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Effect of a *Bombyx mori* Protein Disulfide Isomerase on Production of Recombinant Antibacterial Peptides

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Abstract

The insect baculovirus expression vector system (BEVS) is useful for producing biologically active recombinant proteins. However, the overexpression of heterologous proteins using this system often results in misfolded proteins and the formation of protein aggregates. To overcome this limitation, we developed a versatile baculovirus expression and secretion system using *Bombyx mori* protein disulfide isomerase (bPDI) as a fusion partner. bPDI gene fusion was found to improve the secretions and antibacterial activities of recombinant nuecin and enbocin proteins. Thus, we conclude that bPDI gene fusion is a useful addition to BEVS for the large-scale production of bioactive recombinant proteins.

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Introduction

The production of recombinant proteins from cultured insect cells for use in diagnostics, protein and biomedical research, and as vaccines is increasing, and currently is a major biotechnology research topic (Harrison and Jarvis, 2006; van Oers, 2006). The baculovirus expression vector system (BEVS) is a powerful recombinant protein expression system based on insect cells, which allow posttranslational modifications to occur in the endoplasmic reticulum (ER) (Yun *et al*., 2005a; Yun *et al*., 2005b). However, recombinant proteins obtained using the BEVS system often have low bioactivities and are poorly secreted, because of protein aggregation and degradation in the ER lumen. Endogenous protein biosynthesis in BEVS host cells is essentially shutdown immediately after viral infection because of high levels of exogenous gene production. Moreover, this shutdown leads to molecular chaperone and foldase insufficiencies in the ER, which in turn result in protein aggregation (Fath-Goodin *et al*., 2006; Teng *et al*., 2013).

Protein disulfide isomerase (PDI) catalyzes the oxidation of disulfides and the isomerization of incorrect disulfides in new polypeptides during folding in the oxidizing environment of the ER. PDI consists of four domains (a-b-b'-a'); the a and a' domains contain catalytic CGHC motifs, whereas the b and b' domains have no catalytic activity (Turano *et al*., 2002; Wilkinson and Gilbert, 2004). We previously isolated *Bombyx mori* protein disulfide isomerase (bPDI), which has two thiol oxidoreductase sites and enzymatic activity for reduced and scrambled RNase, like other PDI family members (Goo *et al*., 2002; Goo *et al*., 2008). However, bPDI expression is low in baculovirus-infected cells, especially when the recombinant protein is first expressed. In addition, is possible that PDI exhibits chaperone-like activity, which suppresses

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aggregation, and thus, increases heterologous protein folding and secretion. We previously expressed the *B. mori* antibacterial peptide in insect Sf9 cells using BEVS, but failed to obtained high expression (Yun *et al*., 1997; Yun *et al*., 2002). For the large-scale production of bioactive antibacterial peptides, we constructed a modified BEVS vector that contains nuecin and enbocin fused with the bPDI gene. This modification was found to improve recombinant nuecin and enbocin secretion and antibacterial activity, which suggests that bPDI gene fusion can be used to improve the productions of other biologically active recombinant proteins.

Materials and Methods

Experimental insect cell line

Sf9 cells derived from the pupal ovarian tissue of *Spodoptera frugiperda* were maintained as an adherent cell culture in TC-100 medium (Sigma) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), as described previously (Yun *et al*., 2005a).

Construction of transfer vector plasmid

To construct secreted bPDI, the ER retention signal (KDEL; 5'-AAA GAC GAG TTA-3') in the C terminus of bPDI cDNA was removed by double restriction enzyme digestion with *Bam*HI and *Stu*I. The resulting bPDI fragment was inserted into the pGEM-T vector to form pGEMT-(bPDI-KDEL). To construct the recombinant baculovirus, pGEMT-bPDI(-)KDEL was digested again with *Bam*HI and *Stu*I. The resulting bPDI-KDEL fragment was subcloned into the baculovirus vector pBAC-1 to form pBAC1-(bPDI-KDEL). pBACl-(bPDI-KDEL), pBAC1-nuecin, pBAC1-enbocin or pBAC1-(bPDI-KDEL)-nuecin-enbocin was co-transfected with linearized viral DNA (Novagen) into Sf9 cells, and selection was performed by staining with 50 mg/ml^{-1} neutral red and 250 mg/ml^{-1} X-Gal 3 days post-infection (p.i.). The plaques formed by the recombinant baculoviruses were plaque-purified three times and designated vAc-nuecin, vAc-enbocin or vAc-(bPDI-KDEL)-nuecin-enbocin.

