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BV/ODV-E26: An Envelope Protein for Simple Purification of Fusion Foreign Protein in Baculovirus Expression System

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The baculovirus expression system (BES) is widely used to express the foreign proteins. However, purification of expression products is not only complex and expensive, but also be detracted from high throughput. Here, we investigated the envelope protein BV/ODV-E26 from *Autographa californica multiple nucleopolyherovirus* (AcMNPV) and *Bombyx mori* NPV (BmNPV), with fusion to a marker protein- Enhanced green fluorescent protein (EGFP) at C-terminal, respectively, and expression in *Spodoptera frugiperda* (SF9) and *B. mori* (BmN) cells. Fluorescent particles were observed under fluorescent microscope at 72 h post infection (p.i.). The results indicated that the fusion protein (Da26-EGFP) can be bound to the occluded-bodies (OBs). According to purifying Da26-EGFP particles by ultrasonic disruption and differential centrifugations, these proteins were still binding to the OBs. Furthermore, by treatment with 1 % DTT (w/v) and 2 % SDS (w/v), the fusion proteins could not be eluted from OBs. It indicated that using baculovirus BV/ODV-E26 as fusion protein could make it easy and rapid to purify the foreign proteins by differential centrifugation.

Key words: Purification, BV/ODV-E26, GFP, Baculovirus, Envelope protein.

The expression systems of prokaryotic, yeast and eukaryotic are widely used in producing the recombinant proteins. However, in industrial applications, purification of these recombinant proteins are usually tedious and expensive[1-5]. To place into robust scalable manufacturing strategies, a simple and effective method of purification can be highly improved the efficiency of work in purifying the proteins. The baculovirus expression system (BES) is widely used to recombine the production of proteins[6,7]. BES is great for recombining the frequency and large-scale production, and also for high level of post translational modification as mammalian cells including glycosylation, phosphorylation[8]. Especially expression of a foreign protein within the occlusion body (OB) is able to protect foreign protein away from affects of external environment and also easy to isolate and purify the recombinant virus. However, the polyhedrin gene is missing in the baculovirus genome in most of BES, so the system losses natural selection marker to ensure foreign protein into OBs and observe the expression level and localization. According to the investigated report, gene fusing at C-terminal or N-terminal facilitates the detection and purification of recombinant protein in BES[9]. In addition, N-terminal fusion with chicken avidin, Hydrophobin (HFBI), in aqueous micellar two-phase system (AMPTs) could efficiently purify foreign protein in one-step[10]. The most frequently used purification method is that of conjunction with hemagglutinin (HA), maltose-binding peptide (MBP), and histidine tag (His6), as tag or fusion partner for purification of recombinant proteins[3, 11, 12].

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Baculoviruses are divided into four types: alpha-, beta-, gamma- and delta-baculovirus. Alpha- and beta-baculoviruses infect insects of the order Lepidoptera; gamma-baculoviruses are specific for hymenopteran insects, while delta-baculoviruses could only infect dipteran insects[13]. NPVs are members of alpha-genera containing two types, which are budded viruses (BVs) and occluded-derived viruses (ODVs). BVs are from the surface of infected cells which spread the infection to insect bodies whereas the mature ODVs are occluded in the protein crystal matrix and then are released to the environment to infect other insects, and disintegrated in the alkaline midgut[14-16]. ODVs are the fundamental of functional virulence and host specificity, so they play the major infection role during all the processing. 24 baculovirus proteins as the components of ODV have been identified by multidimensional protein identification technology (MUDPIT), including ODV-E26, ODV-E66, ODV-E28, ODV-E56, ODV-E18, P74, P91, etc.[17]. ODVs could be packaged into the polyhedrin molecule but the mechanism has not clear yet. A previous study found EGFP products in BmNPV infected cell can be packed into the polyhedrin crystallization, however, the EGFP is randomly expressed in the infected cells and only a few of them into the OBs. To date, no evidence indicates that the outer proteins of ODVs can be fused with foreign proteins and bound to the surface of OBs.

Ac16 (bv/odv-e26, da26) in Autographa californica multiple nucleopolyherovirus (AcMNPV) is a homolog of BmOrf8 (bm8) in Bombyx mori NPV (BmNPV), ac16 and bm8 are both envelope proteins of BV and ODV [18, 19]. Different from the other envelope proteins in NPVs expressed early and late in infection, BV/ODV-E26 actually expressed from a cryptic sequence rather than baculovirus early and later promoter[20]. Compared with polyhedrin fusion protein, which is also lead fusion protein into OBs, ODV envelope proteins are easy to observe, separate and purify.

