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Baculovirus Expression and Biochemical Characterization of the *Bombyx mori* Protein Disulfide Isomerase (bPDI)

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Protein disulfide isomerase (PDI) found in the endoplasmic reticulum (ER) catalyzes disulfide bond exchange and assists in protein folding of newly synthesized proteins. PDI also functions as a molecular chaperone and has been found to be associated with proteins in the ER. In addition, PDI functions as a subunit of two more complex enzyme systems: the prolyl-4-hydroxylase and the triacylglycerol transfer proteins. A cDNA that encodes protein disulfide isomerase was previously isolated from *Bombyx mori* (bPDI), in which open reading frame of 494 amino acids contained two PDI-typical thioredoxin active site of WCGHCK and an ER retention signal of the KDEL motif at its C-terminal, and we report its functional characterization here. This putative bPDI cDNA is expressed in insect Sf9 cells as a recombinant proteins using baculovirus expression vector system. The bPDI recombinant proteins are successfully recognized by anti-rat PDI antibody, and shown to be biologically active *in vitro* by mediating the oxidative refolding of reduced and scrambled RNase. This suggests that bPDI may play an important role in protein folding mechanism of insects.

Key words: Baculovirus, Enzymatic activity, bPDI, *Bombyx mori*, PDI, RNase A

Introduction

Protein disulfide bond formation is a rate-limiting step in protein folding and is catalyzed by enzymes belonging to the protein disulfide oxidoreductase superfamily, including PDI in eukaryotes and DsbA in bacteria (Noiva, 1994). PDI catalyzes disulfides oxidation and isomerizes incorrect disulfides on newly synthesized polypeptides undergoing correct folding in the oxidizing ER environments. It is now accepted that PDI is a multifunctional protein that is involved in the folding, assembly, and post-translational modification of many proteins in addition to actin filament polymerization, gene expression, cell-cell interaction and the regulation of the receptor function (Frand *et al.*, 2000). PDIs are known to share a strong DNA sequence homology and their expression is found abundantly in many cell types. The typical PDI structure has led to a five-domain model for enzyme activity, in which two protein-thiol oxidoreductase active site sequences of WCGHCK are shown in both the C-/N-terminal regions, and an ER retention signal of KDEL in the C-terminal. This evidences that the PDI is located/retained in the ER lumen and functions as an ER chaperone (Noiva, 1999; Ciaffi *et al.*, 2001; Warsome *et al.*, 2001).

In the previous study, it has been shown that a PDI homologue from *Bombyx mori* also had two thiol oxidoreductase sites and a KDEL motif in the C-terminal (Goo *et al.*, 2002b). The cDNAs encoding the PDI family have been isolated from a number of organisms or tissues. However, only one of the PDI cDNA sequences has been reported from the insect, *Drosophila melanogaster* and limited information on the PDI in insects is available (McKay *et al.*, 1995). It has already been demonstrated that bPDI expression depends on ER stress and hormones in the Bm5 cell line derived from *Bombyx mori* (Goo *et al.*, 2002a). In addition, most recent studies have demonstrated that coexpressed or overexpressed PDI increased

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the folding and secretion of heterologous proteins from the used cells (Ailor and Betenbaugh, 1999; Hsu *et al.*, 1996; Ritchie *et al.*, 1999). To establish that the forced expression of PDI in a cell is effective in enhancing the folding and secretion of heterologous proteins, it is essential to understand the enzymatic activity of bPDI protein. For this purpose, bPDI cDNA was expressed in Sf9 cells using baculovirus expression vector system, and we identified its enzymatic activity in this study here. This is the first report of biochemical characterization of PDI from insect.

Materials and Methods

Experimental insects and cells

Silkworms, *Bombyx mori* (Jam 306), were reared on an artificial diet at 24 – 27°C with 70 – 90% humidity. The culture cell line Bm5 derived from *B. mori* was cultured at 27°C in a TC-100 medium (Sigma Chemical, St. Louis, MO) with 10% fetal bovine serum (GIBCO) using the standard method.