SDS-PAGE and Western blots

Sf9 insect cells were mock-infected, or infected with wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) or recombinant AcNPVs at a multiplicity of infection of 10^6 in a 35-mm dish. After incubation at 27°C, cells were harvested 1, 2, 3, 4, or 5 days post-infection. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the cell lysates or media, uninfected Sf9 cells and virus-infected cells were washed twice with phosphate-buffered saline (PBS), mixed with protein sample buffer, and boiled. Total cellular lysates and media were then subjected to 12.5% (v/v) SDS-PAGE. After electrophoresis, gels were fixed and stained with 0.1% (v/v) Coomassie Brilliant Blue R-250. Proteins were blotted onto polyvinylidene (PVDF) membranes (Amersham Biosciences) in transfer buffer [25 mM Tris-HCl, pH 7.6, and 192 mM glycine in 20% (v/v) methanol] at 30 V, overnight at 4°C. Membranes were then blocked in l% bovine serum albumin (BSA) for 2 h at room temperature, and incubated with anti-6×His-tag (Invitrogen) anti-serum (1:1,000 v/v) for 1 h at room temperature. After washing in TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.05% Tween 20), membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:10,000 v/v; Clontech) for 30 min at room temperature. After repeated washing, substrate solution (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5 mM $MgCl_2$) containing Nitro-Blue Tetrazolium and 5-bromo-4-chloroindoyl phosphate was added. The reaction was quenched with distilled water.

Antibacterial assays of recombinant proteins

Recombinant proteins were tested for antibacterial activity using the inhibition zone assay (Ponti *et al*., 1999). Briefly, bacterial strains in the logarithmic phase were grown on LB medium. Sf9 cell supernatants were collected at 4 days post-infection. Supernatants were concentrated and poured onto small paper disks (3 mm diameter, 1 mm deep) placed on thin agar in LB medium containing test bacteria, and then incubated at 37°C for 18 h. Antibacterial activity was identified by the formation of clear zones around wells after incubation.

Results and Discussion

The ER contains molecular chaperones that optimize the folding and assembly of newly synthesized secretory or cytoplasmic proteins. However, little is known about the role of PDI. In a

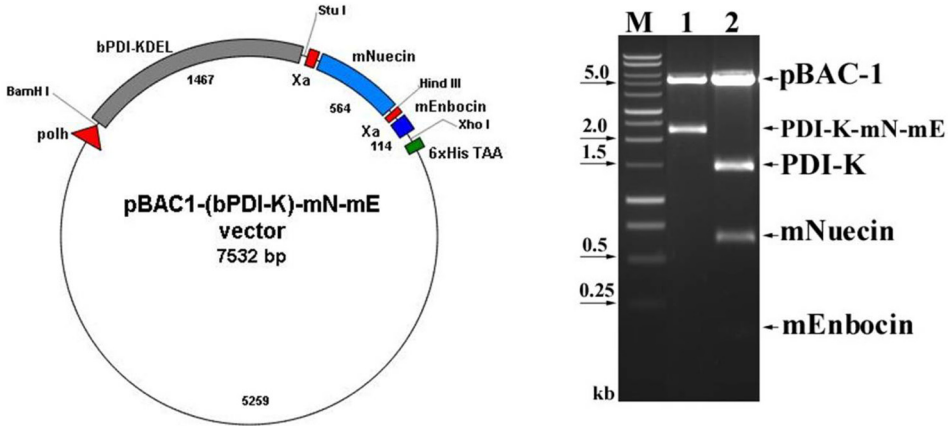

Fig. 1. Construction of baculovirus transfer vector for production of chemeric mature nuecin and enbocin fused bPDI lacking the ER retention signal KDEL. The open reading frame of bPDI lacking the ER retention signal KDEL (bPDI-KDEL) was subcloned into the *Bam*HI and *Stu*I site in baculovirus transfer vector pBAC-1. The coding sequence of mature nuecin (mNuecin) and enbocin (mEnbocin) linked factor Xa cleavage site was flanked by *Stu*I and *Xho*I site, and then inserted into the pBAC1-(bPDI-KDEL) vector with *Stu*I and *Xho*I. The baculovirus transfer vector pBAC1-(bPDI-KDEL)-mNuecin-mEnbocin was disgested with *Bam*HI/*Xho*I (lane 1) and *Bam*HI/*Stu*I/ *Hind*III/*Xho*I (lane 2). M, 1 kb ladder DNA markers.

