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Immobilization of foreign protein in BmNPV polyhedra by fusion expression with partial polyhedrin fragments

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A B S T R A C T

Bombyx mori nucleopolyhedrovirus (BmNPV) produces large, proteinaceous crystal matrix named polyhedra, which occlude progeny virions which are produced during infection and protect virions from hostile environmental conditions. In this study, five overlapping N-terminal fragments of the BmNPV polyhedrin ORF were cloned and ligated with the foreign gene *egfp*, and five recombinant baculoviruses were constructed by BmNPV(Polh$^+$) Bac-to-Bac baculovirus expression system was used to co-express the polyhedrin and fused protein. The results showed that the fusion proteins were highly expressed, and the foreign proteins fused with the 100aa fragment of polyhedrin could be embedded into polyhedra at a higher ratio. This study provides a new method for efficient preservation of useful proteins for the development of new biopesticide with toxin protein and delivery vector system of vaccines.

1. Introduction

Insect baculovirus produces stable infectious micro-crystals called polyhedra which function to protect the virus after the death of infected larvae (van Oers and Vlak, 2007). Polyhedra form within infected cells and contain numerous virus particles embedded in a crystalline lattice of the viral protein polyhedrin. Polyhedrin, the major protein component of polyhedra, is produced in high-level during viral infection (Guarino, 2007; Slack and Arif, 2007). It has been shown that the polyhedra were trimeric building blocks connected by extensive interactions, many involving the polyhedron H1-helix (Coulibaly et al., 2007).

Apart from the use of polyhedrin promoter for the expression of foreign proteins, utilization of polyhedrin as the signal for foreign protein immobilization of was also reported. In 1991, Jarvis et al. (1991) reported that the supramolecular assembly of polyhedrin into nuclear occlusion-like particles required the domain between amino acids 19 and 110. Ikeda et al. (2001, 2006) demonstrated that the N-terminal 75 amino acids of *Bombx mori* cypovirus (BmCPV) turret protein(VP3) can function as a polyhedrin recognition signal leading to the incorporation of foreign proteins into polyhedra. Enhanced green fluorescence protein (EGFP) and a variety of human proteins tagged with the VP3 polyhedrin recognition signal have been incorporated successfully into polyhedra by co-expression with polyhedrin in insect cells (Ijiri et al., 2009). Proteins immobilized into polyhedra were protected from dehydration and were resistant against higher temperature. Mori et al. (2007) showed that BmCPV polyhedrin H1-helix (residues 42–93) could also function as a polyhedrin recognition signal and used like the VP3 N-terminal sequence to target foreign proteins into BmCPV polyhedra. Fibroblast growth factor-2 (FGF-2), FGF-7 and epidermal growth factor (EGF) have been immobilized successfully into polyhedra with the H1 signal. These reports suggested that the polyhedra can be used for encapsulating and stabilizing bioactive proteins and the development of high-throughput protein microarrays.

In a previous study, EGFP and host-derived proteins were incorporated into BmNPV polyhedra (Liu et al., 2008) where small quantities of proteins were embedded into the polyhedra at random. In order to determine whether BmNPV polyhedrin sequence could be used as an immobilization signal to direct foreign proteins into polyhedra and subsequently determine the key fragment responsible for the immobilization, five different BmNPV polyhedrin partial fragments were fused individually with *egfp* and five recombinant baculoviruses were constructed. This study aimed to determine whether the EGFP tagged BmNPV polyhedrin recognition signal could incorporate into polyhedra by co-expression with polyhedrin in insect cells.

2. Materials and methods

2.1. Viruses and cells

The BmNPV(polh$^+$) Bac-to-Bac baculovirus expression system was constructed as described previously (Cao et al., 2006; Xiang

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Table 1
The primers for cloning partial polyhedrin fragments and EGFP.

Primer	Sequence (5′-3′)	The size of PCR product (bp)
Polh-1 forward	ATTTGTACGTAATTAACGATAC	
PH50 backward	CATGTAGGATCCTAGAAGATC	243
PH100 backward	TCAACGGATCCGGTCCAAG	393
PH150 backward	GATGGATCCGTGGGGCAC	543
PH200 backward	GAAGGATCCCCATATGACG	693
PH245 backward	CAATGTATAGTGGATCCATAC	828
EGFP-forward	AATGGATCCATGGTGAGCAAG	740
EGFP-backward	CACTAGTGACTAGTCTTGTAC	

et al., 2010). In this system, foreign gene and *polyhedrin* are co-expressed. The major components of this system include: donor plasmids for the generation of an expression construct containing the gene of interest, *Escherichia coli* host strain DH10BmBac^P (competent cells) containing a BmNPV bacmid (BmBacmid) and a helper plasmid that allows generation of a recombinant bacmid by site-specific transposition. *Bombyx mori* BmN cells were maintained at 27 °C in TC-100 medium supplemented with 10% fetal calf serum (*GibcoBRL*).

