3.8–4.3-fold higher with the BmNPV-CP⁻ or BmNPV-CP⁻-Chi⁻ bacmid than that with the unmodified BmNPV because of the suppression of degradation by the cysteine protease from BmNPV (Fig. 2). However, no difference in the scFv expression levels between the modified and unmodified bacmids was observed in pupae (Ishikiriya et al. 2009). This result suggests that the baculoviral cysteine protease, v-cath, may not be activated or only a small portion of the cysteine protease may be expressed in pupae. Pupae may be useful for the production of cysteine protease-sensitive proteins.

A hybrid nucleopolyhedrovirus (HyNPV) bacmid was also constructed, by cotransfection with the AcMNPV bacmid and pUC18/BmScH containing the BmScH fragment of the DNA helicase gene from BmNPV (Deo et al. 2006). This bacmid allows baculoviral amplification in AcMNPV and protein expression in BmNPV. To obtain a high virus titer, this HyNPV bacmid is very helpful.

Molecular chaperones play an important role in protein folding at the endoplasmic reticulum. Bacmids allow easy coexpression of a chaperone with a target protein. The expression level of a recombinant GFP_{uv}-human α1,4-*N*-acetylglucosaminyltransferase (α4GnT) fusion protein was enhanced in silkworm larvae, by coexpression of a human molecular chaperone, human calnexin (CNX), or immunoglobulin heavy-chain binding protein (BiP; Nakajima et al. 2009). The expression of CNX and BiP under the control of the polyhedrin promoter enhanced the α4GnT activity by 1.4- and 2.0-fold, respectively. When CNX or BiP under the control of the *ie-2* promoter was expressed during the early stage of baculovirus infection, the α4GnT activity in the hemolymph was also enhanced significantly. In silkworm larvae, the coexpression of a chaperone with the target protein effectively increases the quality of the recombinant protein.

Protein production using transgenic silkworms

Transgenic silkworms have also been used for recombinant protein production. To establish transgenic silkworms, two different systems, involving the use of an attenuated recombinant baculovirus or a *piggylBac* transposon-derived

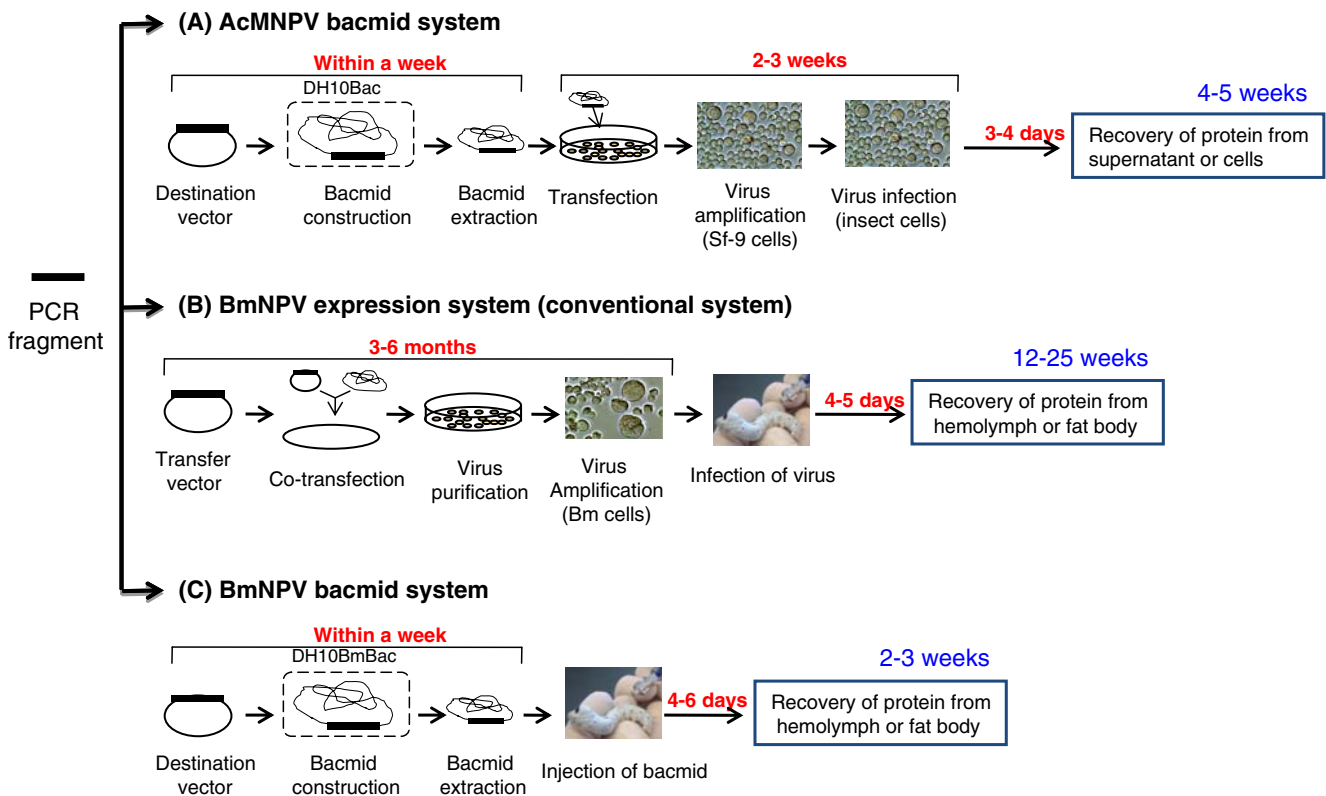


Fig. 1 Baculovirus gene expression system in insect cells and silkworm larvae. **a** AcMNPV expression system using the AcMNPV bacmid. The target gene was incorporated into the AcMNPV bacmid. This extracted recombinant AcMNPV bacmid from *E. coli* was transfected into insect cells. The resulting recombinant AcMNPV was purified, amplified, and increased in titer and then was used for

insect cell infection. **b** Conventional BmNPV expression system. The target gene and the wild-type BmNPV gene were cotransfected into *B. mori* cells, and the recombinant BmNPV was obtained. This recombinant BmNPV bacmid from *E. coli* was injected directly into silkworm larvae. After 4–6 days postinjection, the recombinant protein was harvested

vector, were adopted (Tamura et al. 2000; Yamao et al. 1999). A method combining the two systems was also established (Yamamoto et al. 2004). Human type III procollagen and feline interferon were produced in cocoons using transgenic silkworms (Kurihara et al. 2007; Tomita et al. 2003). Human μ -opioid receptor was expressed in the silk glands and fat bodies of transgenic silkworms, which were screened by the *GAL4/UAS* system (Tateno et al. 2009). Its expression level was comparable to that obtained in the baculovirus expression system using Sf-9 cells.