Construction of Expression vector for bPDI and Western blot analysis

The cDNA encoding bPDI was amplified using a sense primer (5'-CGGGATCCCGGAAATGCGTGTTTTAATT TTCACG-3'; underline indicates the initial codon) and an antisense primer (5'-GAAGGCCTTCTAACTCGTCTTT GGCAGGC-3'; underline indicates the stop codon that was modified from the original TAA). The PCR products were ligated once to a TA cloning vector, pGEM-T (Promega). The pGEM-bPDI was digested with BamH I/ Stu I and subcloned into the baculovirus vector, pBAC1-bPDI. After incubation for 15 min, a mixture pBAC1-bPDI/BacVector-3000 Triple Cut Virus DNA (Novagen) with Eufectin (Novagen) was inoculated at 27°C for 5 hrs at the Sf-9 cell line. The infected cells were collected after 3 days and digested with lysis buffer (6.25 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% DTT). Electrophoresis was performed in 7.5% polyacrylamide gels. Protein transfer to a PVDF membrane was carried out in a semi-dry system from Bio-Rad. The PVDF membrane was quenched for 30 min in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 containing 2% non-fat dry milk (TBS-milk). Immunological detection was done by first incubating the PVDF membrane for 1 hr at room temperature with the primary antiserum in TBS containing 0.05% Tween 20. Subsequently, the PVDF membrane was incubated for 1 hr with peroxidase-conjugated secondary immunoglobulins. The PVDF membrane was washed extensively between each step with TBS-milk.

Purification of the PDI-His_6 fusion protein

To facilitate the isolation of recombinant *B. mori* bPDI proteins, the C-terminal of bPDI was His_6-tagged. The histidine tagged bPDI fusion proteins were purified by IMAC using a Ni^{2+}-immobilized resin (HisTrap chelating column; Pharmacia Biotech). The column was equilibrated with start phosphate buffer (20 mM phosphate, 0.5 M NaCl, 10 mM Imidazole, pH 7.4), and the homogenized/clarified sample was applied. Several fractions (0.5 mL) were collected as a flow through. The column was washed with start phosphate buffer, and sample fractions were collected as a wash sample. The fusion proteins were eluted by elution phosphate buffer of different imidazole concentration (50, 100, 200, 300, 400, and 500 mM). Fractions were monitored at A_{280} using the corresponding elution buffer as a blank and also detected by fluorescence intensity using a fluorescence spectrometer. Most bound proteins were eluted at imidazole concentration between 100 and 200 mM.

RNase activity assay

Protein disulfide isomerase activity was measured by determining its ability to renature "reduced and scrambled" RNase A. The reactivation of denatured RNase A was monitored as described by Lyles and Gilbert (1991) with modification. In brief, 5 mg of RNase A (Type XII-A. Sigma) were incubated 1 ml of 100 mM Tris-acetate, pH 8.0, 4 mM EDTA, 6 M guanidine hydrochloride, and 140 mM DTT at 25°C overnight to reduce the RNase A. Then guanidine hydrochloride and DTT were removed by passage through Bio-Gel P4 (Bio-Rad) that had been equilibrated with 0.1% acetic acid. The scrambled RNase A, which had exchanged cysteins, was purchased from Takara Shuzo Co. The activation of reduced and scrambled RNase were assayed with the purified bPDI recombinant protein by a time-course incubation during which aliquots were removed and RNase activity towards RNase was measured (Hillson *et al.*, 1984). Briefly, 0.5 mg/ml reduced and scrambled RNase were mixed with the purified bPDI recombinant protein or 5 μg/ml bovine liver PDI (Takara) and 10 μM 1,4-dithiothreitol (DTT) in 50 mM sodium phosphate buffer (pH 7.5) and incubation at 30°C. RNase was added at a concentration of 50 μg/ml. Aliquots were removed at different time intervals and abosorbance change was measured at 260 nm.

Results and Discussion

A *Bombyx mori* PDI homologue (bPDI) was previously isolated from the culture cell line Bm5, which was treated tunicamycin using a differential screening method (Hoog,

1991), and both strands sequenced the cDNA clone (Goo *et al.,* 2002a). The sequence data of the bPDI was submitted to Genbank under the accession number AF325211. Although a cDNA encoding bPDI showed a high sequence variation compare with known PDI cDNAs, the bPDI protein had two well conserved thiol oxidoreductase sites and a KDEL motif in the C-terminal, which is similar to the other PDIs (Goo *et al.,* 2002b). However, there is as yet no evidence regarding whether bPDI protein has enzymatic activity, as do other PDI family proteins. In order to characterize the enzymatic activity of bPDI protein, the cDNA corresponding to the bPDI protein was expressed in Sf9 cells as a C-terminal polyhistidine fusion protein.