previous study, we isolated and characterized bPDI (Goo *et al*., 2002), and because the over- or co-expression of chaperone proteins is known to enhance protein secretion, we attempted to increase secretory protein production by co-expressing bPDI in BEVS.

We previously showed that that removing the ER retention signal (KDEL) from bPDI cDNA increased bPDI secretion. We first removed the KDEL by digestion with *Bam* HI and *Stu* I, and then inserted the resulting bPDI fragment into the pGEM-T vector to form pGEMT-(bPDI-KDEL). After a second *Bam*HI and *Stu*I digestion, the bPDI-KDEL fragment was subcloned into the baculovirus transfection vector pBAC-1 to form pBAC1-(bPDI-KDEL), which contained an open reading frame for bPDI lacking the KDEL sequence. The coding sequence of mature nuecin (mNuecin) and enbocin (mEnbocin) linked factor Xa cleavage site was flanked by *Stu*I and *Xho*I site, and then inserted into the pBAC1-(bPDI-KDEL) vector with *Stu* I and *Xho*I. The baculovirus transfer vector pBAC1-(bPDI-KDEL)-mNuecin-mEnbocin was disgested with *Bam*HI/*Xho*I (Fig. 1. lane 1) and *Bam*HI/*Stu*I/ *Hind*III/*Xho*I (Fig. 1. lane 2). We have confirmed that the (bPDI-KDEL)-mNuecin-mEmbocin was correctly inserted into pBAC-1 vector (Fig. 1). We then tested whether bPDI-KDEL improves the production of nuecin and enbocin by SDS-PAGE (Fig. 2A) and Westen blotting (Fig. 2B). Cells infected with vAc-(bPDI-KDEL)-nuecin-enbocin caused large amounts of nuecin and enbocin to be produced (Fig. 2A. lane 3, Fig. 2B. lane 3). Mis- or unfolded secretory proteins are known to be retained in the ER by ER chaperones like Bip and GRP94 (Kim *et al*., 1996). Thus, these results suggest that bPDI assists in the folding of newly synthesized poly-polypeptides for

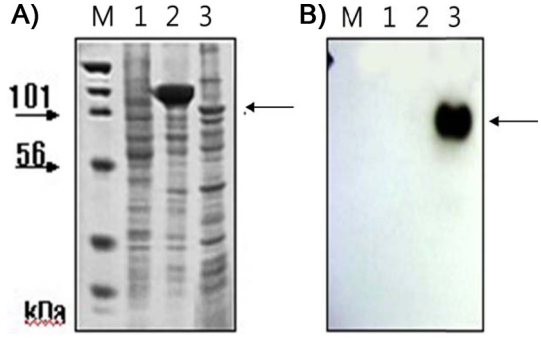

Fig. 2. SDS-PAGE of cell lysates (A) and Western blots of cell culture media (B) for chimeric nuecin and enbocin fused with bPDI-KDEL. Sf9 cells (3.0×10^6) were infected with recombinant baculovirus [vAc-(bPDI-KDEL)-mNuecin-mEnbocin; lane 3)] encoding (bPDI-KDEL)-mNuecin-mEnbocin-His6. Cells and cell culture media were harvested 96 h after infection (A). Western blots were performed using His6-tag antibody (B). Lane 1, proteins extracted from normal cells; lane 2, proteins extracted from cells infected with wild-type baculovirus. Arrows indicate the putative (bPDI-KDEL)-mNuecin-mEnbocin band.