N-glycan structure produced by silkworm larvae

Insect cells can assemble *N*-glycan precursors to produce high mannose or paucimannosidic end products. However, the cells failed to elongate the trimmed *N*-glycan to produce complex products containing terminal galactose and sialic acid (SA) residues (Kost et al. 2005). Enzyme assays revealed that the insect cell lines had little or none of the galactosyltransferase and sialyltransferase activities involved in *N*-glycan elongation.

When human anti-BSA IgG1 was expressed in hemolymph, using the BmNPV-*CP*⁻-*Chi*⁻ bacmid, its main *N*-glycan structure was paucimannosidic (Park et al. 2009). In accordance with this observation, human killer cell Ig-like receptor 2DL1 was expressed using the BmNPV bacmid (see details below), and the protein thus obtained harbored the paucimannose-type oligosaccharides, Man α 1-6Man β 1-4GlcNAc β 1-4GlcNAc and Man α 1-6Man β 1-4GlcNAc β 1-4(Fucal α 1-6)GlcNAc (Sasaki et al. 2009). However, the *N*-glycan of β 3GnT2 purified from the hemolymph of larvae contained terminal *N*-acetylglucosamine (GlcNAc β 1,2Man α 1,3(GlcNAc β 1,4)(Man α 1,6)Man β 1,4GlcNAc β 1,4GlcNA) and galactose residues (Gal β 1,4GlcNAc β 1,2Man α 1,3(GlcNAc β 1,4)(Man α 1,6)Man β 1,4GlcNAc β 1,4GlcNAc), which were not observed in samples from cultured cells (Dojima et al. 2009). These results indicate that β 1,2*N*-acetylglucosaminyltransferase I (GlcNAcT I), β 1,4*N*-galactosyltransferase (GalT), and β 1,4*N*-acetylglucosaminyltransferase III (GlcNAcT III) might exist in silkworm larvae, but not in insect cells (Fig. 3). Therefore, silkworm larvae would be useful for the production of human glycoproteins, if the

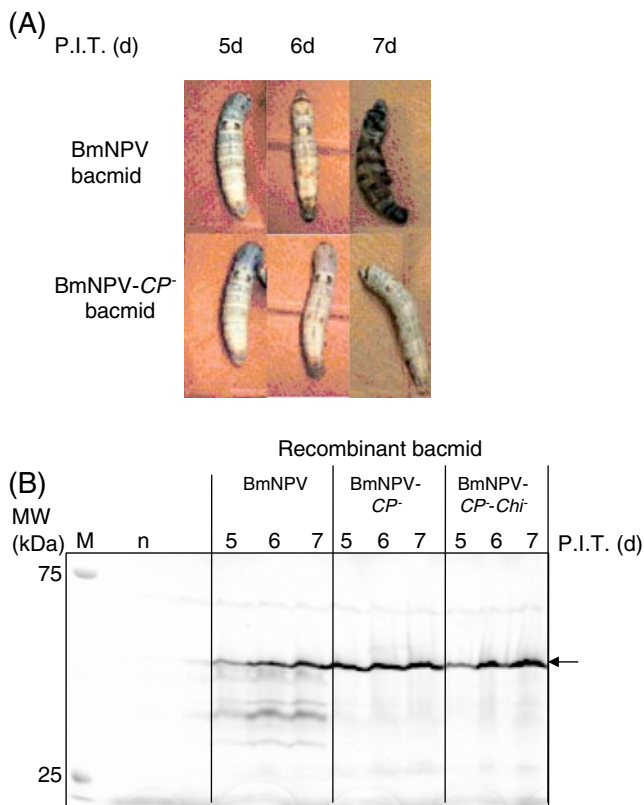


Fig. 2 **a** Comparison of larval liquefaction. Silkworm larvae were injected with BmNPV (WT) and BmNPV-CP⁻ bacmids. **b** GFP_{uv} fluorescence of the GFP_{uv}-13CG2 (scFv) fusion protein expressed in the BmNPV/bx-GFP_{uv}-13CG2-, BmNPV-CP⁻/bx-GFP_{uv}-13CG2-, and BmNPV-CP⁻-Chi⁻/bx-GFP_{uv}-13CG2 bacmid-injected silkworm larvae. Loaded protein concentrations were 65–95 μg. The arrow indicates the molecular mass of the GFP_{uv}-13CG2 fusion protein

glycosylation pathway was reinforced with GlcNAcT II and sialyltransferase.

Expression of glycosyltransferase in silkworm larvae and its application to the synthesis of glycomimetics to block infections by avian and human influenza viruses

Recently, the glycosyltransferase produced by silkworm larvae was applied to the synthesis of sialoglycopolypeptides for blocking influenza virus infection. Influenza viruses infect host cells through the binding of viral hemagglutinins (HAs) to sialoglycoproteins or sialoglycopeptides of the receptors on the host cell surfaces (Suzuki et al. 1986). Since the molecular recognition process leads to the adhesion of the host cells and virus, molecules with a high affinity for the viral HA act as potent inhibitors of infection by influenza viruses (Ogata et al. 2007; Schengrund 2003). The host cell specificity of the virus is dependent not only on the linkage of SAs to the

