Secretion of the Antibacterial Recombinant Protein Enbocin

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

Key words: Baculovirus Expression Vector System (BEVS), *Bombyx mori*, Enbocin, Protein Disulfide Isomerase (PDI)

Introduction

The production of recombinant proteins from cultured insect cells for use in diagnostics, protein and biomedical research, and vaccines is increasing and is a major focus of biotechnology research (Harrison and Jarvis, 2006; van Oers, 2006). The baculovirus expression vector system (BEVS) is a powerful recombinant protein expression system using insect cells that allows posttranslational modification in the endoplasmic reticulum (ER) (Yun *et al*., 2005a, b). However, recombinant proteins in this system often have low bioactivity and poor secretion because of protein aggregation and degradation in the ER lumen. Endogenous protein biosynthesis in the BEVS host cell is essentially shut down just after infection with the virus because of the high levels of exogenous gene production. This shutdown leads to insufficient expression of molecular chaperones and foldases in the ER, resulting in protein aggregation (Fath-Goodin *et al*., 2006).

Protein disulfide isomerase (PDI) catalyzes the oxidation of disulfides and the isomerization of incorrect disulfides on 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 the *Bombyx mori* protein disulfide isomerase (bPDI) (Goo *et al*., 2002), which has two thiol oxidoreductase sites and enzymatic activity for reduced and scrambled (malfolded) RNase, similar to other PDI family members. In addition, bPDI expression is very low in baculovirus-infected cells, especially when the recombinant protein is first being expressed. It is questionable that PDI also exhibits chaperone-like activity, which suppresses the aggregation and increases the folding and secretion of heterologous proteins. We previously expressed the *B. mori* enbocin gene, which encodes an antibacterial protein, in insect Sf9 cells using the BEVS, but we did not achieve high levels of expression (Yun *et al*., 1997, 2002). To allow the large-scale production of bioactive enbocin, we constructed modified BEVS vectors that contained antibacterial enbocin fused with the bPDI gene. This modification improved the secretion and antibacterial activity of recombinant enbocin, indicating that bPDI gene fusion may be useful for the production of other biologically active recombinant proteins.

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Materials and Methods

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). 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*H I and *Stu* I. The resulting bPDI fragment was inserted into the pGEM-T vector (Pharmacia, Uppsala, Sweden) to form pGEMT-bPDI(−)KDEL. To construct the recombinant baculovirus, pGEMT-bPDI(−)KDEL was digested again with *Bam*H I 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-enbocin, or pBAC1-bPDI(−)-KDEL-enbocin were co-transfected with linearized viral DNA (Novagen, Inc., Madison, USA) into Sf9 cells, and selection was performed by staining with 50 mg ml^{-1} Neutral Red and 250 mg ml^{-1} X-Gal on day 3 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-enbocin.

Sf9 insect cells were mock-infected (only vector) or infected with the wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) or the recombinant AcNPVs at a multiplicity of infection in a 35-mm dish (106 p. f.u. cells). After incubation at 27 °C, the cells were harvested on days 1, 2, 3, 4, or 5 p.i. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the cell lysates or media, the uninfected Sf9 cells and virus-infected cells were washed twice with phosphate-buffered saline (PBS), mixed with protein sample buffer, and boiled. The total cellular lysates and media were subjected to 12.5% (v/v) SDS-PAGE. After electrophoresis, the gels were fixed and stained with 0.1% (v/v) Coomassie Brilliant Blue R-250. The proteins were blotted onto a polyvinylidene (PVDF) membrane (Amersham Biosciences, Korea Ltd., Seoul, Korea) in transfer buffer [25 mM Tris (tris(hydroxymethyl)aminomethane)-HCl, pH 7.6, and 192 mM glycine in 20% (v/v) methanol] at 30 V overnight at 4 °C. The membrane was blocked by incubation in 1% bovine serum albumin (BSA) for 2 h at room temperature and then incubated with anti-6 × His-tag (Invitrogen, Co., Carlsbad, USA) 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, 0.05% Tween 20), the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:10,000 v/v; Clontech, Inc., Mountain View, USA) for 30 min at room temperature. After repeated washing, the substrate solution (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$) containing nitro-blue tetrazolium and 5-bromo-4-chloroindoyl phosphate was added. The reaction was quenched with distilled water.

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 4 d p.i. The supernatants were concentrated and poured onto small paper disks, 3 mm in diameter and 1 mm deep, which were placed on thin agar in LB medium containing test bacteria and incubated at 37 °C for 18 h. Antibacterial activity was identified by the formation of clear zones around the wells after incubation.