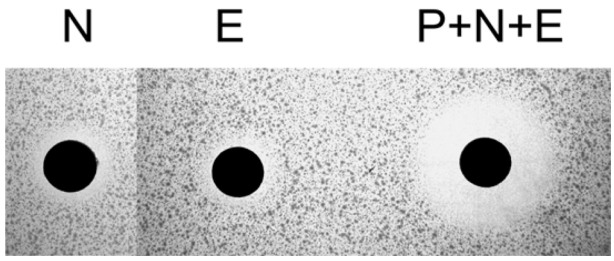

Fig. 3. Antibacterial activity of recombinant nuecin and enbocin protein against Escherichia coli. 5 ml of culture medium (2 x 10^6 cells ml^{-1}) were concentrated to 500 μl using a freezing dryer; 40 μl of concentrated sample were loaded onto a paper disk. N, recombinant nuecin; E, recombinant enbocin; P+N+E, chemric recombinant nuecin and enbocin with bPDI-KDEL.

oxidation and/or disulfide isomerization, which improves nuecin and enbocin production.

Nuecin and enbocin have antibacterial activity against *Escherichia coli*, but studies on the topic have been limited by poor nuecin production (Yun *et al*., 2002). To test the antibacterial activity of recombinant nuecin and enbocin, a supernatant containg the (bPDI-KDEL)-Xa-nuecin-Xa-enbocin fusion proteins were cleavage by the treatment of factor Xa protease, and then performed inhibition zone assays against *E. coli* (Fig. 3).

We performed inhibition zone assays against *E. coli* (Fig. 3). The antibacterial activity of nuecin and enbocin was approximately 24-fold higher than that of nuecin or enbocin alone, indicating that bPDI promotes nuecin and enbocin trafficking and secretion and maintains nuecin activity. We also used inhibition zone assays to test the antibacterial activity of nuecin and enbocin produced against nine bacteria pathogenic in plants, i.e., *Pseudomonas syringae*, *Pseudomonas tolaasii Staphylococcus aureus*, *Agrobacterium tumefaciens*, *Ralstonia solacerum*, *Erwinia mallotivora*, *Pectobacterium carotovorum*, *Erwinia chrysantuemi*, and *Bacillus megaterium* (Fig. 4). Nuecin and enbocin showed the strongest activity against *E. mallotivora*, *E. chrysantuemi*, *R. solacerum*, and *P. carotovorum*. It showed moderate activity against *P. syringae*, *P. tollasii*, and *B. megaterium*, and weak activity against A. tumefaciens. However, Nuecin and enbocin was not ineffective against S. aureus (Fig. 4). These differences might be due to bacterial result from differences in the membrane potential differences of each bacterium.

The expression of exogenous proteins by BEVS often leads to protein aggregation and intracellular accumulation. Therefore, we

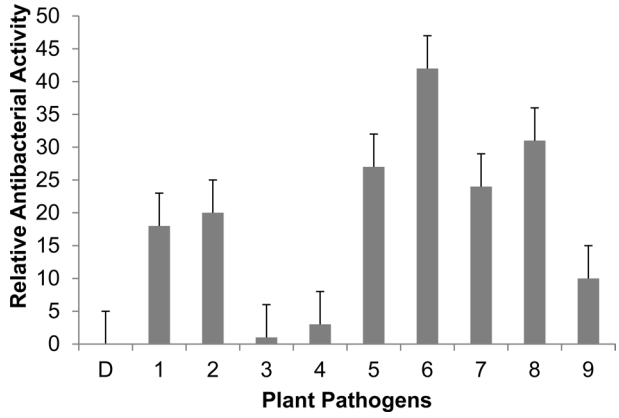

Fig. 4. Antibacterial activity of recombinant nuecin and enbocin against various plant pathogens. Five ml of culture medium (2×10^6 cells ml-1) were concentrated to 500 μl using a freeze-dryer, and 40 μl of concentrated samples were loaded onto paper disk. Values are the mean diameter of the clear zones ± standard deviation from three independent experiments. $P < 0.05$ versus control. D, distilled water (control); 1, *P. syringae*; 2, *P. tollasii*; 3, *S. aureus*; 4, *A. tumefaciens*; 5, *R. solacerum*; 6, *E. mallotivora*; 7, *P. carotovorum*; 8, *E. chrysantuemi*; and 9, *B. megaterium*.

developed a baculovirus expression and secretion system using bPDI as a gene fusion partner. Linking antibacterial peptides to bPDI was found to increase secretion and antibacterial activity, which suggests that bPDI may be useful for the mass productions of other recombinant proteins.

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