penultimate galactose but also on the number of SA residues and the precise nature of the core structure (Gambaryan et al. 2004; Yamada et al. 2006). In order to synthesize an artificial sialoglycoprotein, rat α 2,6-sialyltransferase (ST6Gal I) was expressed in fifth-instar silkworm larval hemolymph, using a recombinant bacmid. Approximately 2.2 mg of ST6GalI was purified from the hemolymph of 11 larvae. The substrate specificity of the ST6GalI purified from hemolymph was the same as that of the native rat ST6GalI. By using this ST6GalI, the synthesis of a α 2,6-sialoglycopolypeptide as a glycoprotein mimetic was successfully achieved. The synthesized α 2,6-sialoglycopolypeptide selectively inhibited hemagglutination induced by *Sambucus nigra* lectin, which specifically binds to the Sia α 2-6Gal(GalNAc) structure, and showed approximately 780-fold higher affinity than fetuin as a control (Ogata et al. 2009b). Moreover, Ogata et al. (2009a) designed a series of γ -polyglutamic acid (γ -PGA)-based glycopolypeptides carrying long/short R2,3/6 sialylated glycans to act as inhibitors of the influenza virus. As an alternative design, sialoglycopolypeptides carrying long-spacer-linked glycans were engineered, by the replacement of the *N*-acetylglucosamine (LN) unit by an alkyl chain. The avian viruses specifically bound to glycopolypeptides carrying a short sialoglycan with higher affinity than to a long glycan. In contrast, human viruses preferentially bound not only to a long R2,3/6 sialylated glycan with LN repeats in the receptors but also to more spacer-linked glycans, in which the inner sugar was replaced by a nonsugar structural unit, such as a pentylamido group (Hidari et al. 2007). A spaced tandem/triplet pentylamido repeat is a good mimetic of a tandem/triplet LN repeat and thus provides a facile way to design strong polymeric inhibitors of infection by avian and human influenza viruses (Fig. 4).

Surface display system and subunit vaccine production in silkworm

Baculovirus particles have become valuable tools in biotechnology and medicine. Even a small virus provides a huge surface, which can be engineered in many ways. Heterologous peptides or proteins can be displayed on the surface of baculovirus particles by fusing the peptide or protein to the baculovirus surface glycoprotein, gp64 (Boublik et al. 1995). Baculoviruses displaying gp64 fusion proteins have proven to be very effective immunogens (Kost et al. 2005). Since this approach was first used to raise monoclonal antibodies against the nuclear receptors LXR β and FXR (Lindley et al. 2000), it has been used for the development of low-cost insect larvae-derived recombinant subunit vaccines. These include H5N1 influenza vaccine (Jin et al. 2008), as well as subunit vaccines against rabbit hemorrhagic disease virus (Pérez-Filgueira

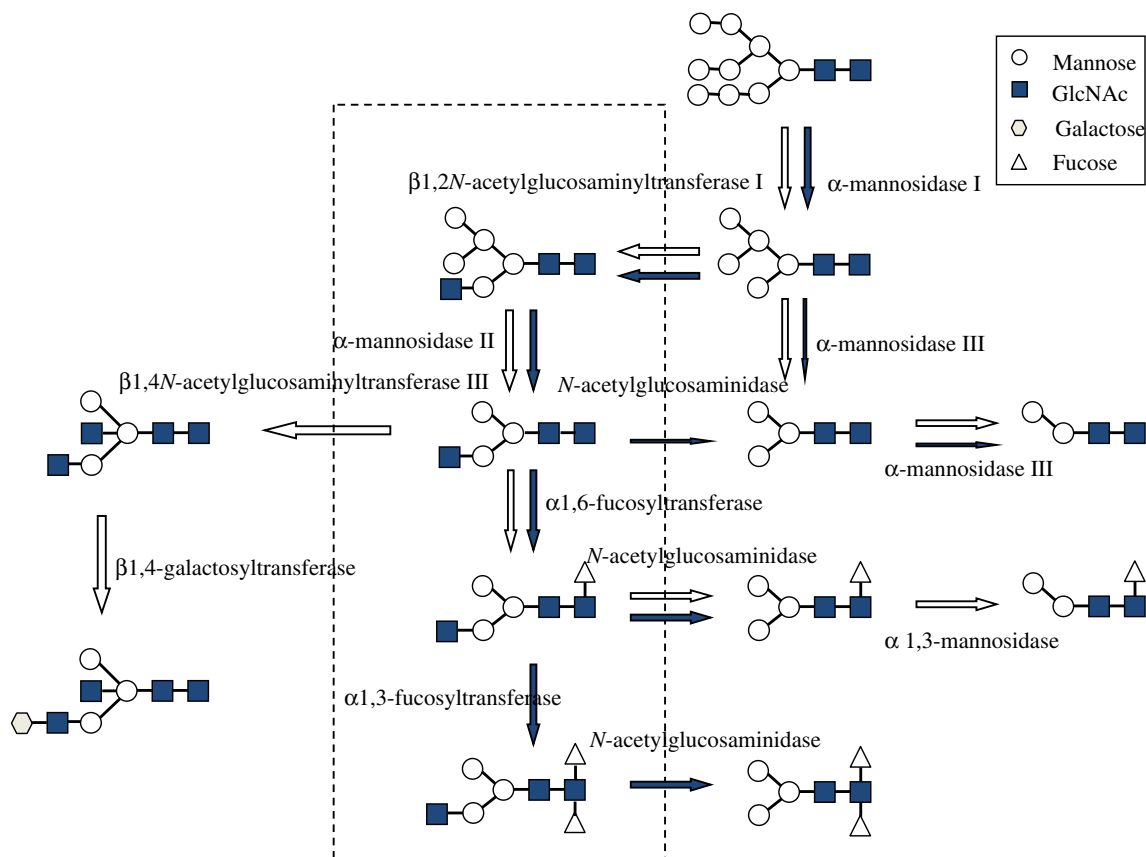
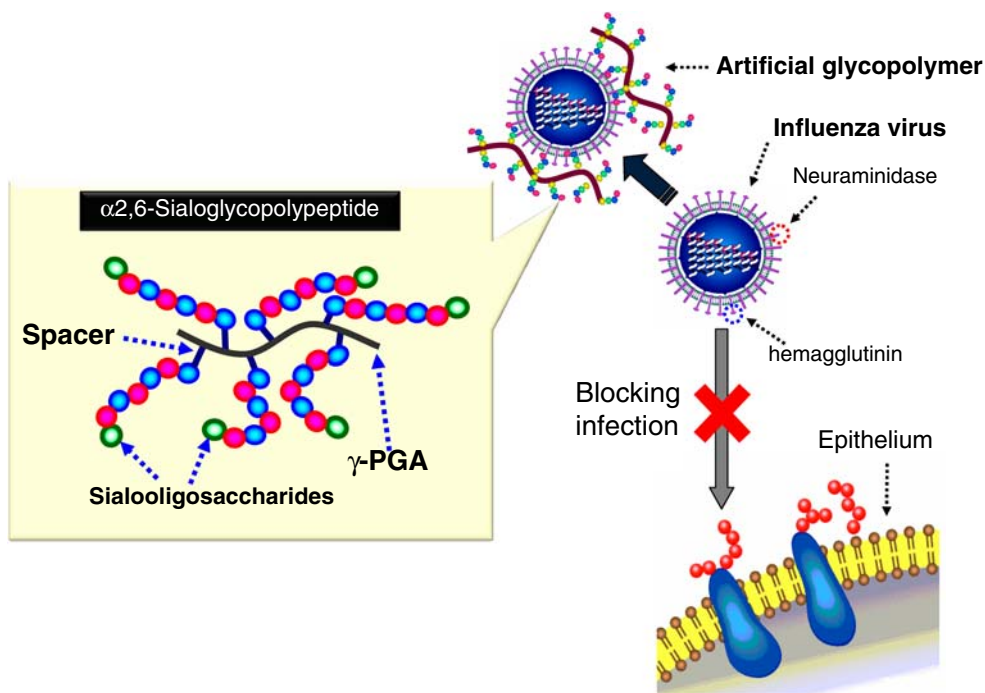


