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Silkworm Baculovirus Expression System for Molecular Medicine

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Abstract

The characterization of immunologically or biologically relevant proteins is prerequisite for the development of effective drugs or therapies. Since large amounts of proteins are often required for this purpose, more effective expression systems for recombinant human proteins must be developed. Among the various expression systems, the baculovirus expression system (BEVS) is an attractive tool with certain advantages for high-level expression and post-translational modifications.

The silkworm BEVS utilizes silkworm larvae or pupae as bioreactors programmed by recombinant Bombyx mori nucleopolyhedrovirus (BmNPV) or bacmid DNA. Recombinant human protein production is generally higher in silkworm BEVS than insect cell line BEVS. In addition, transgenic silkworm technology has been developed for stable recombinant protein expression. In this review, we introduce the use of this silkworm BEVS for two human proteins that are potential targets for drug development, the ectodomains of immunoreceptors (i.e. IL-4Ra, IL13Ra, KIR2DL1 and Fas) and guanine nucleotide-binding protein (G-protein) coupled receptors (GPCR) (i.e. μ-opioid receptor and nociceptin receptor). These proteins were successfully expressed in the silkworm BEVS, showing the advantages in both the expression level and simpler manipulation, as compared to other systems. The silkworm BEVS is expected to be convenient for recombinant protein production for molecular medical studies.

Keywords: BEVS; BmNPV: Bacmid DNA; Silkworm; Immunoreceptors; GPCR

Recombinant Protein Production for Biomedical Sciences

The rapid evolution of biotechnology in recent years has enabled the identification of various proteins responsible for several diseases. To develop drugs or therapies targeting these proteins, precise understanding of their functions is crucially important. Therefore, many researchers are utilizing combinations of several methods to characterize the molecular basis of the functions of these proteins. However, such biomedical research generally requires large amounts of proteins, and thus efficient protein production is becoming more of a concern. In addition, medically important proteins themselves are expected to be employed as biopharmaceuticals [1]. In this regard, efficient recombinant protein production will presumably reduce the cost.

Among the several recombinant protein expression systems currently available, the *Escherichia coli* (*E. coli*) expression system is one of the attractive tools for large-scale recombinant protein expression. This system has the advantages of low cost, simplicity, and high expression level; however, it also has some disadvantages for the expression of many human proteins, such as the lack of posttranslational modifications (i.e. intramolecular disulfide-bond, glycosylation, and phosphorylation). Recently, some improved *E. coli* systems with phosphorylation or disulfide-bond ability have been developed [2], but they are not always successful. On the other hand, eukaryotic cells, including mammalian cells (i.e. human 293, hamster CHO etc.) and yeast (i.e. *Pichia pastoris, Saccharomyces cerevisiae* etc.), are appropriate hosts for the production of these "difficult-to-express" proteins [3-6]. Many human proteins with post-translational modifications and proper conformations have been produced by these systems. However, the costs are usually quite high and the expression levels are generally low.

Among the available expression systems, the baculovirus expression vector system (BEVS) has many advantages for the expression of these proteins, including 1) high level expression by strong promoters (Polyhedrin and P10), 2) post-translational modifications similar to those generated by mammalian cell expression, and 3) more reasonable cost than mammalian cell culture. The commercially available BEVS utilizes Autographa californica nucleopolyhedrovirus (AcNPV) and insect cell lines (High five™, Sf9 and Sf21) [7]. This system employs a very useful bacmid DNA comprising the AcNPV DNA genome with the *E. coli* origin and transposition sequences. Therefore, we can construct recombinant AcNPV viruses by simple molecular biological techniques, because the AcNPV bacmid DNA can replicate in E. coli, and the recombinant virus can be expressed by direct transfection into an insect cell line. However, this still requires the time-consuming procedures of virus amplification, handling, and large-scale cultivation.

