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Specific expression of GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 fusion protein in fat body of *Bombyx mori* silkworm larvae using signal peptide

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Abstract

Bombyxin (bx) and prophenoloxidase-activating enzyme (ppae) signal peptides from *Bombyx mori*, their modified signal peptides, and synthetic signal peptides were investigated for the secretion of GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 (GGT2) fusion protein in *B. mori* Bm5 cells and silkworm larvae using cysteine protease deficient *B. mori* multiple nucleopolyhedrovirus (BmMNPV-CP^-) and its bacmid. The secretion efficiencies of all signal peptides were 15–30% in Bm5 cells and 24–30% in silkworm larvae, while that of the +16 signal peptide was 0% in Bm5 cells and 1% in silkworm larvae. The fusion protein that contained the +16 signal peptide was expressed specifically in the endoplasmic reticulum (ER) and in the fractions of cell precipitations. Ninety-four percent of total intracellular β1,3-*N*-acetylglucosaminyltransferase (β3GnT) activity was detected in cell precipitations following the 600, 8000, and 114,000*g* centrifugations. In the case of the +38 signal peptide, 60% of total intracellular activity was detected in the supernatant from the 114,000*g* spin, and only 1% was found in the precipitate. Our results suggest that the +16 signal peptide might be situated in the transmembrane region and not cleaved by signal peptidase in silkworm or *B. mori* cells. Therefore, the fusion protein connected to the +16 signal peptide stayed in the fat body of silkworm larvae with biological function, and was not secreted extracellularly.

Keywords: Bacmid; Baculovirus expression system; BmMNPV; Signal sequence; Silkworm; β13-*N*-acetylglucosaminyltransferase 2

Recently, it has been shown that insect larvae can be used in the baculovirus expression system instead of insect cell culture for large-scale protein production. Scale-up is performed more easily in insect larvae than in insect cells [1–3]. Moreover, the *Bombyx mori* multiple nucleopolyhedrovirus (BmMNPV) bacmid system was established [4] and time-consuming manipulation including virus amplification and virus titer determination can be omitted by using this bacmid system.

To improve the production of proteins in insect cells, various genetic manipulations have been performed; random and site-directed mutagenesis [5], signal peptide and promoter exchange [6], and increase of plasmid copy number [7]. In the case of secreted proteins, processing of signal peptide and glycosylation may be crucial. In both insect cell-based expression systems, enforcement of the secretory pathway in host cells and overexpression of molecular

Abbreviations: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; BmMNPV, *Bombyx mori* multiple nucleopolyhedrovirus; BmMNPV-CP^-, cysteine protease deficient *Bombyx mori* multiple nucleopolyhedrovirus; bx, bombyxin; ER, endoplasmic reticulum; GFPuv, green fluorescent protein when excited with long-wave UV light; β3GnT2, β1,3-*N*-acetylglucosaminyltransferase 2; GGT2, GFPuv-β3GnT2; HMM, hidden Markov model; PCR, polymerase chain reaction; ppae, *Bombyx mori* prophenoloxidase-activating enzyme.

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chaperones such as calnexin and calreticulin, enhance the secretion of recombinant proteins [8–12].

In this study, using the BmMNPV bacmid, the GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 (β3GnT2) fusion protein was expressed in *B. mori* Bm5 cells and silkworm larvae. In a previous report, its fusion protein was expressed using the *B. mori* bombyxin (bx) and *B. mori* prophenoloxidase-activating enzyme (ppae) signal peptide from *B. mori,* which was functional in Bm5 cells [13]. In order to investigate secretory efficiency, synthetic signal peptides was predicted by a computer program (SignalP ver.3.0) [14] and its effects on the secretion of the fusion protein were examined in Bm5 cells and silkworm larvae. Further, characterization of the ability of signal peptides to secretion fusion protein was performed.

Materials and methods

Signal peptide design. Signal peptides of bx and ppae from *B. mori* were modified with the aid of SignalP 3.0 which is based on artificial neutral network and HMM [14]. Signal peptides +38, +34, +28, and +16 were used for reference [15].

Construction of recombinant BmMNPV and BmMNPV bacmids. Fundamentally, construction of recombinant baculoviruses was performed as described in a previous report [13]. Recombinant baculovirus was constructed using the BmMNPV-*CP*$^-$ Bac-To-Bac system [16]. GFP_{uv}-β3GnT2 (GGT2) fusion genes containing different signal sequences were amplified by polymerase chain reaction (PCR) using the primers shown in Table 1. Finally, recombinant BmMNPV-CP$^-$/(signal peptide)-GGT2 and its bacmid were constructed. Eight different types of signal peptides were used.