In this study, we expressed the fusion protein AC16 and Bm8 fusing with EGFP at C-terminus, respectively. The particles with fluorescence collected in the nuclear of the infected cells. These Particles were purified after ultrasonic lysis and differential centrifugation. SDS-PAGE and Western blot analyses showed that the EGFP fusion products bound to the OBs.

MATERIALS AND METHODS

Virus, Insect cell lines and DH10Bac competent cells

The Sf9 and BmN cells were maintained at 28°C in TC-100 insect medium supplemented with 10% fetal bovine serum (Gibco). BmNPV recombinant in *Escherichia coli* DH10B cells was supplied by Prof. E.Y. Park[6].

Plasmid construction, generation recombinant baculovirus

The genes of *Bm8* and *Ac16* were PCR-amplified with the following primers: Forward, 5'-CGT GGATCC ATG AAT TCT GTT CAC ACG-3'; Reverse, 5'-AGA CTCGAG TAC AGT GCG TCC TTT CGT-3' and Forward 5'-CGC GGATCC ATG GAG TCT GTT CAA ACG CGC TTG-3'; Reverse, 5'-AGA CTCGAG ATA GGC GTT AAT ATC ACT TTG AGA TTC using BmNPV and AcMNPV genomic DNA as templates, respectively. The fragments were cloned into vector pFastBac1-EGFP at *Bam*H I/*Xho* I, generating the fusion fragments of PFB1-*Bm8-EGFP* and PFB1-*Ac16-EGFP*, respectively.

The Bac to Bac system was used to integration of foreign genes into the baculovirus genome. The pFB1-*Bm8-EGFP* and pFB1-*Ac16-EGFP* plasmids were transformed in *E. coli* (AcMNPV or BmNPV-DH10Bac) competent cells, generating the recombinant bacmids, AC-*AC16-EGFP* and Bm-*Bm8-EGFP*, respectively. After investigated by PCR, the positive bacmid DNAs were purified for transfection in SF9 or BmN cells according to the virus type.

Production of recombinant baculovirus and western blotting

The BmN and SF9 cells were seeded to a concentration of 1.0×10^6cells/ml and were infected with the recombinant virus and wild-type virus (ratio 1:1) at a multiplicity of infection (MOI) of 10. BVs of the recombinant viruses were harvested at 72 h post infection (p.i.).

The infected cells were resuspended in lysis buffer and solubilized with Laemmli sample buffer, and heated at 100°C for 10 min. SDS-PAGE was performed using 10% separating gel in Mini-Protein system (Bio-Rad)[21].

For western bolt assay, the proteins were transferred onto nitrocellulose membranes (Millipore) with anti-EGFP (1:1,000, Santa Cruz)

antibody at overnight (4!) then use anti-rabbit HRP-conjugated secondary antibodies (1:10,000, Santa Cruz) to detect primary antibody for 1 h at room temperature. Enhance Chemiluminescence System (ECL) was used to detect the signals.

Purification of recombinant proteins

The SF9 and BmN cells were infected with recombinant viruses AC-AC16-EGFP and Bm-Bm8-EGFP in 75mm^2 cell Flask, respectively, and then, were collected at 120 h p.i., and resuspended in 10 mL PBS (20 mmol/L NaH_2PO_4, 20 mmol/L Na_2HPO_4 150 mmol/L NaCl; pH 7.2) By ultrasonic disruption, the cell lysate was centrifuged at 15 000 r/min for 10 min to remove cell debris. Afterward the deposit was resuspended in 500 uL Percoll solution mixture (10X *PBS* into *percoll* solution as *1*:*9* ratio, pH 7.2) and purified by differential centrifuged at 12 000 r/min for 20 min. The purified virus particles were disjected in 100 uL PBS (pH 7.2) for SDS-PAGE and Western blot analysis.