Results and Discussion

The ER contains molecular chaperones that optimize the folding and assembly of newly synthesized secretory or cytoplasmic proteins. Little is known about the role of PDI. We isolated and characterized PDI from the silk worm, *Bombyx mori* (bPDI) (Goo *et al.*, 2002). Because over- or co-expression of chaperone proteins enhances protein secretion, we attempted to increase the secretory protein production through co-expression of bPDI in the BEVS.

We previously showed that removing the ER retention signal (KDEL) from bPDI cDNA increases bPDI secretion. We first removed the KDEL by digestion with *Bam*H I and *Stu* I and then inserted the resulting bPDI fragment into the pGEM-T vector to form pGEMT-bPDI(−)KDEL (Fig. 1). After a second *Bam*H I and *Stu* I digestion, the bPDI(−)KDEL fragment was subcloned into the baculovirus transfection vector pBAC-1 to form pBAC1-bPDI(−)KDEL, which contains an open reading frame for bPDI lacking the KDEL sequence. We have confirmed that the bPDI(−)-KDEL was correctly inserted into the pBAC-1 vector (Fig. 1) by RT-PCR and the expression of the modified bPDI [*i.e.*, bPDI(−)KDEL] by West-

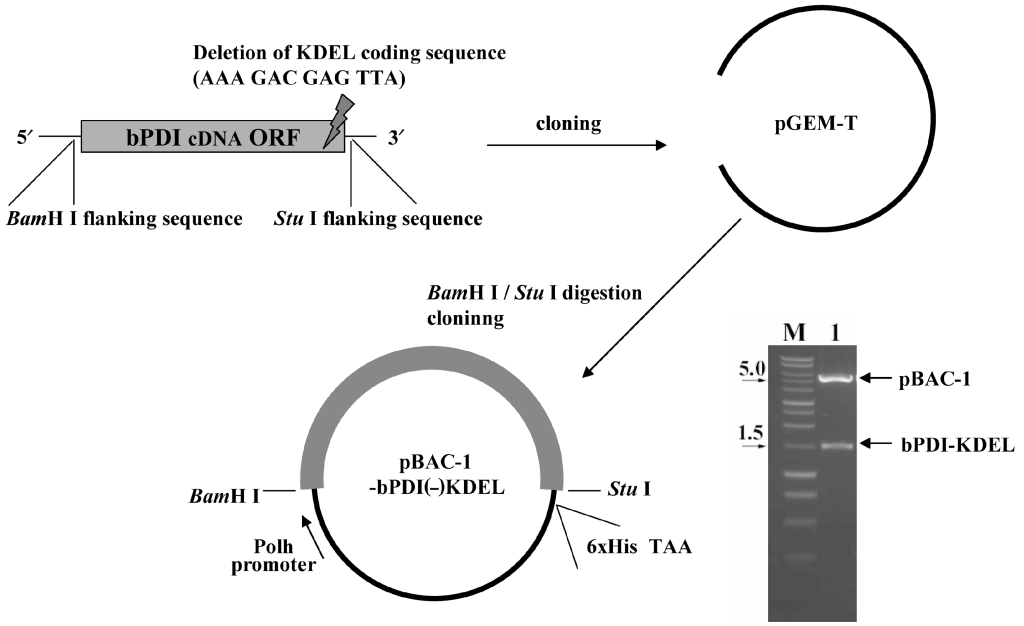

Fig. 1. Preparation of recombinant secretory bPDI lacking the ER retention signal KDEL. The open reading frame of bPDI with its ER retention signal sequence was digested at the *Bam*H I and *Stu* I sites and then inserted into the pGEM-T vector. pGEMT-bPDI(–)KDEL was digested again with *Bam*H I and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

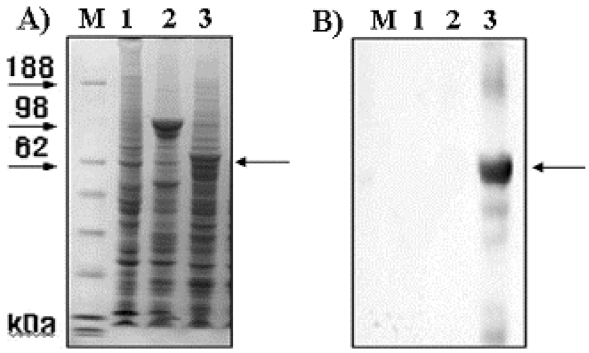

Fig. 2. (A) SDS-PAGE and (B) Western blots of chimeric enbocin fused with bPDI(–)KDEL. Sf9 cells ($3.0 \cdot 10^6$) were infected with recombinant baculovirus [vAc-bPDI(–)KDEL-enbocin; lane 3] encoding bPDI-(–)KDEL-nuecin-His_6. Cells and cell culture media were harvested 96 h after infection (A, lane 3). Western blots were performed using His_6-tag antibody (B, lane 4). Lane 1, protein extracted from normal cells; lane 2, protein extracted from cells infected with wild-type baculovirus. Panel A and B are the results of SDS-PAGE using cell lysates and Western blots using cell culture media, respectively. Arrows indicate the putative bPDI(–)KDEL-enbocin band.

