J. Biotechnol.

Enhanced production of secretory
 β 1,3-*N*-acetylglucosaminyltransferase 2 fusion protein into
hemolymph of *Bombyx mori* larvae using recombinant BmNPV
bacmid integrated signal sequence

Enoch Y. Park^{1,2*}, Ayano Kageshima¹, Mi-Sun Kwon¹, Tatsuya Kato¹

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

² Department of Bioscience, Graduate School of Science and Technology, Shizuoka
University, 836 Ohya Suruga-ku, Shizuoka 422-8529, Japan;

**Abbreviations:* AcNPV, Autographa californica nuclear polyhedrosis; Bacmid, baculovirus shuttle vector; BES, baculovirus expression system; BmNPV, *Bombyx mori* nucleopolyhedrovirus; *bx*, signal sequence from *Bombyx mori* bombyxin; CBB, Coomassie Brilliant Blue; *ce*, signal sequence from cecropin B; *dh*, signal sequence from silkworm diapause hormone precursor; GFP_{uv}, green fluorescent protein when excited with long-wave UV light; β 3GnT2, β 1,3-*N*-acetylglucosaminyltransferase 2; *gp*, signal sequences from the 64K envelope protein of AcNPV; *jhe*, signal sequences from a silkworm juvenile hormone esterase; *me*, signal sequence from honeybee prepromelittin; PCR, polymerase chain reaction; *ppae*, signal sequence from *B. mori* phenoloxidase-activating enzyme

**Corresponding author.* Tel./fax: +81-54-238-4887. *E-mail address:* yspark@agr.shizuoka.ac.jp (EY Park).

Abstract

The enhanced secretion of β 1,3-*N*-acetylglucosaminyltransferase 2 (β 3GnT2) fusion protein into the hemolymph of *Bombyx mori* larvae was studied using a recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid integrating seven signal sequences. When the BmNPV bacmid encoding the signal sequences from the silkworm *B. mori* bombyxin (*bx*) and *B. mori* prophenoloxidase-activating enzyme (*ppae*) was injected into silkworm larvae, 56.1 and 51.5 mU/ml β 3GnT2, respectively, were secreted into the hemolymph of silkworm larvae. For *bx*, 97.3% of the total β 3GnT2 activity was secreted into hemolymph, and only 1.1% remained in the intestines of silkworm larvae. For *ppae*, 90.8% of the total ~~β 3GnT2 activity~~ β 3GnT activity was secreted to the hemolymph, but 7.8% remained in the intestines of silkworm larvae. Using the BmNPV bacmid encoding *bx*, the amount of secreted β 3GnT2 was 91 μ g per larva, which was 2.5% of the total amount of protein in the hemolymph.

Keywords: *Bombyx mori* nucleopolyhedrovirus; bacmid; *Bombyx mori*; signal peptide; β 1,3-*N*-acetylglucosaminyltransferase

書式変更: フォント : Times New Roman

1. Introduction

The baculovirus expression system (BES) is widely used for the expression of the recombinant proteins at high levels in cultured insect cells, owing to it being driven by the high-power polyhedrin promoter. It also has advantages in expressing eukaryotic proteins, which cannot be expressed to bacterial expression systems, because the insect cells can post-translationally modify onto recombinant proteins similarly to their natural proteins and can be handled easily (Luckow, 1991; Luckow et al., 1993; O'Reilly et al., 1992). There are two baculoviruses used in BES, the *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the *Bombyx mori* nuclear polyhedrosis virus (BmNPV). AcNPV can infect only *Spodoptera frugiperda* and *Trichoplusia ni*, however, BmNPV can only infect the silkworm and its cell lines. The levels of protein expression using the silkworm or its pupa is 10-100-fold higher than that using *B. mori* cells, indicating that the silkworm or its pupa is an optimal system for the mass production of recombinant proteins. From this point of view, BmNPV BES using *B. mori* silkworm larvae or pupae is the most suitable combination for the large-scale production of eukaryotic proteins. However, the conventional preparation of a recombinant baculovirus that express exogenous genes requires a lot of time and effort, because multiple rounds of purification and amplification of viruses are necessary.