Cultivation of Bm5 cells, bacmid transfection, and baculovirus infection. Culture of Bm5 cells was carried out in 25 cm^2 T-flasks with Sf-900 II Serum Free Medium (SFM) (Invitrogen) supplemented with 1% antibiotic–antimycotic (Invitrogen). Transfection of Bm5 cells with recombinant bacmids was performed according to the procedure described in the Invitrogen manual. Bm5 cells, at a density of approximately 1.0×10^6 cells/ml, were infected with the viral transfection solution. Cell viability and cell number were measured using the 0.4% trypan blue staining method.

Recombinant BmMNPV bacmid injection to silkworm larvae. The *B. mori* fifth-instar larvae (Ehime Sansyu, Ehime, Japan) were reared for 1 day on an artificial feed (Silkmate 2, NOSAN Co., Yokohama, Japan) in a 27 °C incubator, and were injected with 50 μl bacmid solution consisting of 8 μg bacmid DNA in 45 μl of SF900 SFM and 5 μl DMRI-C (Invitrogen) per larva. Hemolymph was collected at 1 day after emission of GFP_{uv} fluorescence in silkworm larvae. Five millimolars of phenyl-thiourea was immediately added into collected hemolymph to prevent melanization. Fat body was collected after dissection of silkworm larvae and suspended in 50 mM Tris–HCl (pH 8.0) and then disrupted using a sonicator (VC 130PB, Sonic and Materials, Newtown, CT, USA) for three 30 s periods with 1 min intervals. Its suspension was used as the fat body extraction.

Confocal laser scanning microscopy analysis. Fat body was collected from silkworm larvae and immediately observed with the confocal laser-scanning microscope (TCS SP2, Leica Microsystems Heidelberg, Germany) equipped with an imaging system.

Cell fractionation. BmMNPV-*CP*$^-$/(signal peptide)-GGT2-infected Bm5 cells and fat body from BmMNPV-*CP*$^-$/(signal peptide)-GGT2 bacmid injected silkworm larvae were collected and suspended in 50 mM Tris–HCl (pH 8.0). The suspension was homogenized and the homogenate was spun at 600*g* for 5 min using a centrifuge (3700, Kubota, Tokyo). The supernatant was then centrifuged at 8000*g* for 5 min. Finally, the supernatant was spun at 114,000*g* for 1 h using a centrifuge (himac CS150GXS, Hitachi Koki Co., Ltd., Tokyo). All precipitates were resuspended in 50 mM Tris–HCl (pH 8.0). Supernatants and suspension of precipitation were analyzed for GGT2 expression.

SDS–PAGE. The protein contained in the hemolymph was subjected to SDS–PAGE on a 10% polyacrylamide gel using the Mini-protean II system (Bio-Rad, Hercules, CA). To detect directly the molecular band of a specific GGT2 protein on SDS–PAGE gel using Molecular Imager FX (Bio-Rad), the samples were mixed with sample buffer, but not boiled.

β3GnT assay. After cultivation, the culture broth was centrifuged at 20,000*g* for 5 min, and the supernatant was used for the enzyme assay. A lysis buffer containing 50 mM Tris–HCl (pH8.0) and 1% Triton X-100 was added to the cell pellet and the mixture was placed on ice for 10 min. This cell lysate was used for the measurement of intracellular enzyme activity. The β3GnT activity assay was carried out according to the protocol described previously [12].

Table 1
PCR primers

Name	Sequence (5′–3′)
38-F	CACCATGTGGTGGAGGCTTTGGTGGCTTCTTCTTCTTCTTCTTCTTTGGCCCATGGTGTGGGCCAAACC GCGGGGTTCTCATCATC
34-F	CACCATGACGCCCACATGGGCTTGGTGGCTTTTCCTTGTGCTTCTTCTTGCCCTTTGGGCTCCCGCTACGGCT AAACCGCGGGGTTCTCATCATC
28-F	CACCATGATGTGGCTTTGGCTTGTGCTTCTTCTTCTTTGCCTTGCCGGCAACGTGCAGGCCAAACCGCGGGG TTCTCATCATC
16-F	CACCATGAAGCTTCTTCTTATCTTCTTCGTGCTTGTGGTGTGGATGGGCCCCGCCCATCGCAAACCGCGGGG TTCTCATCATC
ppae-1-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCAAAAGTGCTGTTCAACCGCG GGGTTCTCATCATC
ppae-12-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCCTGAGTGCTGTTCAACCGCG GGGTTCTCATCATC
ppae-123-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCCTGAGTGCTCAACCGCGGG GTTCTCATCATC
bx-12-F	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGGTAATGTGGGTGTCAGCTGCTCCGCGGGGTTCTC ATCATC
bx-F	CACCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACACAACCGCGGG GTTCTCATCATC
ppae-F	CACCATGTTTTTAATTTGGACATTCATCGTGGCTGTTCTGGCGATCCAGACCAAAAGTGTTGTTCAACCGCG GGGTTCTCATCATC
β3GnT2-R	CGGAATTCTGAAGGGTTTAGAGGCCCTCAAATGGG

Underlined sequences indicate putative signal peptide coding sequences.