Silkworm Expression System

The silkworm (Bombyx mori) expression system is a BEVS that uses the silkworm, in place of cell lines, as a bioreactor for the production of recombinant proteins. A quarter-century ago, Maeda et al. [8] reported the secreted production of human interferon α in the haemolymph of silkworm larvae, using recombinant Bombyx mori nucleopolyhedrovirus (BmNPV) encoding human α-interferon driven by the Polyhedrin promoter, as in the AcNPV system. That was the first report of the recombinant production of a medically relevant protein in

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silkworm. The expression levels of recombinant proteins in silkworm are generally higher than those in cultured cells [9], and this provides the biggest advantage. Moreover, the procedures required for this system are relatively easy, because the silkworm larvae do not require sterile facilities and large-scale cultivation in flasks or culture bags. The recent progress in artificial diet production is also helpful for feeding larvae, and eliminates the need for prior experience in silkworm breeding and cultivation.

However, the construction, amplification, and purification of recombinant BmNPV virus using the silkworm cell line are also time-consuming, and require specific techniques as well as the AcNPV system. As a solution to this problem, transgenic silkworm technology is available without virus handling, for the stable expression of recombinant proteins [10], although it requires special skills and substantial time is needed to establish the transgenic lines.

We recently developed BmNPV bacmid DNA systems, based on BmNPV genomic DNA, to solve these labor problems [11-14]. The BmNPV bacmid DNA is the simplest tool among silkworm systems, because the recombinant proteins are expressed in the silkworm by the direct injection of recombinant bacmid DNA in larvae or pupae, without the preparation of a recombinant BmNPV virus [11].

In order to use recombinant proteins in biomedical studies, the profiling of post-translational N-glycosylation is an important analysis. The population of N-linked glycans on recombinant proteins expressed by silkworms is often investigated. Misaki et al. [15] reported that mouse interferon-β, expressed in silkworm larvae using the BmNPV virus, had variable sets of high and paucimannose-type N-linked sugars. On the other hand, in the case of the BmNPV-bacmid expression system, Ishikiriyama et al. [16] demonstrated that the IgG protein consisted of only two paucimannose-type oligosaccharides, Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (77.5%) and Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (12.7 %), and Sasaki et al. [17] showed that the human KIR2DL1 ectodomain also had only two paucimannose-types, Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc and Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc. Thus, these sugar profiles on proteins expressed in the silkworm are nearly identical to those produced by other insect cell lines [18,19]. In human, sialylated complex type N-linked glycans are predominant in glycoproteins, while proteins expressed in insect cells and silkworm lack the sialylated complex, but have high-mannose or paucimannose-type oligosaccharides [19]. Glycosylation assists in both the adoption of the proper conformation and stabilization after purification. However, if glycosylation is not necessary for their functions, then the recombinant proteins with heterologous glycans will have endogenous activity. In addition, the small and relatively homogeneous N-linked sugar modifications in the BmNPV bacmid-silkworm system would be an advantage for structural studies.

The silkworm is expected to become one of the attractive systems for the expression of recombinant proteins with posttranslational modifications for biomedical sciences, and in fact, many human proteins have already been expressed using silkworm [9,20]. The following three silkworm systems are available now: 1) infection with recombinant BmNPV virus, 2) injection of BmNPV bacmid DNA, and 3) construction of a transgenic silkworm (Figure 1). In this review, we describe the successful silkworm expression of the following typical biomedical targets, the ectodomains of human immunoreceptors and human guanine nucleotide-binding protein (G-protein) coupled receptors (GPCRs).