Recently, a bacmid (a baculovirus shuttle vector) system has been developed for BmNPV (Motohashi et al., 2005). The BmNPV bacmid can be replicated in *Escherichia coli* as a large plasmid, generates recombinant virus DNA by site-specific transposition in *E. coli*, and remains infectious in insect cells. Because this method eliminates the need for multiple rounds of purification and amplification of viruses, it markedly decreases the

1 technical difficulty and the time required to select and purify recombinant viruses. Using
2 this system, we demonstrated a high-level expression of the green fluorescent protein
3 (GFP) gene in larvae and pupae of the *B. mori* silkworm by directly injecting recombinant
4 bacmid DNA, and in *B. mori* cells by transfecting recombinant bacmid DNA (Motohashi et
5 al., 2005). However, complex proteins are generally not well expressed as biologically
6 functional proteins. The reason for this is unknown, but it is possible that signal peptides
7 might be inefficiently recognized by the protein translocation machinery in insect cells.
8 Tessier et al. (1991) reported that insect cells infected with a baculovirus recombined with
9 the gene encoding propapain fused to the honeybee melittin signal peptide secreted more
10 than fivefold the amount of the papain precursor than those infected the gene encoding a
11 plant signal peptide. We could not express functional human
12 β 1,3-*N*-acetylglucosaminyltransferase 2 (β 3GnT2) using BES without encoding a signal
13 sequence, but could express β 3GnT2 in a functionally active form using BES with
14 encoding the honeybee melittin signal peptide (Kato et al., 2004a). Those secreted proteins
15 represent about 10% of all the proteins in a genome, and have particular values in medical
16 and industrial applications. It is important for an efficient expression of biologically active
17 protein in BES to efficiently and accurately recognize and identify this class of proteins.

18 In this paper, we explore the effect of various signal peptides on the expression of
19 functional human β 3GnT2 in the BmNPV bacmid system. The new processing signals
20 were derived from cecropin B, a native secretory protein of the lepidopteran insect
21 *Hyalophora cecropia* (van Hofsten et al., 1985); a *B. mori* juvenile hormone esterase (Hirai
22 et al., 2002); bombyxin, an insulin-like brain secretory peptide of the silkworm *B. mori*
23 (Adachi et al., 1989); a diapause hormone precursor, a secretory neuropeptide from the
24 subesophageal ganglion of the *B. mori* (Sato et al., 1993); a prophenoloxidase-activating

enzyme of *B. mori* (Sato et al., 1999); and the 64K envelope protein of AcNPV (Volkman, 1986). We also used the signal peptide from honeybee prepro-melittin (Vlasak et al., 1983), which was able to enhance the secretion of propapain (Tessier et al., 1991) and β 3GnT2 (Kato et al., 2004a) in BES. The constructed BmNPV bacmid encoding different signal sequences for driving the expression of functional human β 3GnT2 was injected into the silkworm larvae for biologically active β 3GnT2 expression and production. Here, we report the establishment of the first practical BmNPV bacmid system encoding signal sequences. Using this system, we demonstrated high-level secretion of β 3GnT2 in silkworm hemolymph by directly injecting the recombinant bacmid DNA.

2. Materials and methods

2.1. Insect, cell line, media, and cell culture

The *B. mori* fifth-instar larvae (Ehime Sansyu, Ehime, Japan) were reared on an artificial feed (Silkmate 2, NOSAN Co., Yokohama, Japan) at 27°C incubator. The Bm5 cell line was provided by Dr. Boo of Insect Pathology Laboratory in Seoul National University. Sf900II serum-free medium (Invitrogen) and TNM-FH medium (Sigma, St. Louis, MO, USA) supplemented with 0.35 g/l NaHCO₃ and 10% heat-inactivated FBS (Sigma, St. Louis, MO, USA) were used for culture of Bm5 cells. Both media were supplemented with 1% antibiotic-antimycotic (Invitrogen) before use. Viable cells (6×10^5 cells/ml) were cultured in a 25 cm² tissue culture flask at 27°C in a mixture of Sf900 II and TNM-FH media at 4:1. For a suspension culture of Bm5 cells, viable cells (6×10^5 cells/ml) were cultured in a 100-ml flat-bottom flask with a working volume of 20 ml of a mixture

of Sf900 II and TNM-FH media at 9:1 supplemented with 1 g/l Pluronic F-68 (Invitrogen) and 1 g/l HyPep 1510 (Quest Int'l., Naarden, Netherlands).