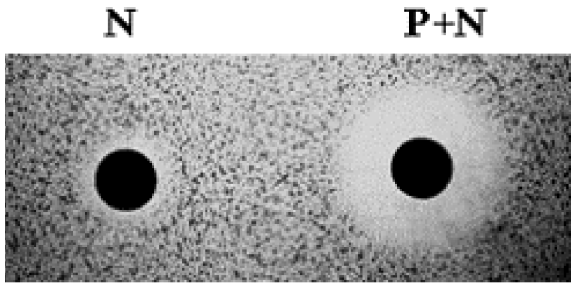

Fig. 3. Antibacterial activity of recombinant enbocin proteins against *Escherichia coli*. 5 ml of culture medium ($2 \cdot 10^6$ cells ml^{-1}) were concentrated to 500 μl using a freezing dryer; 40 μl of concentrated sample were loaded onto a paper disk. N, recombinant enbocin; P+N, chimeric recombinant enbocin fused with bPDI(–)KDEL.

ern blots (data not shown). We then tested whether bPDI(–)KDEL improved the production of enbocin or not (Fig. 2). Cells infected with vAc-enbocin without bPDI(–)KDEL did not produce a detectable amount of enbocin protein (Fig. 2, lane 2). However, transfection with vAc-bPDI(–)-KDEL-enbocin produced a large amount of enbocin (Fig. 2, lane 3). These results suggest that

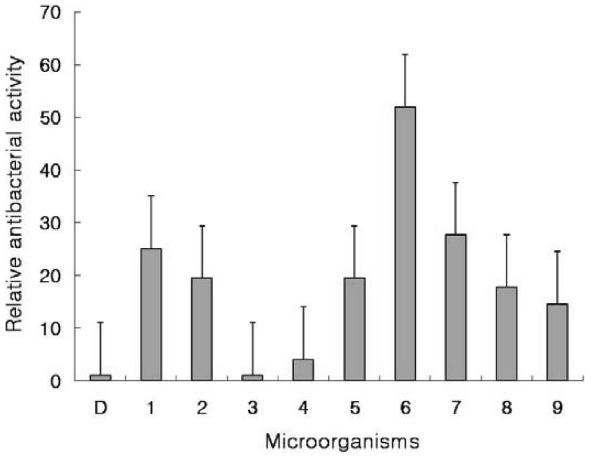

Fig. 4. Antibacterial activity of recombinant enbocin proteins against various plant pathogens. 5 ml of culture medium ($2 \cdot 10^6$ cells ml^{-1}) were concentrated to 500 μl using a freezing dryer; 40 μl of concentrated sample were loaded onto a paper disk. Values are the mean diameter of the clear zone ± standard deviation calculated 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. chrysanthemi;* 9, *B. megaterium.*

PDI assists the folding of newly synthesized polypeptides for the oxidation or/and isomerization of disulfides, which improves the enbocin production.

Enbocin shows antibacterial activity against *Escherichia coli*, but studies have been limited because of poor enbocin production (Yun *et al.*, 2002). To test the antibacterial activity of recombinant enbocin fused with bPDI(−)KDEL, we performed an inhibition zone assay against *E. coli* (Fig. 3). The antibacterial activity of enbocin fused with bPDI(−)KDEL was approx. 20 times stronger than that of bPDI alone, indicating that bPDI promotes enbocin trafficking and secretion without loss of activity. As shown in Fig. 4, we also used the inhibition zone assay to test the antibacterial activity of enbocin against nine plant pathogenic bacteria, including *Pseudomonas syringae, Pseudomonas tollasii, Staphylococcus aureus, Agrobacterium tumefaciens, Ralstonia solacerum, Erwinia mallotivora, Pectobacterium carotovorum, Erwinia chrysanthemi*, and *Bacillus megaterium*. Enbocin fused with bPDI(−)KDEL showed the strongest activity against *R. solacerum, E. mallotivora, P. carotovorum,* and *E. chrysanthemi*. It showed moderate activity against *P. syringae* and, *P. tollasii,* and weak activity against *A. tumefaciens.* Enbocin was not effective against *S. aureus.* These differences may result from differences in the membrane potential of each bacterium.

The expression of exogenous proteins by the BEVS often leads to protein aggregation and intracellular accumulation. We therefore developed a baculovirus expression and secretion system using bPDI as a gene fusion partner. Enbocin linked to bPDI showed increased secretion and antibacterial activity, suggesting that bPDI may be useful for the mass production of other recombinant proteins.

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