Fig. 3 Proposed *N*-glycan processing pathway in the *T. ni* cell line and silkworm larvae. *Open* and *closed* arrows indicate silkworm larvae and the *T. ni* cell line, respectively. *N*-glycans enclosed by *dotted* lines were not detected by the HPLC mapping

Fig. 4 Blocking influenza virus infection using an α 2,6-sialoglycopolypeptide. The α 2,6-sialoglycopolypeptide was enzymatically synthesized from an (γ -PGA)-based glycopoly-peptides carrying α 2,6-sialylated glycans acted as inhibitors of influenza virus because human viral HAs bind to α 2,6-linked sialosides in the core glycan structures



et al. 2007) and pancreatic necrosis virus (Shivappa et al. 2005).

Recently, Li et al. (2008) reported that the capsid proteins of foot-and-mouth disease virus (FMDV) were produced in the hemolymph of silkworm larvae, and cattle vaccinated with hemolymph, diluted 30-fold, were protected from challenge with FMDV.

The ability of BmNPV to display gp64 fusion proteins also offers the possibility of using this silkworm as a host for the production and display of a wide range of antigenically important proteins on the cell or virus surface. Using silkworm larvae, rather than insect cell lines, as a host for recombinant virus amplification and recombinant protein production has advantages, in terms of their size and easiness to rear. The purified virus particles displaying the cloned foreign protein on the viral envelope could be used directly for functional analysis, without isolating the proteins. Since the presence of the gp64 fusion protein did not alter the growth and yield of the virus, the availability of the gp64-fusion-protein-displayed virus will also provide insight into the virus–host cell interaction, and this system may provide a fundamental platform for large-scale vaccine production. The BmNPV-based system is an economic process for the large-scale production of surface-displayed baculoviruses and safe subunit vaccines for animals, since the silkworm larvae, which are easy to rear on a synthetic or natural diet, can be used instead of the cultured cell lines.

Expression of pharmaceutically relevant proteins, including cell/viral surface proteins

The infection of silkworms with recombinant BmNPV virus is a very attractive strategy for protein production, especially for cell surface receptors and secreted proteins, which often require posttranslational modifications, including sugar modifications. Silkworm expression is used for the expression of pharmaceutically relevant proteins. Immune cell and viral surface molecules and guanine nucleotide-binding protein (G protein) coupled receptors (GPCR) are the main drug targets because they regulate a broad range of physiological functions (Kristiansen 2004). The production of cell surface proteins using the silkworm-BmNPV expression system is summarized in Table 2. The expression levels ranged from several micrograms to 0.5 mg per larva. The proteins normally had signal sequences and thus were secreted in the hemolymph when properly processed. The obtained proteins were basically functional, as confirmed by either ligand binding assays or ELISA. Human killer cell immunoglobulin-like receptor, KIR2DL1, which regulates natural killer cell function by recognizing human leukocyte antigen C molecules, was expressed successfully (Sasaki et al. 2009). The expression level was high, 0.2 mg/larva, either by direct injection of bacmid DNA or by injection of collected body fluid containing the recombinant virus. Furthermore, the protein was easily purified using Ni-NTA affinity chromatography.

Table 2 Expression of cell/viral surface proteins and GPCRs in silkworm

Receptor	Gene transfer	Yield	Expression level	Application	Functional assay	Function	Reference
KIR2DL1	BmNPV Bacmid	~0.2 mg/larva		SPR, sugar characterization		Confirmed	Sasaki et al. 2009
IL4-R α	BmNPV	0.11 mg/ml hemolymph		SPR, sugar characterization		Confirmed	Honjo et al. 2008
IL4-R α -Fc ^a		0.053 mg/ml hemolymph		Gel filtration			
IL13-R α 1		0.55 mg/ml hemolymph					
IL13-R α 1-Fc ^a		0.33 mg/ml hemolymph					
Influenza hemagglutinin	BmNPV	0.4–4 μ g/larva		ELISA		Not confirmed	Sugiura et al. 2001
Nociceptin receptor (G α 1) ^b	BmNPV bacmid		Not determined		[³⁵ S]GTP γ S binding assay (EC ₅₀ =9.3 \pm 3.4 nM)		Kajikawa et al. 2009
μ -Opioid receptors	Transgenic		150–250 ng/larva		[³ H]diprenorphine saturation analysis (K _d =1.4–2.1 nM)		Tateno et al. 2009

Expression of immune cell and viral surface receptors using silkworm

SPR surface plasmon resonance analysis

^a Fc fusion proteins

^b G α -fusion proteins

The sugar modifications of the obtained KIR2DL1 protein revealed that the protein harbored the relatively small paucimannose-type oligosaccharides and thus seemed to be appropriate for structural and binding studies of cell surface receptors.