2.2. Construction of recombinant *BmNPV* bacmid encoding signal sequences

β 3GnT2 cDNA from the cDNA of Quick-Clone human fetal brain cDNA (Clontech, Palo Alto, CA) was inserted between the *Bam*H I and *Eco*R I sites of pBlueBacHis2-GFP_{uv} to yield pBlueBacHis2-GFP_{uv}- β 3GnT2 (Kato et al., 2003). To enable the introduction of a new signal-peptide-coding region, the GFP_{uv}- β 3GnT2 fusion fragment was amplified using a PCR primer set (Table 1). The signal sequence from cecropin B (van Hofsten et al., 1985) is designated *ce*. The signal sequences from a silkworm juvenile hormone esterase (Hirai et al., 2002), a silkworm bombyxin (Adachi et al., 1989), a silkworm diapause hormone precursor (Sato et al., 1993); and a silkworm prophenoloxidase-activating enzyme (Sato et al., 1999) are designated *jhe*, *bx*, *dh*, and *ppae*, respectively. The signal sequences from the 64K envelope protein of AcNPV (Volkman, 1986) and honeybee prepromelittin (Vlasak et al., 1983) are designated *gp* and *me*, respectively. This PCR product including the signal sequences was cloned into the Gateway cloning vector pENTR/D-TOPO (Invitrogen) according to the protocol provided for the TOPO cloning system. The isolated plasmid, pENTR/D/signal-GFP_{uv}- β 3GnT2 was recombined with pDEST8. The resultant plasmid was designated pDEST/signal-GFP_{uv}- β 3GnT2. Transposition was carried out by transforming the donor plasmid pDEST/signal-GFP_{uv}- β 3GnT2 into *E. coli* BmDH10Bac cells harboring a transposition helper plasmid. White kanamycin-, gentamycin-, and tetracycline-resistant colonies were

selected and then, the BmNPV bacmid was isolated and designated BmNPV
bacmid/signal-GFP_{uv}-β3GnT2.

2.3. Recombinant virus production in Bm5 cell

The approximately 100 ng of BmNPV bacmid/signal-GFP_{uv}-β3GnT2 DNA was
transfected into Bm5 cells using Cellfectin (Invitrogen) according to the protocol provided
by Invitrogen. After 4 days, the cultured medium was harvested and centrifuged at 2000 *g*
for 5 min, and then the supernatant was collected, and the resulting recombinant virus
vBmNPV/signal-GFP_{uv}-β3GnT2 was stored at 4°C. This recombinant virus was amplified
fivefold and virus titer was determined by a tissue culture infectious dose 50 (TCID₅₀)
method, which is based on end-point dilution.

2.4. Expression of GFP_{uv}-β3GnT2 in B. mori silkworm larvae using either vBmNPV/signal-GFP_{uv}-β3GnT2 or BmNPV bacmid/signal-GFP_{uv}-β3GnT2 DNA

GFP_{uv}-β3GnT2 fusion protein expression in silkworm larvae was carried out using
two methods. One involved a direct injection of BmNPV bacmid/signal-GFP_{uv}-β3GnT2
DNA and the other involved an infection with recombinant
vBmNPV/signal-GFP_{uv}-β3GnT2. For the direct injection, 45 µl of BmNPV
bacmid/signal-GFP_{uv}-β3GnT2 DNA was suspended in 5 µl of
1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRI)-C reagent
(Invitrogen) and incubated at room temperature for 45 min. Forty microliters of this
bacmid DNA mixture was injected into the dorsal of the larvae using a syringe with a

26-gauge beveled needle. For the BmNPV/signal-GFP_{uv}-β3GnT2 virus infection, 40 μl of virus suspension ranging from 4.0x10³ to 2.0x10⁴ pfu/ml was injected into the larvae using the syringe.

After either bacmid DNA injection or virus infection, hemolymph was collected with 5 mM 1-phenyl-2-thiourea on ice. Then, the harvested hemolymph was centrifuged at 25000 g for 10 min at 4°C, and the supernatant was stored for further assay. Proteins from the fat body, the silk gland, Malpighian tubules, and the middle gut were extracted for an assay of enzyme activity. The silk gland from one larva was sliced and mixed with 2 ml of 20 mM Tris-HCl (pH 7.5) containing 0.4 M NaCl and 1 mM 2-mercaptoethanol, and then the mixture was mashed. The fat body and middle gut were collected and mixed with 2 ml of 20 mM Tris-HCl (pH 7.5) containing 0.4 M NaCl and 1 mM 2-mercaptoethanol, whereas Malpighian tubules were collected and mixed with 0.5 ml of the same solution. These tubes were sonicated four times for 30 sec with a one-minute interval. These samples were then transferred to either 50-ml or 15-ml falcon tubes and centrifuged twice at 9000 g for 20 min at 4°C. The supernatant after the second centrifugation was collected and stocked for further assay.