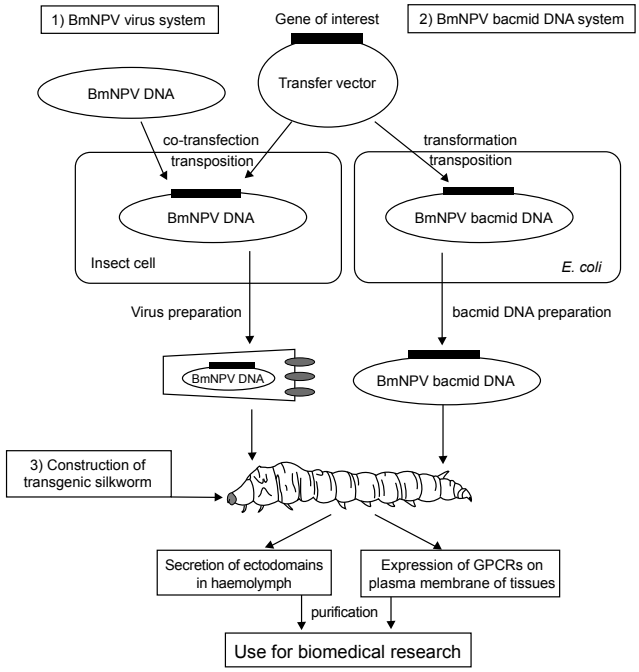

Figure 1: Schematic representation of silkworm expression system. Following three methods are available:1) BmNPV virus infection, 2) BmNPV bacmid injection, and 3) construction of transgenic silkworm. Pupae are also used instead of larvae for the recombinant production of membrane proteins or intracellular proteins.

Ectodomains of Immunoreceptors

Human immunoreceptors expressed on the cell surface regulate various immunological functions via specific binding to their physiological ligands. Immunoreceptors are often directly involved in immune-related diseases. Therefore, they are important for drug development, and moreover, they have potential not only as effective immunotherapeutic targets but also as biopharmaceuticals by themselves. In fact, the soluble ectodomains of such immunoreceptors or specific antibodies against them could become attractive biopharmaceutical drugs for immune regulation [21-23]. However, it is generally difficult to obtain sufficient amounts of functional immunoreceptors for biomedical research, because they frequently require some posttranslational modifications, such as glycosylation, as described above. Moreover, even if these modifications are not required, many immunoreceptor ectodomains require intramolecular-disulfide bonds to form the proper conformation; therefore, these proteins are expressed as inclusion bodies in the reductive environment of the *E. coli* cytoplasm. In this case, an appropriate refolding procedure is required to utilize these proteins in subsequent experiments. On the other hand, the BEVS are quite useful for the preparation of post-translationally modified recombinant ectodomains of immunoreceptors. We will introduce successful examples of the use of the silkworm system for the expression of immunoreceptor ectodomains.

Interleukin-13 Receptor Using BmNPV Virus

Interleukin (IL)-4Rα and IL-13Rα1 form a heterodimeric receptor on the surface of immune cells, and mediate signal transduction to regulate inflammatory responses [24,25]. In 2008, Honjo et al. [26] reported the expression and purification of the extracellular region of the human IL-4 receptor α chain (IL-4Rα) and human IL-13 receptor α1

chain (IL-13Rα1) by a silkworm-baculovirus system. They constructed the recombinant BmNPV virus [27] encoding the cDNA of IL-4Rα or IL13Rα1 with the Fc domain of murine IgG2a, and injected it into the body cavity of silkworm larvae. Each recombinant protein was purified from haemolymph by sequential chromatography using Protein-A resin and anion exchange resin. Finally, 0.19 mg of IL13Rα1 and 0.014 mg of IL-4Rα without the Fc domain were successfully purified from 1 ml of haemolymph (Table 1). As reported previously [28], these receptors formed a heterodimer and bound to IL-13. In addition, a surface plasmon resonance (SPR) analysis was performed, and the K_d value of IL-13 against IL-13Rα1-Fc was determined (2.3 nM) [26]. This was consistent with the previously reported K_d value (≈ 4 nM) calculated by a radiolabeled IL-13 binding assay, using IL-13Rα1 expressed on mammalian CHO cells [29].

