

Domain a' of *Bombyx mori* Protein Disulfide Isomerase Has Chaperone Activity

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Protein disulfide isomerase (PDI) is an endoplasmic reticulum (ER)-localized multifunctional enzyme that can function as a disulfide oxidase, a reductase, an isomerase, and a chaperone. The domain organization of PDI is abb'xa'c, with two catalytic (CxxC) motifs and a KDEL ER retention motif. The members of the PDI family exhibit differences in tissue distribution, specificity, and intracellular localization. We previously identified and characterized the PDI of *Bombyx mori* (bPDI) as a thioredoxin-like protein that shares primary sequence homology with other PDIs. Here we compare the reactivation of inactivated rRNase and sRNase by bPDI and three bPDI mutants, and show that bPDI has mammalian PDI-like activity. On its own, the N-terminal a domain does not retain this activity, but the a' domain does. This is the first report of chaperone activity only in the a' domain, but not in the a domain.

Key words: Protein Disulfide Isomerase (PDI), *Bombyx mori*, PDI Activity

Introduction

Protein disulfide isomerase (PDI; EC 5.3.4.1) was found to catalyze oxidative protein folding in the lumen of the endoplasmic reticulum (ER) in eukaryotic cells more than 40 years ago, and it constitutes ~0.8% of the total cellular protein in mammalian cells (Dias-Gunasekara and Benham, 2005). PDI can catalyze both the oxidation and reduction of disulfides, as well as disulfide isomerization, under physiological conditions, in addition to several other functions (Ellgaard and Ruddock, 2005). PDI normally has six structural domains: a, b, b', x, a', and c. The a and a' domains each contain a CxxC active-site sequence motif similar to that in thioredoxin, which is required for disulfide bond formation in the cell. Domains b and b' are generally referred to as being important for substrate recognition and binding. The c domain contains a highly acidic calcium-binding site followed by a KDEL ER retention sequence (Gruber *et al.*, 2006; Noiva, 1999; Wang, 1998).

We previously isolated a PDI homolog from *Bombyx mori* (bPDI), which is highly expressed in

the fat body. The cDNA contains an open reading frame of 494 amino acids that encompasses two PDI-typical CxxC thioredoxin active sites in both the a and a' domains, as well as a KDEL ER retention signal in its C-terminal region. Drugs that increase ER stress (*e.g.*, A23187, tunicamycin, and DTT) increase *bPDI* mRNA expression, as do juvenile hormone and exogenous bacterial infections (Goo *et al.*, 2002a). In our previous study, we did not measure the bPDI activity, nor did we address whether both the a and a' thioredoxin active sites (CxxC) are required for PDI activity. Therefore, in this study, we generated three bPDI constructs and tested their PDI activity using an RNase activity assay.

Materials and Methods

Experimental cells

Sf9 cells, derived from *Spodoptera frugiperda*, were cultured at 27 °C in TC-100 medium containing 10% (v/v) heat-inactivated fetal bovine serum, as described previously (Yun *et al.*, 2005).

Purification of the bPDI-His₆ fusion protein

To isolate our recombinant bPDI proteins, we generated a C-terminal His₆-tagged version of bPDI, cloned it into the pBAC-1 baculovirus expression transfer vector (Novagen, Darmstadt, Germany), and co-transfected it with linearized viral DNA (Novagen) into Sf9 cells. The recombinant baculoviruses were plaque-purified three times and were designated as vAc-bPDI-His₆. vAc-bPDI-His₆ was purified by IMAC using an Ni²⁺-immobilized resin (His-Trap chelating column; Amersham Pharmacia Biotech., Uppsala, Sweden). The column was equilibrated with phosphate start buffer (20 mM phosphate, 0.5 M NaCl, and 10 mM imidazole, pH 7.4), and the homogenized/clarified sample was applied. The column was then washed with phosphate buffer and sample fractions (0.5 mL each) were collected. The fusion proteins were eluted with a phosphate buffer containing increasing amounts of imidazole (50, 100, 200, 300, 400, and 500 mM). The fractions were monitored at A₂₈₀ using the corresponding elution buffer as a blank, and detected based on their fluorescence intensity using a fluorescence spectrometer. Most of the bound proteins were eluted between 100 and 200 mM imidazole.

Construction of the expression vectors

The expression plasmid, pGEX-4T-1, was originally purchased from Pharmacia (Uppsala, Sweden). The host cells, *E. coli* BL21, used for gene expression were purchased from Stratagene (West Chester, USA). bPDI was divided into domains as follows: bPDI-Full (residues 1–494), bPDI-1 (residues 1–162), bPDI-2 (residues 163–315), and bPDI-3 (residues 316–494). The four cDNAs were PCR-amplified using the following primers: bPDI-Full-F-*Bam*HI (5'-GGATCCGAAATGCGTGTGTTTAAATTTTC-3') and bPDI-Full-R-*Xho*I (5'-CTCGAGTAACTCGTCTTTGGCAGGC-3'); bPDI-1-F-*Sca*I (5'-AGTACTGAAATGCGTGTGTTTAAATTTTC-3') and bPDI-1-R-*Xho*I (5'-CTCGAGGCTCTGGTCCGAAAA-GAAACC-3'); bPDI-2-F-*Bam*HI (5'-GGATCC-TCAACCAGAGCCAAAATTTTC-3') and bPDI-2-R-*Xho*I (5'-CTCGAGAGACGGGCAGATGGAACCTC-3'); and bPDI-3-F-*Bam*HI (5'-GGA-TCCATCGCCCTTGAACAAGACATG-3') and bPDI-3-R-*Xho*I (5'-CTCGAGTAACTCGTCTTTGGCAGGC-3'). Each DNA fragment was subsequently cloned into pBAC-1 (Novagen) and pu-

rified using the His-Trap chelating column described above.