2.5. Analysis of ~~β3GnT2 activity~~ β3GnT activity

Five microliters of harvested hemolymph was used for enzyme assay. ~~β3GnT2~~ activity β3GnT activity was measured in 50 mM Tris-HCl, pH 8.0, containing 9.5 mM MnCl₂·4H₂O, 12 mM UDP-GlcNAc, 14 mM Galβ1-4 GlcNAcβ-pNP, and 5 μl of the enzyme solution (total volume, 10 μl). The reaction was started by adding the β3GnT2 sample to the reaction mixture and leaving the resulting mixture in a 37°C water bath for 1

書式変更: フォント : Times New Roman

書式変更: フォント : Times New Roman

h. For each sampling, 5 μ l of the reaction mixture was added to 195 μ l of distilled water, and the mixture was boiled for 5 min. The resulting solution was centrifuged at 20000 g for 5 min at 4°C, and then the supernatant (100 μ l) was diluted with distilled water 10-fold, followed by filtration with a 0.45 μ m nitrocellulose filter membrane (ADVANTEC, Tokyo). The filtrates were analyzed by HPLC (LC-10AD_{VP}, Shimadzu Co., Inc., Kyoto, Japan). The Mightysil RP-4 GP 150-4.6 column (5 μ m, Kanto Chem. Co., Inc., Tokyo, Japan) was used. The reaction products were eluted with 10% methanol and detected at an absorbance of 300 nm. HPLC was performed at 40°C at a flow rate of 0.8 ml/min. One unit of enzyme activity is defined as the amount of enzyme capable of catalyzing the transfer of 1 μ mol of GlcNAc per minute.

2.6. SDS-PAGE and fluorescent image analysis

To detect the secretion of the recombinant fusion protein into the hemolymph of the silkworm larvae, the supernatants were subjected to SDS-PAGE on a 12% polyacrylamide gel using the Mini-protean II system (Bio-Rad). Six microliters of hemolymph was mixed with the same amount of sample buffer, and then applied to a 12% polyacrylamide gel. For the detection of fluorescent GFP_{uv}- β 3GnT2 protein on the SDS-PAGE gel, the samples were only mixed with the sample buffer without boiling and green fluorescent bands were then directly determined using Molecular Imager FX (Bio-Rad). Nonspecific proteins on the SDS-PAGE gel were detected with Coomassie brilliant blue (CBB) R-250.

3. Results and discussion

3.1. Influence of heterologous signal peptides on expression and secretion of *GFP_{uv}-β3GnT2* fusion protein

Recombinant BmNPV bacmid/signal-GFP_{uv}-β3GnT2 encoding heterologous signal sequences was constructed and transfected into Bm5 cells. Produced recombinant virus vBmNPV bacmid/signal-GFP_{uv}-β3GnT2 was infected to Bm5 cells to investigate the effect of signal sequences encoded in BmNPV on protein secretion. The secreted GFP_{uv}-β3GnT2 fusion protein from Bm5 cells to each encoded signal sequence is shown in Fig. 2. When vBmNPV with the signal sequences of *gp*, *ce*, and *me* were used, the fusion protein was expressed intracellularly, and was not detected as an extracellular fusion protein (Fig. 2A). However, when the two signal sequences *bx* and *ppae* were used, the fusion protein was secreted PITs 3 d and 5 d (Fig. 2B). *jhe* also showed very weak band intensity. This indicates that the fusion protein could be secreted extracellularly, suggesting that the signal sequences *bx* and *ppae* are capable of secreting the fusion protein into the hemolymph of the silkworm.

3.2. Secretion of *GFP_{uv}-β3GnT2* fusion protein using recombinant vBmNPV and BmNPV bacmid

The effects of three signal sequences on the secretion of the fusion protein were investigated using both recombinant baculovirus infection and the bacmid injection. The

1 optimum amount of vBmNPV/signal-GFP_{uv}-β3GnT2 injection was determined to be 160
2 pfu/larva. When vBmNPV with 800 pfu/larva was injected into the larvae, they were
3 markedly weak 3 days postinjection, and one-half of the larvae were dead 4 days
4 postinjection (data not shown). When vBmNPV ranging from 160 pfu/larva to 400
5 pfu/larva was injected, a sufficient amount of hemolymph was harvested. For the
6 recombinant bacmid, the optimum bacmid DNA was determined to be in the range of
7 50-100 ng/larva by a preliminary experiment.

