

Bombyx mori protein disulfide isomerase enhances the production of nuecin, an antibacterial protein

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The insect baculovirus expression vector system (BEVS) is useful for producing biologically active recombinant proteins. However, the overexpressions of foreign 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 proteins. Thus, we conclude that bPDI gene fusion is a useful addition to BEVS for the large-scale production of bioactive recombinant proteins. [BMB reports 2008; 41(5): 400-403]

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 (1, 2). 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; 3, 4). 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 (5).

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 (6, 7).

We previously isolated *Bombyx mori* protein disulfide isomerase (bPDI; 8), which has two thiol oxidoreductase sites and enzymatic activity for reduced and scrambled RNase, like other PDI family members. However, bPDI expression is low in baculovirus-infected cells, especially when the recombinant protein is first expressed. In addition, it is possible that PDI exhibits chaperone-like activity, which suppresses aggregation, and thus, increases heterologous protein folding and secretion.

We previously expressed the *B. mori* nuecin gene, which encodes an antibacterial protein, in insect Sf9 cells using BEVS, but failed to obtain high expression (9, 10). For the large-scale production of bioactive nuecin, we constructed a modified BEVS vector that contains nuecin fused with the bPDI gene. This modification was found to improve recombinant nuecin secretion and antibacterial activity, which suggests that bPDI gene fusion can be used to improve the productions of other biologically active recombinant proteins.

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 previous study, we isolated and characterized bPDI (8), 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 found that removing the ER retention signal (KDEL) from bPDI cDNA increased bPDI secretion. KDEL was removed by digestion with *Bam*HI and *Stu*HI, and the resulting bPDI fragment was inserted into a pGEM-T vector to form pGEMT-bPDI(-)KDEL (Fig. 1). 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. Gel electrophoresis was used to confirm that bPDI(-)

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Received 14 January 2008, Accepted 15 February 2008

Keywords: *Bombyx mori*, Nuecin, Protein disulfide isomerase (PDI)

KDEL was correctly inserted into pBAC-1 vector (Fig. 1).

To confirm the expression of the modified bPDI [i.e., bPDI(-)KDEL], Sf9 cells were infected with vAc-bPDI or vAc-bPDI(-)KDEL, and cell lysates were subjected to Western blotting (Fig. 2). Cells infected with bPDI(-)KDEL were found to produce more bPDI (Fig. 2, lane 4) than cells infected with intact bPDI (Fig. 2, lane 3).

We then examined whether bPDI(-)KDEL improves nuecin production by SDS-PAGE (Fig. 3A) and Western blotting (Fig. 3B). Cells infected with vAc-nuecin without bPDI(-)KDEL produced undetectable levels of nuecin (Fig. 3B, lane 2). However, transfection with vAc-bPDI(-)KDEL-nuecin caused large amounts of nuecin to be produced (Fig. 3B, lane 3). Mis- or unfolded proteins are known to be retained in the ER by ER chaperones like Bip and GRP94 (11). Thus, these results suggest that bPDI assists in the folding of newly synthesized poly-

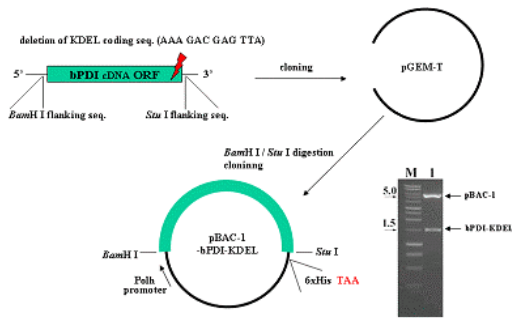


Fig. 1. Preparation of recombinant secretory bPDI lacking the ER retention signal KDEL. The open reading frame of bPDI with its ER retention signal sequence was digested at *Bam*HI and *Stu*I sites, and then inserted into pGEM-T vector. pGEMT-bPDI(-)KDEL was re-digested with *Bam*HI and *Stu*I, and the cDNA fragment obtained was subcloned into the *Bam*HI and *Stu*I site in baculovirus transfer vector pBAC-1. pBAC1-bPDI(-)KDEL was also digested with *Bam*HI and *Stu*I (lane 1). M, 1-kb DNA marker.

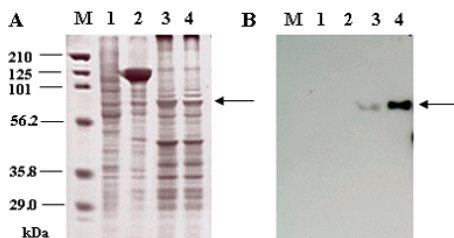


Fig. 2. SDS-PAGE of cell lysates (A) and Western blotting of cell culture media (B) of secretory bPDI. Sf9 cells (3.0×10^6) were infected with the recombinant baculovirus vAc-bPDI (lane 3) or with vAc-bPDI(-)KDEL (lane 4) encoding bPDI-His₆ and bPDI(-)KDEL-His₆, respectively. Cells and cell culture media were harvested 96 h after infection. Western blots were performed using His₆-tag antibody. Lane 1, proteins extracted from normal cells; lane 2, proteins extracted from cells infected with wild-type baculovirus. Arrows indicate the bPDI(-)KDEL band.