To produce the bPDI recombinant proteins using baculovirus expression vector system (BEVS), baculovirus expression transfer vector (pBAC1-bPDI) was constructed (Fig. 1). The constructed pBAC1-bPDI and BacVector-3000 Triple Cut Virus DNA was co-transfected into Sf9 cells, then recombinant baculovirus, vAc-bPDI was selected by plaque assay. To analysis whether the bPDI gene was correctly introduced in wild type AcNPV viral genome, vAc-bPDI viral DNA was extracted from Sf9 cells, which harvested 72 hrs after vAc-bPDI infection. The extracted vAc-bPDI viral DNA used as template for PCR screening with sense and antisence primer used in construction of the baculovirus expression transfer vector, pBAC1-bPDI.

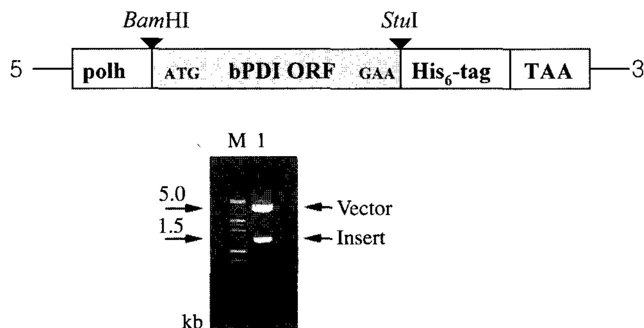

Fig. 1. Cloning of the open reading frame of the bPDI gene. M, 1 kb ladder DNA maker; lane 1, bPDI gene inserted in pBAC-1 baculovirus transfer vector digested with *BamHI/Stu*, and sample was analyzed on 1.0% agarose gel.

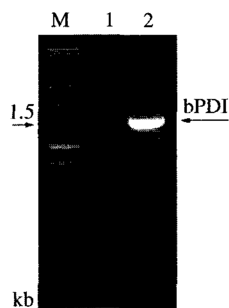

Fig. 2. PCR screening of the recombinant baculovirus, vAc-bPDI. PCR was performed with sense and antisense primer used in construction of the baculovirus transfer vector. M, 1 kb ladder DNA maker; lane 1, wild type baculovirus (AcNPV) viral DNA as template used for PCR screening; lane 2, vAc-bPDI viral DNA as template used for PCR screening.

As shown in Fig. 2, the PCR product of 1,500 bp size is correctly amplified in band when vAc-bPDI viral DNA as template used for PCR screening (lane 2), whereas no band estimated ATFC appeared when AcNVP viral DNA as template used for PCR screening (lane 1). The result suggests that the polyhedrin gene of AcNVP be replaced correctly with the *B. mori* bPDI gene. To confirm whether or not the cDNA encoding bPDI translates correctly *in vivo* by bPDI, the recombinant baculovirus (vAc-ATFC) was translated in the culture insect Sf-9 cell line.

As shown in Fig. 3, the cDNA encoding the *Bombyx mori* bPDI was successfully translated in bPDI (line 3 in panel A), which was also recognized by anti-rat PDI antibodies (line 3 in panel B). No band estimated PDI appeared between the normal cells and the cells infected with the wild type baculovirus (line 1 and 2 in panel A and B). In addition, to facilitate the isolation of the bPDI recombinant protein, the C-terminal of bPDI was His_6-tagged (Fig. 1). That is, the bPDI recombinant proteins were expressed from Sf9 as fusion polyhistidin-binding protein (bPDI-His_6), the bPDI-His_6 fusion proteins were purified by IMAC using a Ni^{2+}-immobilized resin (Fig. 3) and tested for PDI activity by using the reduced and scrambled RNase folding method. The ability to refold RNase that has been reduced, and denatured is classically used as a measure of protein disulfide isomerization (Lyles and Gilbert, 1991). While the spontaneous refolding of

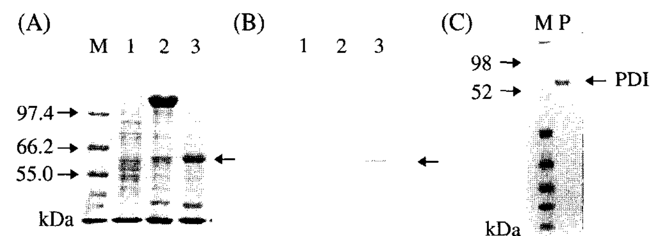

Fig. 3. SDS-PAGE (A), Western blot analysis (B) and purification of bPDI recombinant protein (C). Sf-9 cells (3.0×10^6) were infected with recombinant baculovirus (vAc-bPDI) encoding ATFC-His_6 at a total m.o.i of 5. Cells were harvested at 72 hrs after infection (lane 4). Western blot analysis was performed using a anti-rat PDI antibody. The bPDI was expressed and purified from Sf-9 cells infected with vAc-bPDI as fusion protein (bPDI-His_6). Lane 2 (Mo), protein extracted from normal cells; lane 3 (Wt), protein extracted from cells infected with wild type baculovirus; M, mid range protein size maker. Arrows indicate prepro bPDI protein.