8 For *bx* and *ppae*, the fusion protein was secreted into the hemolymph 2 days
9 postinjection and increased 3 and 4 days postinjection (Fig. 3A). However,
10 low-molecular-weight bands appeared 4 days postinjection, which might be due to the
11 proteolytic activity of a cysteine protease originating from the baculovirus. This means that
12 the optimal harvest time of the hemolymph was 3 or 4 days postinjection. On the other
13 hand, when the recombinant bacmid was injected into the larvae, the secretion of the fusion
14 protein began 4 days postinjection and reached maximum activity 5 days postinjection (Fig.
15 3B). Six days postinjection, the secreted fusion protein was degraded, possibly owing to
16 the same reason as that for the baculovirus injection shown in Fig. 3A. This proteolytic
17 degradation occurred irrespective of signal sequences and might be a common
18 phenomenon.

19 For recombinant vBmNPV, the signal sequence *bx* showed a strong secretory effect on
20 the fusion protein; the β3GnT2 activity was 38.3 mU/ml (Table 2), which was the
21 maximum. *ppae* also showed a secretory effect on the fusion protein, but the efficiency
22 was lower than that of *bx*. When the recombinant bacmid was used *bx* and *ppae* showed

high secretory efficiencies, namely β 3GnT2 activities of 56.1 and 46.7 mU/ml, respectively (Table 2). To our knowledge the β 3GnT2 activity using *bx* is the highest reported activity, and is twofold as high as the published data of Kato et al. (2005). Kato et al. (2005) obtained a β 3GnT2 activity of 22 mU/ml using a stable cell line with the coexpression of a chaperone. *jhe* was not suitable for secreting the fusion protein in the silkworm (Table 2), showing only 5 to 20% compared with those of *bx* and *ppae*.

Expression with the use of recombinant bacmid was delayed by one day to compare the use of recombinant baculovirus. However, the striking advantage of using recombinant bacmid is the expression of β 3GnT2 in hemolymph, producing 91 μ g/larva which is almost twofold higher than that of the recombinant baculovirus (Table 2). This shows that silkworm larvae produced almost 1 mg of the secreted biologically active protein.

Moreover, in the case of using bacmid, troublesome procedures including transfection, virus amplification, and virus titer determination can be omitted. By using this system provides the rapid protein production in silkworm as long as 10 days, is free from biohazard.

3.3. Distribution of fusion protein in silkworm larval tissue

β 3GnT2 activity was measured in the hemolymph, fat body, and the silk gland of the silkworm larvae. When the recombinant vBmNPV was infected, only the fusion protein was detected in the hemolymph (Fig. 5A), which contained 99% of the total β 3GnT2 activity (Fig. 5B). The degraded fusion protein was detected in the fat body (Fig. 5A). However, when the recombinant BmNPV bacmid was used, 88% of the total amount of

1 fusion protein was secreted in the hemolymph (Fig. 5B). Slightly degraded protein was
2 detected in the hemolymph and fat body, but 12% of the total β 3GnT2 activity remained in
3 the fat body. The proteolytic degradation of the fusion protein using vBmNPV was more
4 marked than that of the BmNPV bacmid (a in Fig. 5A). Kato et al. (2003) reported that
5 because β 3GnT2 is sensitive to a cysteine protease, it is difficult to express it without
6 adding a protease inhibitor. This also causes the deactivation of β 3GnT2 in the hemolymph
7 and fat body. For the BmNPV bacmid no degraded fusion proteins were found in the
8 hemolymph or fat body. The remaining fusion protein in the fat body might be present
9 owing to the rate-limiting of the secretion process.

10 The effect of the signal sequences on the secretion of the fusion protein into silkworm
11 tissue was measured. The use of the *bx* resulted in the secretion of 97.3% of the total
12 amount of the fusion protein into the hemolymph, whereas the use of *ppae* resulted in that
13 of 90.8% (Fig. 6B). The fusion protein was detected in the fat body and intestines, but not
14 in the silk gland (Fig. 5A). The fat body and intestines of the silkworm larvae accumulated,
15 the degraded fusion protein with a low molecular weight of 72kDa (a and b in Fig. 6A),
16 which might be due to the photolytic activity of protease. For *ppae* 7.8% of the total
17 amount of fusion protein was detected in intestines, which was eightfold that for *bx*.

18 The amount of the secreted protein was 130 μ g/ml, assuming a hemolymph volume of
19 700 μ l/larva, but the total protein amount was 44.7 mg/ml (Table 2). This means that 3%
20 of the total amount of protein is the secreted target protein. For the use of silkworm larvae
21 for recombinant protein production, the development of a highly efficient purification
22 method for protein secreted into the hemolymph of the silkworm is urgent.