GPCRs are seven-transmembrane proteins and thus are quite difficult to express due to their low solubility and instability. The expression of a GPCR (human μ -opioid receptor) in silkworms was first reported using a transgenic technique, but the expression level was rather low, and many technical steps were required (Tateno et al. 2009). The expression of other GPCRs using other expression systems including Sf9 and CHO cells was reviewed by Massotte (2003) and Sarramegna et al. (2003). Human GPCR (human nociceptin receptor) expression was reported recently by the simple injection of BmNPV bacmid DNA into the silkworm (Kajikawa et al. 2009). Human nociceptin receptor, which is an inhibitory GPCR for an exogenous opioid peptide, nociceptin, involved in pain control (Meunier 1997), was actually produced as a membrane protein in microsomal fractions of the fat bodies of silkworms. The microsomal fractions including functional GPCRs could be very easily prepared by a simple operation and centrifugation. Interestingly, the protein was also expressed in the BmNPV viral fraction, which was consistent with that previously reported for the GPCR expression in Sf9 cells (Masuda et al. 2003). GPCRs physiologically bind at the cytoplasmic region to a trimeric G protein, composed of alpha ($G\alpha$), beta ($G\beta$), and gamma ($G\gamma$) subunits (Wess 1997). The ligand binding to GPCRs enhances the guanosine triphosphatase (GTPase) activity of the trimeric G protein, resulting in its dissociation from GPCRs by exchanging $G\alpha$ -bound GTP to GDP, which triggers the G-protein-mediated signal transduction (Liebmann and Bohmer 2000). The nociceptin receptor fused with the G protein alpha subunit ($G_i\alpha$), whose ligand recognition can be detected by measuring the [35 S]GTP γ S binding, was also successfully expressed in the same system as the nonfused protein as described above. This expression system exhibited sufficient production for 500 binding assays using the radiolabeled compound, [35 S]GTP γ S. The expression level of the nonfusion type of nociceptin receptor was much higher than that of the fusion type.

Silkworms also have the capacity to produce functional membrane proteins in the same way as secretory proteins. Many membrane proteins have been produced in the hemolymph and pupae of silkworms as secretory proteins. Recently, human (pro)renin receptor (hPRR) and its complex with human prorenin were expressed in silkworm larvae, with the same expression level as some secretory proteins, and were purified from the fat bodies of larvae (Du et al. 2008, 2009a). This hPRR may be helpful for the development of an hPRR blocker.

Conclusions and future developments

The BmNPV bacmid system is innovative and easy to use for producing large amount of recombinant protein, without any laborious techniques. Many BmNPV DNAs for recombinant protein expression can be constructed rapidly by the BmNPV bacmid system, based on the Bac-to-Bac system. Moreover, expression of multi-subunit complexes can be achieved simply by injection of a mixture of BmNPV bacmids into silkworms, without prior virus amplification using cultured cells. The protein expression levels in bacmid-injected silkworm larvae are very high, which reduces the cost of large-scale production. This BmNPV bacmid-silkworm expression strategy is very successful for expression of membrane proteins including GPCRs which are major drug targets. Therefore, this system is very promising and will be useful for high-throughput drug screening of many transmembrane proteins and orphan GPCRs for which physiological ligands have not yet been identified (Takeda et al. 2002; Wise et al. 2004).

In Asian countries, silkworms are abundantly available, and many laboratories have experience in rearing and maintaining larvae. Thus, the opportunity to utilize the long-forgotten resourceful silkworm for producing therapeutically important proteins, vaccines, and biomaterials is here. By the spurt in influenza viruses, vaccines for influenza viruses are being actively produced in insect cell system. More and more patents are being procured for customized insect cell lines and their products are making inroads into the market which until now had been dominated by other production systems. With the genomic elucidation of silkworm, even more laboratories around the world will be interested in its utilization. We expect that the glory of silkworm is returning back, and it will be once more associated with wealth and prosperity.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

- Boublik Y, Bonito PD, Jones IM (1995) Eukaryotic virus display: engineering the major surface glycoprotein of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) for the presentation of foreign proteins on the virus surface. *Bio/Technol* 13:1079–1084
- Chen J, Wu XF, Zhang YZ (2006) Expression, purification and characterization of human GM-CSF using silkworm pupae (*Bombyx mori*) as a bioreactor. *J Biotechnol* 123:236–247