KIR2DL1 Using BmNPV Bacmid DNA

Human killer cell immunoglobulin-like (KIR) family genes, encoding transmembrane-type Ig-like glycoproteins, are expressed on natural killer cells and some T cell subsets [30] and mediate several forms of immune regulation [31]. Our group succeeded in the high-level expression of a KIR family ectodomain, KIR2DL1, by using the BmNPV bacmid DNA-silkworm larvae system [17]. Using one-step affinity chromatography with a hexahistidine tag, ~ 0.2 mg of highly purified KIR2DL1 ectodomain was successfully obtained from the haemolymph of one larva (Table 1). The SPR analysis revealed that the KIR2DL1 bound to its physiological ligand, HLA-Cw4, with a K_d of 8.6 ± 0.69 μM [17]. This was consistent with the previous report, using refolded KIR2DL1 from an E. coli expression and refolding system (K_d ≈ 3.3 μM) [32]. In addition, a circular dichroism spectrum analysis suggested that the secondary structure is β-sheet rich, indicating the proper folding of the KIR2DL1 ectodomain [33]. The high level expression of the KIR2DL1 ectodomain was previously established with the *E. coli* system and refolding technique. However, the silkworm expression system can provide a sufficient amount of protein for several investigations, without large-scale cultivation and refolding.

Fas Receptor Using BmNPV Virus

The Fas receptor (FasR)-Fas ligand (FasL) system is an effective apoptotic system used by immune cells. The recombinant expression of the ectodomains of these proteins, using various expression systems, was previously reported [34-38]. Muraki and Honda [39,40] applied the silkworm expression system to produce the FasR ectodomain by a simple procedure using silkworm larvae. They generated the recombinant BmNPV virus encoding the fusion protein, composed of the human FasR ectodomain and the Fc domain of human IgG1 (hFasRECD-Ig), based on the BmNPV genome lacking the cysteine protease gene [27]. The recombinant virus was injected into silkworm larvae, and hFasRECD-Ig was successfully secreted as a disulfide-linked dimer in the haemolymph. Using protein G affinity chromatography and anion exchange chromatography, they purified 22.5 mg of hFasRECD-Ig from 26 ml of haemolymph (Table 1). As compared with the Sf9 system, the expression level of hFasRECD-Ig in silkworm haemolymph was 150 times higher. Using the recombinant human FasL ectodomain produced from a *Pichia pastoris* expression system [41,42], they confirmed the binding activity of hFasREDC-IG to hFasL by immunoprecipitation and size exclusion chromatography.

These examples of IL-4R, IL13R, KIR2DL1, and FasR expression clearly showed that the BmNPV bacmid DNA-silkworm expression system is quite useful for the efficient production of ectodomains, as secreted, functional recombinant proteins, in comparison with the insect cell expression system.

GPCRs

GPCRs are seven transmembrane-type proteins with cytoplasmic regions that associate with a trimeric G protein, composed of alpha (Gα), beta (Gβ), and gamma (Gγ) subunits [43]. The binding of specific ligands (peptides, lipids, steroids, etc.) to the extracellular region of GPCRs causes the activation of the GTPase activity of the trimeric G protein, which is released from the GPCRs. This dissociation triggers the following signal transduction to induce several biological events related to diseases [44,45]. Thus, although approximately ~50% of commercial drugs target GPCRs [46], the molecular basis of their effects on GPCRs remains unclear.

To develop new effective drugs as agonists or antagonists of GPCR ligands, many researchers are trying to elucidate the molecular basis of drug effects on GPCRs by physicochemical methods, including the determination of three-dimensional structures. In addition, despite the presence of several hundred GPCRs in the human genome [47], the physiological ligands of many GPCRs remain unknown, and thus these GPCRs are designated as "orphan receptors" [48]. To understand orphan GPCRs, many researchers are searching for physiological GPCR ligands by ligand screening assays. For this purpose, large amounts of recombinant GPCRs are often required; however, GPCRs are quite difficult to express, due to their low solubility and instability. To successfully express sufficient amounts of functional GPCRs, several expression hosts have been employed [49]. Among them, the BEVS are a powerful tool for the large-scale expression of functional GPCRs. In fact, the first-determined three-dimensional structure of a human GPCR, β adrenergic receptor, utilized the recombinant protein produced by BEVS with insect cell lines [50-52]. Silkworm technology is expected to be a simpler, user-friendly alternative method to express GPCRs.

