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.

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
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 obtain 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 *BamHI* and *StuI*. 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 *BamHI* and *StuI*. The resulting bPDI-KDEL fragment was subcloned into the baculovirus vector pBAC1 to form pBAC1-(bPDI-KDEL). pBAC1-(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 1% 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
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 removing the ER retention signal (KDEL) from bPDI cDNA increased bPDI secretion. We first removed the KDEL by digestion with BamHI and StuI, and then inserted the resulting bPDI fragment into the pGEM-T vector to form pGEMT-(bPDI-KDEL). After a second BamHI and StuI digestion, the bPDI-KDEL fragment was subcloned into the baculovirus transfection vector pBAC-1 to form pBAC1-(bPDI-KDEL). The coding sequence of mature nuecin (mNuecin) and enbocin (mEnbocin) linked factor Xa cleavage site was flanked by StuI and XhoI site, and then inserted into the pBAC1-(bPDI-KDEL) vector with StuI and XhoI. The baculovirus transfer vector pBAC1-(bPDI-KDEL)-mNuecin-mEnbocin was digested with BamHI/XhoI (lane 1) and BamHI/StuI/HindIII/XhoI (lane 2). M, 1 kb ladder DNA markers.

![Fig. 1. Construction of baculovirus transfer vector for production of chimeric 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 BamHI and StuI 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 StuI and XhoI site, and then inserted into the pBAC1-(bPDI-KDEL) vector with StuI and XhoI. The baculovirus transfer vector pBAC1-(bPDI-KDEL)-mNuecin-mEnbocin was digested with BamHI/XhoI (lane 1) and BamHI/StuI/HindIII/XhoI (lane 2). M, 1 kb ladder DNA markers.](image1)

![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.](image2)

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.

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
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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