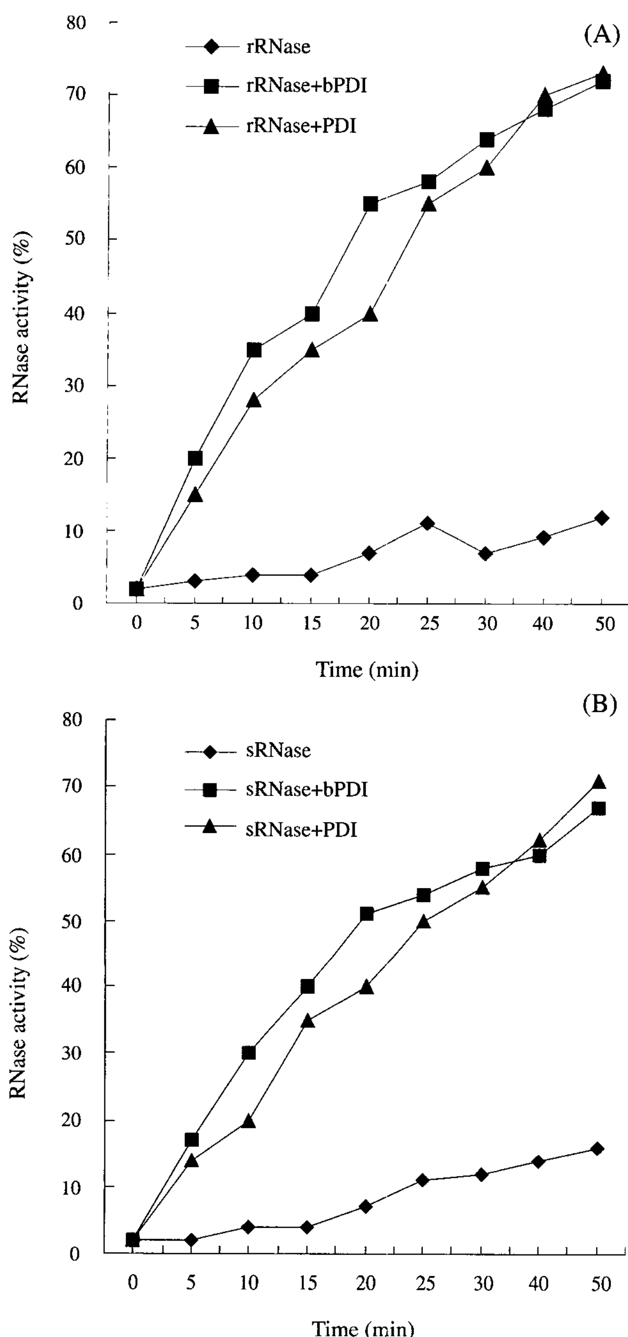

Fig. 4. Enzymatic activity of the recombinant bPDI protein. The assay mixture contained 10 μM DTT, 0.5 mg/ml reduced (rRNase) and scrambled RNase (sRNase) with 5 μg/ml PDI or purified recombinant bPDI protein or no other addition in 50 mM phosphate buffer (pH 7.5). RNase activity is monitored at the indicated time points. The graph shows the percentage refolding by bPDI recombinant protein and PDI (Takara).

reduced and scrambled RNase was scarcely time-dependant increase, the presence of recombinant bPDI and the bovine liver PDI (Takara) showed a time-dependant increase in RNase folding. That is, the bPDI recombinant protein reactivated 72 and 67%, respectively in reduced and scrambled RNase activity, indicating the presence of disulfide isomerase activity. In comparison, the commercially available purified PDI retrieved 73 and 72%, respectively in reduced and scrambled RNase activity. This suggests that bPDI belongs to a PDI family and may play an important role in protein folding mechanism of insects. In addition, further understanding of the enzymatic activity of bPDI among the known PDIs should provide important knowledge of the production of heterologous proteins in eukaryotic environments, in which frequently heterologous proteins form insoluble aggregates or are improperly folded in the ER.

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