Classification	Name	Method	Tissue	Tag	Yield	Ligand-binding affinity (value from other hosts)	Reference
Immunoreceptor	IL4-Rα	virus	haemolymph	Fc	0.19 mg/ml haemolymph	N.D. (Binding activity was confirmed by gel filtration analysis)	[26]
	IL13-Rα	virus	haemolymph	Fc	0.014 mg/ml haemolymph	K_d = 2.3 nM (≈ 4 nM, CHO cells [29])	[26]
	KIR2DL1	bacmid	haemolymph	6xHis	~0.2 mg/larva	K_d = 8.6 ± 0.69 μM (33 μM, *E .coli* [32])	[17]
	FasR	virus	haemolymph	Fc	22.5 mg/26 ml haemolymph	N.D. (Binding activity was confirmed by immunoprecipitation and size exclusion chromatography)	[39]
	FasR	virus	haemolymph	Fc	13.5 mg/25 ml haemolymph	N.D. (Binding activity was confirmed by size-exclusion chromatography)	[40]
GPCR	**u-opioid** receptor	transgenic	fat body	6xHis	150–250 ng/larva*	K_d = 1.4~2.1 nM (0.37 ± 0.09 nM, Sf9 [55])	[54]
	nociceptin receptor	bacmid	fat body	6xHis	N.D.	EC_{50} = 9.3~24 nM (12 nM, Sf9 [62])	[56]

Table 1: Human immunoreceptors and GPCRs expression using silkworm.

μ-Opioid Receptor Using Transgenic Silkworm

In 2009, Tateno et al. [53] generated a transgenic silkworm expressing the μ-opioid receptor, one of the human GPCRs related to the analgesic action of morphine [54], in the silk glands and fat body of larvae. To our knowledge, this is the first report of the successful expression of a recombinant human GPCR in silkworm. The recombinant human μ-opioid receptor exhibited a similar level of diprenorphine-binding activity (K_d = 1.4 ± 0.95 ~ 2.1 ± 1.4 nM) to those produced from Sf9 cells (K_d = 0.37 ± 0.09 nM) and HEK293 cells (K_d = 0.29 ± 0.26 nM) [55]. They estimated that one transgenic larva expresses ~250 ng of μ-opioid receptor (Table 1), equivalent to ~20–30 ml of Sf9 culture. The establishment of transgenic strains is time-consuming and requires laborious technical skills. However, this report clearly showed the potential of the silkworm as an attractive host for the expression of recombinant human GPCRs.

Nociceptin Receptor Using BmNPV Bacmid DNA

Using the BmNPV bacmid system, the human nociceptin receptor was expressed in silkworm larvae [56]. Nociceptin receptor, a member of the opioid receptor family, is expressed in the central nervous system, and its physiological ligand, called nociceptin peptide, binds to the extracellular region to control several neurological responses [57,58]. Upon the injection of the recombinant BmNPV bacmid DNA, silkworm larvae expressed human nociceptin receptor on their fat body. The microsomal fraction expressing recombinant human nociceptin receptor, fused with the Giα subunit at C-terminus [59-62], exhibited [35S] GTPγS-binding activity dependent on the nociceptin concentration. The EC_{50} value (9.3 ± 3.4 nM) was consistent with the previous report using recombinant Giα-fused nociceptin in Sf9 cells (EC_{50} = 12 nM) [62]. The expression level of nociceptin receptor was not determined; however, the microsome fraction from one larva was sufficient to perform 500 ligand-screening assays [56].

Conclusion

The silkworm expression system is a powerful tool for recombinant protein production [9]. In this review, we described the successful expression of human immunoreceptor ectodomains and human GPCRs with posttranslational modifications in silkworm. These proteins are considered to be important targets of biomedical research; however, it is generally difficult to express them by using bacterial and mammalian expression systems. The proteins were expressed in silkworm BEVS at higher expression levels and with simpler methodology than the other BEVS using insect cell lines. Therefore, the silkworm system will open the door to the biomedical analyses of these medicinal targets.

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