Acknowledgment

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

References

- Adachi, T., Takiya, S., Suzuki, Y., Iwami, M., Kawakami, A., Takahashi, S.Y., Ishizaki, H., Nagasawa, H., Suzuki, A., 1989. cDNA structure and expression of Bombyxin, an Insulin-like brain secretory peptide of the silkworm *Bombyx mori*. J. Biol. Chem. 264, 7681-7685.
- Hirai, M., Kamimura, M., Kikuchi, K., Yasukochi, Y., Kiuchi, M., Shinoda, T., [Shiotsuki, T.](#), 2002. cDNA cloning and characterization of *Bombyx mori* juvenile hormone esterase: an inducible gene by the imidazole insect growth regulator KK-42. Insect Biochem. Mol. Biol. 32, 627-635.
- Kato, T., Murata, T., Usui, T., Park, E.Y., 2003. Improvement of GFP_{uv}-β3GnT2 fusion protein production by suppressing protease in baculovirus expression system. Biosci. Biotechnol. Biochem. 67, 2288-2395.
- Kato, T., Murata, T., Usui, T., Park, E.Y., 2004a. Efficient production of human β-1,3-*N*-acetylglucosaminyltransferase-2 fused with green fluorescence protein in insect cell. Biochem. Eng. J. 19, 15–23.
- Kato, T., Murata, T., Usui, T., Park, E.Y., 2004b. Comparative analysis of GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase-2 production in two insect-cell-based expression systems. Protein Expr. Purif. 35, 54–61.

- 1 Kato, T., Murata, T., Usui, T., Park, E.Y., 2005. Improvement of the production of
- 2 GFP_{uv}-β1,3-N-acetylglucosaminyltransferase 2 fusion protein using a molecular
- 3 chaperone-assisted insect-cell-based expression system. *Biotechnol. Bioeng.* 89,
- 4 424-433.
- 5 Luckow, V.A., 1991. Cloning and expression of heterologous genes in insect cells with
- 6 baculovirus vectors. In: Prokop, A., Bajpai, R. K., Ho, C. S., (Eds.), *Recombinant*
- 7 *DNA Technology and Applications*. McGraw-Hill, Inc. New York, pp. 97-152.
- 8 Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. Efficient generation of infectious
- 9 recombinant baculoviruses by site-specific transposon-mediated insertion of foreign
- 10 genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.* 67,
- 11 4566-4579.
- 12 Motohashi, T., Shimojima, T., Fukagawa, T., Maenaka, K., Park, E.Y., 2005. Efficient
- 13 large-scale protein production of larvae and pupae of silkworm by *Bombyx mori*
- 14 nuclear polyhedrosis virus bacmid system. *Biochem. Biophys. Res. Comm.* 326,
- 15 564-569.
- 16 O'Reilly, D.R., Miller, L.K., Luckow, V.A., 1992. *Baculovirus Expression Vectors: A*
- 17 *Laboratory Manual*, Oxford University Press, pp. 216-234.
- 18 Sato, Y., Oguchi, M., Menjo, N., Imai, K., Saito, H., Ikeda, M., Isobe, M., Yamashita, O.,
- 19 1993. Precursor polypeptide for multiple neuropeptides secreted from the
- 20 suboesophageal ganglion of the silkworm *Bombyx mori*: Characterization of the
- 21 cDNA encoding the diapause hormone precursor and identification of additional
- 22 peptides. *Proc. Natl. Acad. Sci. USA* 90, 3251-3255.

- 1 Satoh, D., Horii, A., Ochiai, M., Ashida, M., 1999. Prophenoloxidase-activating enzyme of
2 the silkworm, *Bombyx mori*, ~~Purification~~purification, characterization, and cDNA
3 cloning. J. Biol. Chem. 274, 7441-7453.
- 4 Tessier, D.C., Thomas, D.Y., Khouri, H.E., Laliberte, F., Vernet, T., 1991. Enhanced
5 secretion from insect cells of a foreign protein fused to the honeybee melittin signal
6 peptide. Gene 98, 177-183.
- 7 van Hofsten, P., Faye, I., Kockum, K., Lee, J.Y., Xanthopoulos, K.G., Boman, I.A., Boman,
8 H.G., Engstrom, A., Andreu, D., Merrifield, R.B., 1985. Molecular cloning, cDNA
9 sequencing, and chemical synthesis of ceropin B from *Hyalophora ce*c*ropia*. Proc.
10 Natl. Acad. Sci. USA 82, 2240-2243.
- 11 Vlasak, R., Unger-Ullmann, C., Kreil, G., Frischauf, A.M., 1983. Nucleotide sequence of
12 cloned cDNA coding for honeybee prepromelittin. Eur. J. Biochem. 135, 123-126.
- 13 Volkman, L.E., 1986. The 64K envelope protein of budded *Autographa californica* nuclear
14 polyhedrosis virus. Curr. ~~Topics~~Top. Microbiol. Immunol. 131, 103-118.