- Deo VK, Hiyoshi M, Park EY (2006) Construction of hybrid *Autographa californica* nuclear polyhedrovirus bacmid by modification of *p143* helicase. *J Virol Methods* 134:212–216
- Dojima T, Nishina T, Kato T, Uno T, Yagi H, Kato K, Park EY (2009) Comparison of the N-linked glycosylation of human β 1,3-N-acetylglucosaminyltransferase 2 expressed in insect cells and silkworm larvae. *J Biotechnol* 143:27–33
- Drosophila 12 Genome Consortium (2007) Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* 450:203–218
- Du D, Kato T, Nurun Nabi AHM, Suzuki F, Park EY (2008) Expression of functional human (pro)renin receptor in silkworm (*Bombyx mori*) larvae using BmMNPV bacmid. *Biotechnol Appl Biochem* 49:195–202
- Du D, Kato T, Suzuki F, Park EY (2009a) Binding affinity of full length and extracellular domains of recombinant human (Pro) renin receptor to human renin when expressed in the fat body and hemolymph of silkworm larvae. *J Biosci Bioeng* 108:304–309
- Du D, Kato T, Suzuki F, Park EY (2009b) Expression of protein complex comprising the human prorenin and (pro)renin receptor in silkworm larvae using *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmids for improving biological function. *Mol Biotechnol* 43:154–161. doi:10.1007/s12033-009-9183-7
- Gambaryan AS, Tuzikov AB, Pazygina GV, Webster RG, Matrosovich MN, Bovin NV (2004) H5N1 chicken influenza viruses display a high binding affinity for Neu5AcR2-3Gal₁-4(6-HSO3)GlcNAc-containing receptors. *Virology* 326:310–316
- Gong ZH, Jin HQ, Jin YF, Zhang YZ (2005) Expression of cholera toxin B subunit and assembly as functional oligomers in silkworm. *J Biochem Mol Biol* 38:717–724
- Han J, Zang Y, Lu H, Zhu J, Qin J (2004) A novel recombinant dual human SCF expressed in and purified from silkworm, *Bombyx mori*, possesses higher bioactivity than recombinant monomeric human SCF. *Eur J Haematol* 72:273–279
- Hidari KI-PJ, Shimada S, Suzuki Y, Suzuki T (2007) Binding kinetics of influenza viruses to sialic acid-containing carbohydrates. *Glycoconj J* 24:583–590
- Hiyoshi M, Kageshima A, Kato T, Park EY (2007) Construction of a cysteine protease deficient *Bombyx mori* multiple nucleopolyhedrovirus bacmid and its application to improve expression of a fusion protein. *J Virol Methods* 144:91–97
- Honjo E, Shoyama Y, Tamada T, Shigematsu H, Hatanaka T, Kanaji S, Arima K, Ito Y, Izuhara K, Kuroki R (2008) Expression of the extracellular region of the human interleukin-4 receptor alpha chain and interleukin-13 receptor alpha chain by a silkworm-baculovirus system. *Protein Expr Purif* 60:25–30
- International Silkworm Genome Consortium (2008) The genome of lepidopteran model insect, the silkworm *Bombyx mori*. *Insect Biochem Mol Biol* 38:1036–1045
- Ishihara K, Satoh I, Nittoh T, Kanaya T, Okazaki H, Suzuki T, Koyama T, Sakamoto T, Ide T, Ohuchi K (1999) Preparation of recombinant rat interleukin-5 by baculovirus expression system and analysis of its biological activities. *Biochim Biophys Acta* 1451:48–58
- Ishikiriyama M, Nishina T, Kato T, Ueda H, Park EY (2009) Human single-chain antibody expression in the hemolymph and fat body of silkworm larvae and pupae using BmNPV bacmids. *J Biosci Bioeng* 107:67–72
- Jin R, Lv Z, Chen Q, Quan Y, Zhang H, Li S, Chen G, Zheng Q, Jin L, Wu X, Chen J, Zhang Y (2008) Safety and immunogenicity of H5N1 influenza vaccine based on baculovirus surface display system of *Bombyx mori*. *PLoS ONE* 3:e3933
- Kadono-Okuda K, Yamamoto M, Higashino Y, Taniaki K, Kato Y, Chowdhury S, Xu J, Choi SK, Sugiyama M, Nakashima K, Maeda S, Yamakawa M (1995) Baculovirus-mediated production of the human growth hormone in larvae of the silkworm, *Bombyx mori*. *Biochem Biophys Res Commun* 213:389–396
- Kajikawa M, Sasaki K, Wakimoto Y, Toyooka M, Motohashi T, Shimojima T, Takeda S, Park EY, Maenaka K (2009) Efficient silkworm expression of human (nociceptin receptor) by a *Bombyx mori* bacmid DNA system. *Biochem Biophys Res Commun* 385:375–379
- Kamita SG, Maeda S (1997) Sequencing of the putative DNA helicase-encoding gene of the *Bombyx mori* nuclear polyhedrosis virus and fine-mapping a region involved in host range expansion. *Gene* 190:173–179
- Kost TA, Contreay JP, Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol* 23:567–575
- Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther* 103:21–80
- Kurihara H, Sezutsu TT, Yamada K (2007) Production of an active feline interferon in the cocoon of transgenic silkworms using the fibroin H-chain expression system. *Biochem Biophys Res Commun* 355:976–980
- Lee KS, Je YH, Woo SD, Sohn HD, Jin BR (2006) Production of a cellulose in silkworm larvae using a recombinant *Bombyx mori* nucleopolyhedrovirus lacking the virus-encoded chitinase and cathepsin genes. *Biotechnol Lett* 28:645–650
- Li Z, Yi Y, Yin X, Zhang Z, Liu J (2008) Expression of foot-and-mouth disease virus capsid proteins in silkworm-baculovirus expression system and its utilization as a subunit vaccine. *PLoS ONE* 3:e2273
- Liebmann C, Bohmer FD (2000) Signal transduction pathways of G protein-coupled receptors and their cross-talk with receptor tyrosine kinases: lessons from bradykinin signaling. *Curr Med Chem* 7:911–943
- Lindley KM, Su JL, Hodges PK, Wisely GB, Bledsoe RK, Condreay JP, Winegar DA, Hutchins JT, Kost TA (2000) Production of monoclonal antibodies using recombinant baculovirus displaying gp64-fusion proteins. *J Immunol Methods* 234:123–135
- Luckow VA, Lee SC, Barry GF, Olins PO (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J Virol* 67:4566–4579
- Maeda S, Kawai T, Obinata M, Fujiwara H, Horiuchi T, Saeki Y, Sato Y, Furusawa M (1985) Production of human α -interferon in silkworm using a baculovirus vector. *Nature* 315:592–594
- Maeda S, Kamita SG, Kondo A (1993) Host range expansion of *Autographa californica* nuclear polyhedrosis virus (NPV) following recombination of a 0.6-kilobase-pair DNA fragment originating from *Bombyx mori* NPV. *J Virol* 67:6234–6238
- Massotte D (2003) G protein-coupled receptor overexpression with the baculovirus-insect cell system: a tool for structural and functional studies. *Biochim Biophys Acta* 1610:77–89
- Masuda K, Itoh H, Sakihama T, Akiyama C, Takahashi K, Fukuda R, Yokomizo T, Shimizu T, Kodama T, Hamakubo T (2003) A combinatorial G protein-coupled receptor reconstitution system on budded baculovirus. Evidence for Galpha and Galphao coupling to a human leukotriene B4 receptor. *J Biol Chem* 278:24552–24562
- Meunier JC (1997) Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. *Eur J Pharmacol* 340:1–15
- Mita K, Morimyo M, Okano K, Koike Y, Nohata J, Kawasaki H, Kadono-Okuda K, Yamamoto K, Suzuki MG, Shimada T, Goldsmith MR, Maeda S (2003) The construction of an EST database for *Bombyx mori* and its application. *Proc Natl Acad Sci U S A* 100:14121–14126

- Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T, Kanamori H, Namiki N, Kitagawa M, Yamashita H, Yasukochi Y, Kadono-Okuda K, Yamamoto K, Ajimura M, Ravikummar G, Shimomura M, Nagamura Y, Shin-i T, Abe H, Shimada T, Morishita S, Sasaki T (2004) The genome sequence of silkworm, *Bombyx mori*. DNA Res 11:27–35
- Miyajima A, Schreurs J, Otsu K, Kondo A, Arai K, Maeda S (1987) Use of the silkworm, *Bombyx mori*, and an insect baculovirus vector for high-level of expression and secretion of biologically active mouse interleukin-3. Gene 58:273–281
- Mori H, Nakazawa H, Shirai N, Shibata N, Sumida M, Matsubara F (1992) Foreign gene expression by a baculovirus vector with an expanded host range. J Gen Virol 73:1877–1880
- Motohashi T, Shimojima T, Fukagawa T, Maenaka K, Park EY (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
- Muneta Y, Nagaya H, Minagawa Yu, Enomoto C, Matsumoto S, Mori Y (2004) Expression and one-step purification of bovine interleukin-21 (IL-21) in silkworm using a hybrid baculovirus expression system. Biotechnol Lett 26:1453–1458
- Nagaya H, Kanaya T, Kaki H, Tobita Y, Takahashi M, Takahashi H, Yokomizo Y, Inumaru S (2004) Establishment of a large-scale purification procedure for purified recombinant bovine interferon- τ produced by a silkworm-baculovirus gene expression system. J Vet Med Sci 66:1395–1401
- Nakajima M, Kato T, Kanamasa S, Park EY (2009) Molecular chaperone-assisted production of human α -1,4-N-acetylglucosaminyltransferase in silkworm larvae using recombinant BmNPV bacmid. Mol Biotechnol 43:67–75
- Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, RV Bruggner, J Costas, Coy MR, Crabtree J, Crawford M, Debruyne B, Decaprio D, Eglmeier K, Eisenstadt E, El-Dorri H, Gelbart WM, Gomes SL, Hammond M, Hannick L, Hogan JR, Holmes MH, Jaffe D, Johnson JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, Labutti K, Lee E, Li S, Lovin DD, Mao C, Mauceli E, Menck CF, Miller JR, Montgomery P, Mori A, Nascimento AL, Naveira HF, Nusbaum C, O’leary S, Orvis J, Perteua M, Quesneville H, Reidenbach KR, Rogers YH, Roth CW, Schneider JR, Schatz M, Shumway M, Stanke M, Stinson EO, Tubio JM, Vanzee JP, Verjovski-Almeida S, Werner D, White O, Wyder S, Zeng Q, Zhao Q, Zhao Y, Hill CA, Raikhel AS, Soares MB, Knudson DL, Lee NH, Galagan J, Salzberg SL, Paulsen IT, Dimopoulos G, Collins FH, Birren B, Fraser-Liggett CM, Severson DW (2007) Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 318:1718–1723
- Ogata M, Murata T, Murakami K, Suzuki T, I-PJ HK, Suzuki Y, Usui T (2007) Chemoenzymatic synthesis of artificial glycopolypeptides containing multivalent sialyl oligosaccharides with a γ -polyglutamic acid backbone and their effect on inhibition of infection by influenza viruses. Bioorg Med Chem 15:1383–1393
- Ogata M, I-PJ HK, Murata T, Shimada S, Kozaki W, Park EY, Suzuki T, Usui T (2009a) Chemoenzymatic synthesis of sialoglycopolypeptides as glycomimetics to block infection by avian and human influenza viruses. Bioconjugate Chem 20:539–549
- Ogata M, Nakajima M, Kato T, Obara T, Yagi H, Kato K, Usui T, Park EY (2009b) Synthesis of sialoglycopolypeptide for potentially blocking influenza virus infection using a rat α 2,6-sialyltransferase expressed in BmNPV bacmid-injected silkworm larvae. BMC Biotechnol 9:54
- Palhan VB, Sumathy S, Gopinathan KP (1995) Baculovirus mediated high-level expression of luciferase in silkworm cells and larvae. Biotechniques 19(100):102–104
- Park EY, Kageshima A, Kwon MS, Kato T (2007) Enhanced production of secretory *Bombyx mori* larvae using recombinant BmNPV bacmid integrated signal sequence. J Biotechnol 129:681–688
- Park EY, Abe T, Kato T (2008) Improved expression of fusion protein using cysteine-protease-and chitinase-deficient *Bombyx mori* (silkworm) multiple nucleopolyhedrovirus bacmid in silkworm larvae. Biotechnol Appl Biochem 49:135–140
- Park EY, Ishikiriya M, Nishina T, Kato T, Yagi H, Kato K, Ueda H (2009) Human IgG1 expression in silkworm larval hemolymph using BmNPV bacmid and its N-linked glycan structure. J Biotechnol 139:108–114
- Pérez-Filgueira DM, Resino-Talaván P, Cubillos C, Angulo I, Barderas MG, Barcena J, Escribano JM (2007) Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. Virol 364:422–430
- Qiu P, Ding Y, Qin J, Han KK, Zhu D (1994) Expression of biologically active monomeric form of human M-CSF in baculovirus infected silkworm, *Bombyx mori*. Biol Chem Hoppe Seyler 375:413–418
- Sarramegna V, Talmont F, Demange P, Milon A (2003) Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification. Cell Mol Life Sci 60:1529–1546
- Sasaki K, Kajikawa M, Kuroki K, Motohashi T, Shimojima T, Park EY, Kondo S, Yagi H, Kato K, Maenaka K (2009) Silkworm expression and sugar profiling of human immune cell surface receptor, KIR2DL1. Biochem Biophys Res Commun 387:575–380
- Schengrund C-L (2003) “Multivalent” saccharides: development of new approaches for inhibiting the effects of glycosphingolipid-binding pathogens. Biochem Pharmacol 65:699–707
- Shivappa RB, McAllister PE, Edwards GH, Santi N, Evensen Ø, Vakharia VN (2005) Development of a subunit vaccine for infectious pancreatic necrosis virus using a baculovirus insect/larvae system. Dev Biol Basel Karger 121:165–174
- Sugiura T, Sugita S, Imagawa H, Kanaya T, Ishiyama S, Saeki N, Uchiyama A, Tanigawa M, Kuwano A (2001) Serological diagnosis of equine influenza using the hemagglutinin protein produced in a baculovirus expression system. J Virol Methods 98:1–8
- Suzuki Y, Nagano Y, Kato H, Matsumoto M, Nerome K, Nakajima K, Nobusawa E (1986) Human influenza A virus hemagglutinin distinguishes sialyl oligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection. J Biol Chem 261:17057–17061
- Suzuki T, Kanaya T, Okazaki H, Ogawa K, Usami A, Watanabe H, Kadono-Okuda K, Yamakawa M, Sato H, Mori H, Takahashi S, Oda K (1997) Efficient protein production using a *Bombyx mori* nuclear polyhedrosis virus lacking the cysteine proteinase gene. J Gen Virol 78:3073–3080
- Takeda S, Kadowaki S, Haga T, Takaesu H, Mitaku S (2002) Identification of G protein-coupled receptor genes from the human genome sequence. FEBS Lett 520:97–101
- Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P (2000) Germ line transformation of the silkworm *Bombyx mori* L. using *piggyBac* transposon-derived vector. Nat Biotechnol 18:81–84
- Tateno M, Toyooka M, Shikano Y, Takeda S, Kuwabara N, Sezutsu H, Tamura T (2009) Production and characterization of the recombinant human μ -opioid receptor from transgenic silkworms. J Biochem 145:37–42