2.2. Construction of recombinant viruses

DNA fragments encoding the *polh* promotor followed by 50aa, 100aa, 150aa, 200aa and 245aa of the polyhedrin were amplified respectively by PCR, and marked as PH50, PH100, PH150, PH200 and PH245 (with the corresponding protein labeled as PH50aa, PH100aa, PH150aa, PH200aa and PH245aa). Primers sequence are listed in Table 1. Target genes were amplified using the universal forward primer, Polh-1F, and specific reverse primers PH50B, PH100B, PH150B, PH200B, and PH250B respectively from BmNPV genomic DNA. PCR conditions were as followed: 94 °C for 50 s, 55 °C for 30 s and 72 °C for 1 min; 30 cycles.

The *egfp* gene was amplified by primers EGFP-F and EGFP-B. PCR conditions were as followed: 94 °C for 50 s, 55 °C for 30 s and 72 °C for 40 s; 30 cycles.

Amplified partial fragments of *polh* and *egfp* fragments were digested with *Sna*B I/*Bam*H I and *Bam*H I/*Hin*d III respectively, and subsequently cloned into the corresponding sites of pFastBacHTa (*Invitrogen*). Through gene transposition, recombinant baculoviruses were generated by BmNPV(polh$^+$) Bac-to-Bac expression system. Briefly, the above recombinant donor vectors were transformed into DH10BmBac(Polh$^+$) competent cells, incubated at 37 °C for 4 h, diluted serially using SOC medium and then spread evenly on plates containing kanamycin, tetracycline and X-gal. After 48 h incubation at 37 °C white colonies were picked and cultured overnight in medium containing kanamycin. As described previously (Chen et al., 2012), Bacmid DNA was extracted and transfected into BmN cells to generate five recombinant baculoviruses.

2.3. Purification of polyhedra

BmN cells were inoculated with the recombinant viruses, respectively, and collected at 120 hour post-infection (hpi), followed by washing with PBS (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 150 mM NaCl, pH 7.2). Cells were further disrupted by ultrasonic wave and precipitated by centrifugation at 15,000 × *g* for 10 min. Pellets containing polyhedra were purified further by Percoll density gradient centrifugation at 15,000 × *g* for 20 min. Purified polyhedra was washed several times with PBS and finally suspended in TE buffer.

2.4. Fluorescence measurement of immobilized EGFP in polyhedra

Equal amounts of purified polyhedra were suspended in 50 mM carbonate buffer (100 mM Na_2CO_3, 100 mM $NaHCO_3$, 10 mM NaCl, pH 11.0). After 30 min incubation at room temperature, EGFP fluorescence was measured by excitation at 395 nm and emission at 510 nm using an F-4600 fluorescence spectrophotometer. All experiments were repeated three times.

2.5. SDS-PAGE and Western-blot analysis

Protein samples were electrophoresed by 12% SDS-PAGE gels and subsequently transferred to PVDF membrane, according to manufacturers' recommended protocols (Bio-Rad). Primary antibody was anti-EGFP rabbit monoclonal antibody (*Cell Signaling*). Secondary antibody was goat anti-rabbit IgG (H + L), horseradish peroxidase (HRP) conjugated, and DAB was used for color display.

3. Results

3.1. Construction of recombinant viruses and BmN cells infection

Five recombinant viruses, designated vBmBac(polh$^+$)-PH50-, 100-, 150-, 200- and 245-EGFP were prepared by BmNPV(polh$^+$) Bac-to-Bac expression system. The predicted molecular weight of fusion protein expressed in the above five viruses was 33.19, 39.04, 45.08, 50.81 and 55.88 kDa, respectively.

In the vBmBac(polh$^+$)-PH50-, 100-, 150-, 200- and 245-EGFP infected BmN cells, a large amount of polyhedra were formed with strong green fluorescence (Fig. 1). Interestingly, the fluorescence distribution pattern varied. Cells infected with vBmBac(polh$^+$)-PH50-EGFP gave the strongest signal among the five infected groups (Fig. 1), where fluorescence was evenly distributed in infected BmN cells. However, in vBmBac(polh$^+$)-PH100, 150, 200 or 245-EGFP infected cells there was a clear distinction between nuclear and cytoplasm distribution where a great deal of green fluorescence was observed in the cytoplasm with only a small amount observed in the nucleus.

Proteins were extracted from the BmN cells infected by five recombinant baculoviruses and electrophoresed by using 12% SDS-PAGE gels (Fig. 2). Both polyhedrin and fusion proteins and were clearly shown with sites of recombinant fusion proteins closed to the respective predicted molecular weight. Both polyhedrin and recombinant fusion proteins were high-level expressed in BmN cells.

3.2. Analysis of immobilized EGFP in polyhedra

Polyhedra were purified from infected BmN cells, suspended in PBS (pH 7.2) and examined by fluorescence microscopy (Fig. 3). Different levels of green fluorescence were observed, predominantly on the surface of polyhedra.

After suspension in 50 mM carbonate buffer, the strength of green fluorescence of polyhedra was also determined (Fig. 4). The results showed that the 100aa fragment and the full-length of polyhedrin constructs presented strongest fluorescence, indicating that these two fusions had better incorporation of the fusion protein into the polyhedra.

The relative amount of EGFP immobilized into polyhedra was confirmed further by SDS-PAGE and Western blot analysis (Fig. 5). The predicted bands can be clearly observed on the gel stained by Commassie blue. It was confirmed by Western blot with anti-EGFP antibody. In terms of polyhedra amount, there was no noticeable difference between BmN cells infected with the five different