Legends for figures

Fig. 1. Scheme for construction of recombinant vBmNPV/signal-GFP_{uv}-β3GnT2 and recombinant BmNPV bacmid/signal-GFP_{uv}-β3GnT2.

Fig. 2. Fluorescence image of GFP_{uv}-β3GnT2 fusion proteins produced by Bm5 cells infected with vBmNPV/signal-GFP_{uv}-β3GnT2. (A): GFP_{uv}-β3GnT2 fusion proteins produced with *gp*, *me*, and *ce*. (B): GFP_{uv}-β3GnT2 fusion proteins produced with *dh*, *bx*, and *ppae*. Three and five days postinfection of recombinant vBmNPV/signal-GFP_{uv}-β3GnT2 (1×10^5 pfu/ml) to Bm5 cells, fusion proteins were separated by SDS-PAGE (12% gel) and detected using a fluorescence image analyzer. The arrow indicates the molecular weight of the fusion protein.

Fig. 3. Secretion of GFP_{uv}-β3GnT2 fusion protein using vBmNPV/signal-GFP_{uv}-β3GnT2 (A) and BmNPV bacmid/signal-GFP_{uv}-β3GnT2 (B). Three signal sequences (*bx*, *ppae*, and *jhe*) were encoded in the recombinant vBmNPV and BmNPV bacmid. Fusion proteins were analyzed using GFP_{uv} fluorescence (a) and by CBB staining (b). The numbers on the lane denote either postinfection time or postinjection time.

Fig. 4. Difference in secretion of GFP_{uv}-β3GnT2 fusion protein between vBmNPV/*bx*-GFP_{uv}-β3GnT2 and BmNPV bacmid/*bx*-GFP_{uv}-β3GnT2. vBmNPV/*bx*-GFP_{uv}-β3GnT2 and BmNPV bacmid/*bx*-GFP_{uv}-β3GnT2 were injected

into silkworm larvae, and the hemolymph, fat body, and silk gland were harvested 4 and 5 days postinfection. (A) Fusion proteins were detected by SDS-PAGE with GFP_{uv} fluorescence (a) and CBB staining (b). M, m, v, and b on the lane denote a protein molecular weight marker, mock, vBmNPV/*bx*-GFP_{uv}-β3GnT2, and BmNPV bacmid/*bx*-GFP_{uv}-β3GnT2, respectively. The arrow indicates the GFP_{uv}-β3GnT2 fusion protein. (B) Distribution of β3GnT2 activity secreted in silkworm tissue. Black, white, dotted, slashed, and horizontal bars denote the hemolymph, fat body, silk gland, intestines, and Malpighian tubules of silkworm larvae, respectively.

Fig. 5. Influence of signal sequences on secretion of GFP_{uv}-β3GnT2 fusion protein into silkworm larval tissue. BmNPV bacmid/(*bx* or *ppae*)-GFP_{uv}-β3GnT2 was injected into silkworm larvae, and the hemolymph, fat body, intestines, and silk gland were harvested at five days postinfection. (A) Fusion proteins from silkworm larval tissue were detected by GFP_{uv} fluorescence (a) and CBB staining (b). M, m, *bx*, and *ppae* on the lane denote protein molecular weight marker, mock, and signal sequences *bx*, and *ppae*, respectively. The arrow indicates the GFP_{uv}-β3GnT2 fusion protein. (B) Distribution of β3GnT2 activity secreted in silkworm tissue. Black, gray, dotted, and slashed bars denote the hemolymph, fat body, intestines, and silk gland of silkworm larvae, respectively.

(A) Recombinant vBmNPV infection

(B) Recombinant BmNPV bacmid injection

(A)

(a) GFP_{uv} fluorescence

(b) CBB stain

(B)

(A)

(a) GFP_{uv} fluorescence

(b) CBB stain

(B)

1 **Table 1.** Primers set for PCR.