- Tomita M, Munetsuna H, Sato T, Adachi T, Hino R, Hayashi M, Shimizu NN, Tamura T, Yoshizato K (2003) Transgenic silkworms produce recombinant human type III procollagen in cocoons. *Nat Biotechnol* 21:52–56
- Wang Y, Wu X, Liu G, Cao C, Huang H, Xu Z, Liu J (2005) Expression and porcine lactoferrin by using recombinant baculovirus in silkworm, *Bombyx mori* L., and its purification and characterization. *Appl Microbiol Biotechnol* 69:385–389
- Wei WL, Qin JC, Sun MJ (2000) High-level expression of human butyrylcholinesterase gene in *Bombyx mori* and biochemical–pharmacological characteristic study of its product. *Biochem Pharmacol* 60:121–126
- Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J* 11:346–354
- Wise A, Jupe SC, Rees S (2004) The identification of ligands at orphan G-protein coupled receptors. *Annu Rev Pharmacol Toxicol* 44:43–66
- Xia Q, Zhou Z, Lu C, Cheng T, Dai F, Li B, Zhao P, Zha X, Cheng T, Chai C, Pan G, Xu J, Liu C, Lin Y, Qian J, Hou Y, Wu Z, Li G, Pan M, Li C, Shen Y, Lan X, Yuan L, Li T, Xu H, Yang G, Wan Y, Zhu Y, Yu M, Shen W, Wu D, Xiang Z, Yu J, Wang J, Li R, Shi J, Li H, Li G, Su J, Wang X, Li G, Zhang Z, Wu Q, Li J, Zhang Q, Wei N, Xu J, Sun H, Dong L, Liu D, Zhao S, Zhao X, Meng Q, Lan F, Huang X, Li Y, Fang L, Li C, Li D, Sun Y, Zhang Z, Yang Z, Huang Y, Xi Y, Qi Q, He D, Huang H, Zhang X, Wang Z, Li W, Cao Y, Yu Y, Yu H, Li J, Ye J, Chen H, Zhou Y, Liu B, Wang J, Ye J, Ji H, Li S, Ni P, Zhang J, Zhang Y, Zheng H, Mao B, Wang W, Ye C, Li S, Wang J, Wong GKS, Yang H (2004) A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*). *Science* 306:1937–1940
- Xia Q, Cheng D, Duan J, Wang G, Cheng T, Zha X, Liu C, Zhao P, Dai F, Zhang Z, He N, Zhang L, Xiang Z (2007) Microarray-based gene expression profile in multiple tissue of the domesticated silkworm *Bombyx mori*. *Genome Biol* 8:R162
- Yamada S, Suzuki Y, Suzuki T, Li MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Hiromoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y (2006) Hemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. *Nature* 444:378–382
- Yamamoto M, Yamao M, Nishiyama H, Sugihara S, Nagaoka S, Tomita M, Yoshizato K, Tamura T, Mori H (2004) New and highly efficient method for silkworm transgenesis using *Autographa californica* nucleopolyhedrovirus and *piggyBac* transposable elements. *Biotechnol Bioeng* 88:849–853
- Yamao M, Katayama N, Nakazawa H, Yamakawa M, Hayashi Y, Hara S, Kamei K, Mori H (1999) Gene targeting in the silkworm by use of a baculovirus. *Genes Dev* 13:511–516
- Yue W, Miao Y, Li X, Wu X, Zhao A, Nakagaki M (2006) Cloning and expression of manganese superoxide dismutase of the silkworm, *Bombyx mori* by Bac-to-Bac/BmNPV baculovirus expression system. *Appl Microbiol Biotechnol* 73:181–186
- Zhang Y, Hu J, Miao Y, Zhao A, Zhao T, Wu D, Liang L, Miiikura A, Shiomi K, Kajiura Z, Nakagaki M (2008) Expression of EGFP-spider dragline silk fusion protein in BmN cells and larvae of silkworm showed the solubility is primary limit for dragline protein yield. *Mol Biol Rep* 35:329–335
- Zhang Y, Xia Q, Xu J, Chen J, Nie Z, Wang D, Zhang W, Chen J, Zheng Q, Chen Q, Kong L, Ren X, Wang J, Lv Z, Yu Wei, Jiang C, Liu, L, Sheng Q, Jin Y, Wu X (2009) Aligning the proteome and genome of the silkworm, *Bombyx mori*. *Funct Inter Genomics*. doi:10.1007/s10142-009-0127-x