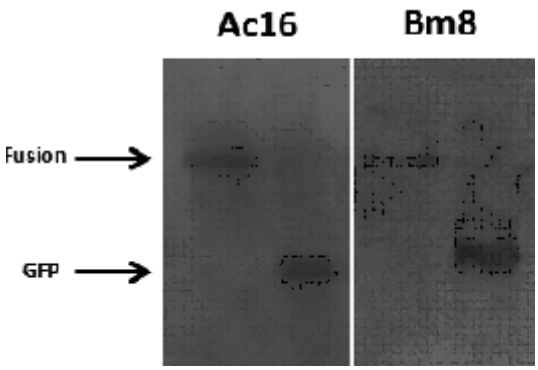
RESULTS

Expression of ac16-EGFP and Bm8-EGFP fusion proteins

Under the Polyhedrin (*Polh*) promoter control, fusing with EGFP at C-terminal, the recombinant bacmids over-expressed Ac16 and Bm8 were constructed. The cells infected with recombinant viruses AC-*AC16-EGFP* and Bm-*Bm8-EGFP*, respectively, were observed under fluorescent microscope. The fluorescence appeared in particles. It indicated that the fusion proteins can bind to the OBs (Fig. 1). Ac-*Ac16-EGFP* and Bm-*Bm8-EGFP* were predominantly located in foci of nucleus, while Ac-EGFP and Bm-EGFP were detected both in the nucleus and cytoplasm of the infected insect cells, respectively. To confirm the fusion proteins, Western blotting using GFP-antibody was carried out, and the expected bands band appeared at 53 KDa (Ac16-EGFP, Bm8-EGFP). The controls, Ac-EGFP, and Bm-EGFP at 27 KDa were detected (Fig. 1B).

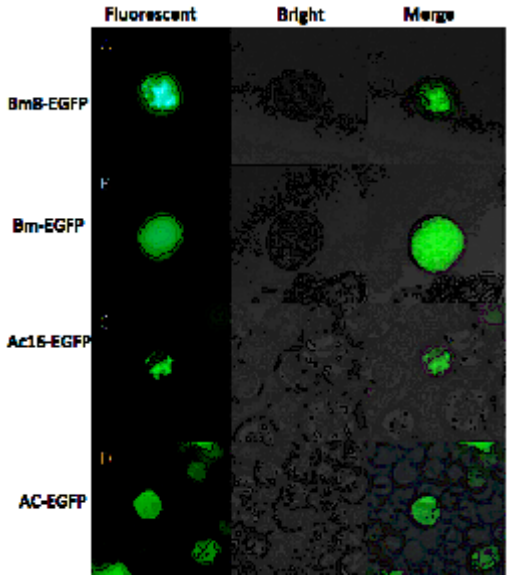
Cellular distribution of the fusion proteins

The cellular distribution of the recombinant proteins was analyzed under fluorescent microscope. Ac-*Ac16-EGFP* and Bm-*Bm8-EGFP* were observed at 72h p.i.. Ac-*Ac16-EGFP* and Bm-*Bm8-EGFP* were predominantly located in the nucleus (Fig. 2) while Ac-EGFP and Bm-EGFP were detected both in the nucleus and cytoplasm of the infected cells, respectively.



SDS-PAGE and Western blot were used to analyze the precipitated proteins. The fusion products of Ac16-EGFP and Bm8-EGFP were detected using anti-GFP antibody. The lysate of the infected cells at 72h p.i were collected to test, respectively. M, mock infected sf9 and BmN cells

Fig. 1. Expression of Ac16-EGFP and BM8-EGFP fusion protein in insect cells



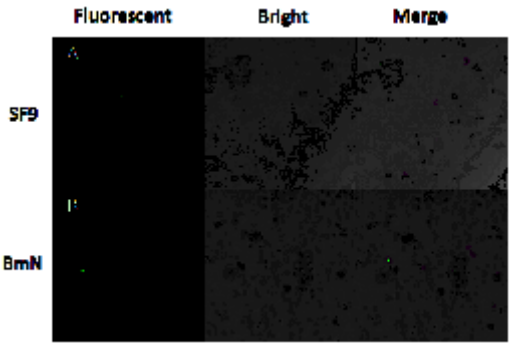
A and B, BmN cells infected with the recombinant viruses which express Bm-EGFP and Bm8-EGFP under control of the polyhedron promoter, respectively; C and D, Ac16-EGFP and Ac-EGFP infected SF9 at 72h p.i.. (Magnification: ×400)

Fig. 2. Cellular localization of Ac16-EGFP in sf9 cells and Bm8-EGFP in BmN cells by infection with viruses

Purification of Da26-EGFP fusion protein

The OBs were purified by ultrasonic disruption and differential centrifugation as described previously. The particles with bright green fluorescence were observed under fluorescent microscope (Fig. 3). It indicated that the fusion proteins can bind to the OBs.