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

Nuecin has antibacterial activity against *Escherichia coli*, but studies on the topic have been limited by poor nuecin production (10). To test the antibacterial activity of recombinant nuecin fused with bPDI(-)KDEL, we performed inhibition zone assays against *E. coli* (Fig. 4A). The antibacterial activity of nuecin fused with bPDI(-)KDEL was approximately 20-fold higher than that of nuecin alone, indicating that bPDI promotes nuecin trafficking and secretion and maintains nuecin activity. We also used inhibition zone assays to test the antibacterial activity of nuecin produced against nine bacteria pathogenic in plants, i.e., *Pseudomonas syringae*, *P. tolasii*, *Staphylococcus aureus*, *Agrobacterium tumefaciens*, *Ralstonia solanaceum*, *Erwinia mallotivora*, *Pectobacterium carotovorum*, *Erwinia chrysanthemi*, and *Bacillus megaterium* (Fig. 4B). Nuecin fused with bPDI(-)KDEL showed the strongest activity against *R. solanaceum*, *E. mallotivora*, *P. carotovorum*, and *E. chrysanthemi*. It showed moderate activity against *P. syringae*, *P. tolasii*, *E. mallotivora*, and *P. carotovorum*, and weak activity against *A. tumefaciens*. However, Nuecin it was not ineffective against *S. aureus*. 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 developed a baculovirus expression and secretion system using bPDI as a gene fusion partner. Linking nuecin 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.

MATERIALS AND METHODS

Cell culture

Sf9 cells derived from the pupal ovarian tissue of *Spodoptera*

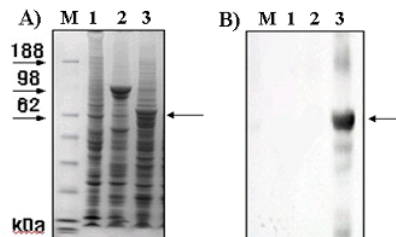


Fig. 3. SDS-PAGE of cell lysates (A) and Western blots of cell culture media (B) for chimeric nuecin fused with bPDI(-)KDEL. Sf9 cells (3.0×10^6) were infected with recombinant baculovirus [vAc-bPDI(-)KDEL-nuecin; lane 3] encoding bPDI(-)KDEL-nuecin-His₆. Cells and cell culture media were harvested 96 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-nuecin band.

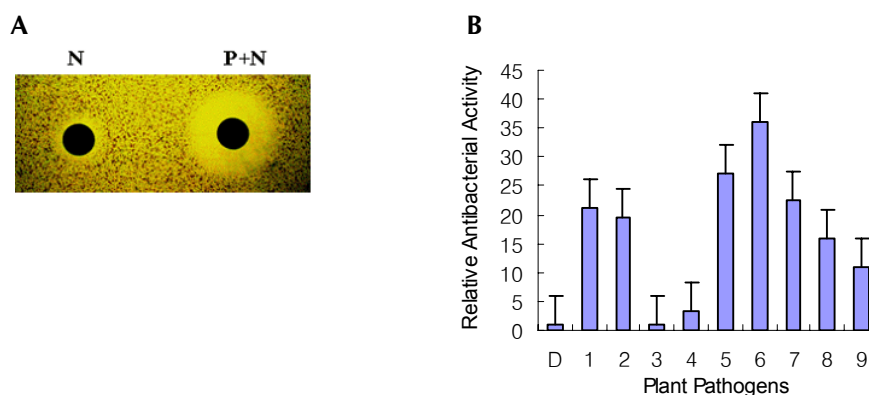


Fig. 4. Antibacterial activity of recombinant nuecin against *E. coli* versus various plant pathogens. Five ml of culture medium (2×10^6 cells ml^{-1}) were concentrated to 500 μl using a freeze-dryer, and 40 μl of concentrated samples were loaded onto paper disks. (A) N, recombinant nuecin; P + N, chimeric recombinant nuecin fused with bPDI(0)KDEL. (B) The mean diameter of clear zones (\pm standard deviation from three independent experiments). $P < 0.05$ versus control. D, distilled water (control); 1, *P. syringae*; 2, *P. tolasii*; 3, *S. aureus*; 4, *A. tumefaciens*; 5, *R. solanaceum*; 6, *E. mallotivora*; 7, *P. carotovorum*; 8, *E. chrysanthemi*; and 9, *B. megaterium*.

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 (3).

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. pBAC1-bPDI(-)KDEL, pBAC1-nuecin, or pBAC1-bPDI(-)KDEL-nuecin 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-bPDI(-)KDEL or vAc-bPDI(-)KDEL-nuecin.

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 106 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 1% bovine serum albumin (BSA) for 2 h at room temperature, and incubated with anti-6 \times 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 (12). 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.

Acknowledgments

This work was supported by a grant (Code # 20070401034024) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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