Primer	Sequence
Forward primers	
<i>me</i> -GFP _{uv} -β3GnT2	5'-CACC <u>ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTATGGTCGTATACATTTCTTACATCTATGCCGG</u> <u>CCCGCGGGGTTCTCATCATC</u> -3'
<i>gp</i> -GFP _{uv} -β3GnT2	5'-CACC <u>ATGCCCATGTTAAGCGCTATTGTTTTATATGTCCTTTTGGCGGCGGCGGCATTCTGCCTTTG</u> <u>CGCCCCCGCGGGGTTCTCATCATC</u> -3'
<i>ce</i> -GFP _{uv} -β3GnT2	5'-CACC <u>ATGAATTTCTCAAGCATATTTTCTTCGTGTTTCGCTTTGGTTCTGGCTTTGTCAACAGTTTCGG</u> <u>CTGCACCGGAGCCGAAACCGCGGGGTTCTCATCATC</u> -3'
<i>ppae</i> -GFP _{uv} -β3GnT2	5'-CACC <u>ATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCAAAAGTGTTGTTCAA</u> <u>CCGCGGGGTTCTCATCATC</u> -3'
<i>jhe</i> -GFP _{uv} -β3GnT2	5'-CACC <u>ATGTGTACCAAATTCGCTGTATTACTATATTACGTTATCGTGGGCTCAGTAAGGGCATACCCGC</u> <u>GGGGTTCTCATCATC</u> -3'
<i>dh</i> -GFP _{uv} -β3GnT2	5'-CACC <u>ATGTATAAAACCAACATTGTTTTCAACGTTTTAGCTTTGGCATTGTTTCAGTATTTTCTTCGCGA</u> <u>GTTGCACGCCGCGGGGTTCTCATCATC</u> -3'
<i>bx</i> -GFP _{uv} -β3GnT2	5'-CACC <u>ATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACAACAACCG</u> <u>CGGGGTTCTCATCATC</u> -3'
Reverse primer	5'-CGGAATTCTGAAGGGTTTAGAGGCCCTCAAATGGG-3'

2 Thin and double underlines indicate signal sequences and the part of GFP_{uv}-β3GnT2 sequences, respectively.

3

4

Table 2. β 3GnT2 activity and specific activity in hemolymph of silkworm larvae.

Signal sequence	Postinjection time (day)	β 3GnT2 activity (mU/ml)*	Protein concentration (mg/ml)*	Specific activity (mU/mg protein)	β 3GnT2 amount (μ g/larva) [†]
<i>BmNPV/signal-GFP_{uv}-β3GnT2 infection</i>					
none	3	0.0 $\pm$ 0.00	24.2 $\pm$ 0.18	0.0	0.00 $\pm$ 0.00
	2	0.0 $\pm$ 0.00	28.5 $\pm$ 1.52	0.0	0.00 $\pm$ 0.00
<i>bx</i>	3	3.6 $\pm$ 1.86	25.6 $\pm$ 0.97	0.1	5.25 $\pm$ 1.45
	4	38.3 $\pm$ 6.60	19.0 $\pm$ 0.73	2.0	56.30 $\pm$ 5.15
<i>ppae</i>	2	0.0 $\pm$ 0.00	30.2 $\pm$ 1.89	0.0	0.00 $\pm$ 0.00
	3	0.9 $\pm$ 1.23	21.2 $\pm$ 0.85	0.0	1.09 $\pm$ 0.81
	4	29.2 $\pm$ 0.60	19.8 $\pm$ 0.67	1.5	41.90 $\pm$ 0.43
<i>jhe</i>	3	6.1 $\pm$ 8.62	24.6 $\pm$ 1.10	0.3	7.82 $\pm$ 5.86
<i>BmNPV bacmid/signal-GFP_{uv}-β3GnT2 injection</i>					
none	3	0.0 $\pm$ 0.00	24.2 $\pm$ 0.18	0.0	0.00 $\pm$ 0.00
<i>bx</i>	3	0.0 $\pm$ 0.00	36.5 $\pm$ 0.55	0.0	0.00 $\pm$ 0.00
	4	56.1 $\pm$ 0.86	44.7 $\pm$ 0.97	1.3	91.0 $\pm$ 0.74

	5	50.8 ± 4.06	37.1 ± 0.49	1.4	63.2 ± 2.68
	3	0.0 ± 0.00	31.6 ± 0.24	0.0	0.00 ± 0.00
<i>ppae</i>	4	46.7 ± 3.34	40.0 ± 1.34	1.2	74.20 ± 2.87
	5	51.5 ± 8.00	32.4 ± 2.50	1.6	52.50 ± 4.32
<i>jhe</i>	4	2.1 ± 1.46	41.4 ± 0.97	0.1	3.61 ± 1.34

* Harvested hemolymph from 10 silkworm larvae was analyzed three times and their average and standard deviation are shown.

† Five silkworm larvae were used for β 3GnT2 assay and their average and standard deviation are shown. β 3GnT2 amount was obtained from the correlation between enzyme activity and purified protein amount (Kato et. al., 2004b).