To identify if the fusion proteins can be washed out from the OBs, the Purified recombinant particles were treated with 1% DTT (w/v) or 2%



A, from SF9 cells; B, from BmN cells (Magnification: ×400)

Fig. 3. Localization of purified the OB particles containing Ac16-EGFP and Bm8-EGFP in sf9 and BmN, respectively

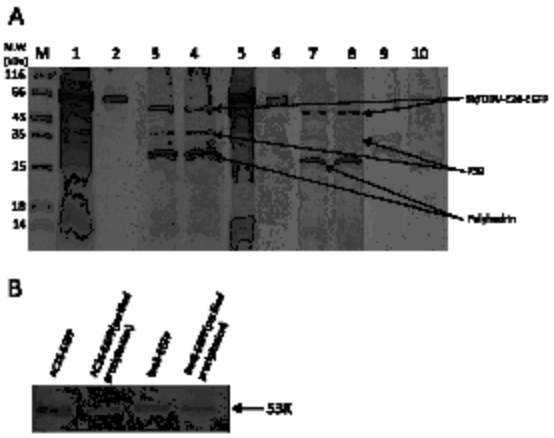


Fig. 4. Analysis of purified particles contents by SDS-PAGE and Western blot

(A) SDS-PAGE analysis of Ac16-EGFP and Bm8-EGFP infected cell lysates. M, Marker Lane 1 and 2 represent sample of sf9 cells were infected with Ac16-EGFP and purified supernatant sample of Ac16-EGFP infected sf9 cells. Lane 3 and 4 represent the purified precipitation sample of Ac16-EGFP infected sf9 cells treatment with 1% DTT and 2% SDS. Lane 5 to 8 SDS-PAGE analysis of Bm8-EGFP infected cell lysate samples ordered as AC16-EGFP infected SF9. Lane 9 and 10 are the purified precipitation samples of WT AcMNPV and WT BmNPV, respectively. (B) Western Blot analysis was used to confirm Ac16-EGFP, purified precipitation of Ac16-EGFP, Bm8-EGFP and purified precipitation of Bm8-EGFP

SDS (w/v), respectively. After centrifugation, the OB lysate were loading on 10% SDS-PAGE. The recombinant products were apparently detected in the purified precipitate, but hardly could be found in the supernatant. (Fig. 4A) This result implied these reagents cannot reduce the binding activity of the fusion products. The fusion proteins were further verified by western blot (Fig. 4B). Besides the recombinant proteins, a 39K protein also was noticed in the purified precipitate lanes.

DISCUSSION

Recombinant baculovirus system is a kind of stable, simple, effective, versatile technology for quantities of foreign protein expression in

Lepidopteran cells. Previous researches have demonstrated that foreign proteins as a content of NPVs must have two characteristics: the first one is proteins have to possess a nuclear localization signal (NLS) sequence, which is necessary for the transportation of foreign protein from the cytoplasm to the nucleus[22]. Another one is the signal of NPV guide, which is also indispensable, due to this signal can be identified as the structure protein in NPV[23].

Progeny nucleocapsids assembled in the nucleus have two fortunes. During the early phase of infected viral DNA are targeted to nucleocapsids then mature at the surface of cell to produce BV whereas ODVs, highly stable in the environment, mature later in the process of infection by obtaining a viral envelope from an intranuclear source of membrane microvesicles. The formation of intranuclear membranes is the result of budding of discrete regions of the inner nuclear membrane (INM) into the nucleoplasm[24]. ODV envelope protein ODV-E66 processes an important sequence INM-sorting motif (INM-SM) which is effective to traffic fusion proteins to intranuclear membranes and the ODV envelope. Similar with E66, BV/ODV-E26 also can transport foreign protein from the ER to the nuclear envelope and utilize the INM-SM to load them to intranuclear membranes[25, 26].

In consideration of this feature, we constructed the recombinant baculoviruses expressing the fusion protein of BV/ODV-E26 and EGFP. These fusion products were obtained in the OBs from both SF9 and BmN insect cells. In another experiment, we also tried to construct a fusion protein GP41 with EGFP, but the fluorescence cannot be found to localize in OBs at 72h p.i. (Data not shown). The reason might be that GP41 is a protein inside of ODV and locates in the tegument region between the envelope membrane and the capsid, whereas, BV/ODV-E26 is mainly resides in the outer envelope. By SDS-PAGE analysis of the OB contents, another major nucleocapsid protein VP39 was found in purified precipitate which is respond for infection. In previous study, this protein was able to bind to cellular actin without assistance, resulting in its rearrangement to form the cable, and leading to cytoskeleton changes in favor of the virus-encoded protease hydrolysis[27, 28]. We inferred that this protein might be used for foreign gene fusing expression as well.

The strong detergents (SDS and DTT) cannot wash out the fusion products from the OBs. This result indicated that the OBs containing BV/ODV-E26 fusion products can be purified from infected cells or insects by washing with detergents. Beyond this expression technical, a protease-recognized sequence is introduced between the bv/odv-e26 and foreign gene, it might be easier for product purification by protease digestion from the OBs. Further evidence should be investigated.

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