RNase activity assay

PDI activity was measured by the ability to renature reduced or scrambled RNase A (rRNase and sRNase). The reactivation of denatured RNase A was monitored as described by Lyles and Gilbert (1991) with modifications. In brief, 5 mg of RNase A (Type XII-A, Sigma-Aldrich) were reduced overnight at 25 °C in 1 mL of 100 mM Tris-acetate (pH 8.0), 4 mM EDTA, 6 M guanidine hydrochloride, and 140 mM DTT. Then, guanidine hydrochloride and DTT were removed by passage through a Bio-Gel P4 column (Bio-Rad, Hercules, USA) that had been equilibrated with 0.1% acetic acid. The scrambled RNase A, which had exchanged cysteines, was purchased from TaKaRa (Shiga, Japan). Activation of the reduced and scrambled RNase was assayed by mixing it with each purified bPDI and measuring the amount of RNase activity in small aliquots. Briefly, 0.5 mg/mL reduced and scrambled RNase were mixed with 5 g/mL bPDI or 5 g/mL bovine liver PDI (TaKaRa) and 10 μM DTT in 50 mM sodium phosphate buffer (pH 7.5) and incubated at 30 °C. RNase was subsequently added at a concentration of 50 g/mL. Aliquots were removed at several time points and the absorbance was measured at 260 nm.

Results and Discussion

bPDI was originally isolated from tunicamycin-treated Bm5 cells using a differential screening method, and both strands of the cDNA clone were sequenced (GenBank accession number AF325211) (Goo *et al.*, 2002a). Despite some sequence variation between bPDI and other PDIs, two conserved thiol oxidoreductase sites and a KDEL motif were identified in the C-terminus of bPDI; however, the enzymatic activity of bPDI was not investigated.

We first used isolated bPDI cDNA to express His₆-tagged bPDI (bPDI-His₆) via recombinant baculovirus (vAc-bPDI) transfection into Sf9 cells. The proteins were purified by IMAC using an Ni²⁺-immobilized resin and detected with anti-rat PDI antibodies which have already demonstrated their specificity for bPDI (Fig. 1) (Goo *et al.*, 2002b).

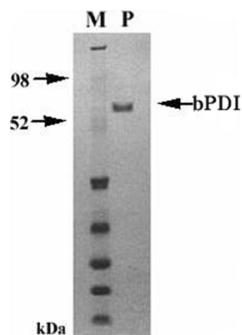


Fig. 1. Purification and Western blot analysis of bPDI. Sf9 cells were infected with a recombinant baculovirus (vAc-bPDI) encoding bPDI-6×His at a total m.o.i. of 5. The cells were harvested 72 h after infection and the recombinant protein was purified. Western blot analysis was performed using rat anti-PDI antibodies, with bPDI indicated by an arrow. M, protein size marker; P, bPDI protein.

We next used the reduced and scrambled RNase folding method to test the enzymatic activity of bPDI (Xiao *et al.*, 2001). The ability to refold reduced and denatured RNase molecules is a classic measure of protein disulfide isomerization. RNase showed time-dependent, spontaneous refolding and recovery of function after reduction (Fig. 2, left panel) and scrambling (Fig. 2, right panel). Recombinant bPDI restored the RNase activity to

72% (reduced) and 67% (scrambled) of the control level, compared to 73% and 72%, respectively, for bovine liver PDI (Fig. 2). This indicates that the recombinant bPDI protein retained disulfide isomerase activity.

To clarify which domain of bPDI has isomerase activity, we made four bPDI constructs (Fig. 3A) by systemic genetic engineering: bPDI-Full (residues 1–494), bPDI-1 (residues 1–162; domain a + 1/2 domain b), bPDI-2 (residues 163–315; 1/2b + 1/2b'), and bPDI-3 (residues 316–494; 1/2b' + a' and including c) (Fig. 3B). The recombinant proteins were expressed in *E. coli* and purified using an N-terminal His₆-tag; all were soluble and sufficiently purified to characterize the isomerase activity (Fig. 4). bPDI activity was measured by catalysis of disulfide isomerization using sRNase. In other PDIs, the a and a' domains have been shown to have isomerase activity (Pirneskoski *et al.*, 2001); however, we found that the isomerase activity of bPDI-3, which included the a' domain, was comparable to that in wild-type PDI, but that bPDI-1, which contained only domain a, lacked isomerase activity (Fig. 5). This is the first evidence that domain a' alone, but not together with domain a, mediates the isomerase activity of wild-type PDI.

Thus, the PDI family member bPDI may play an important role in protein folding and assembly

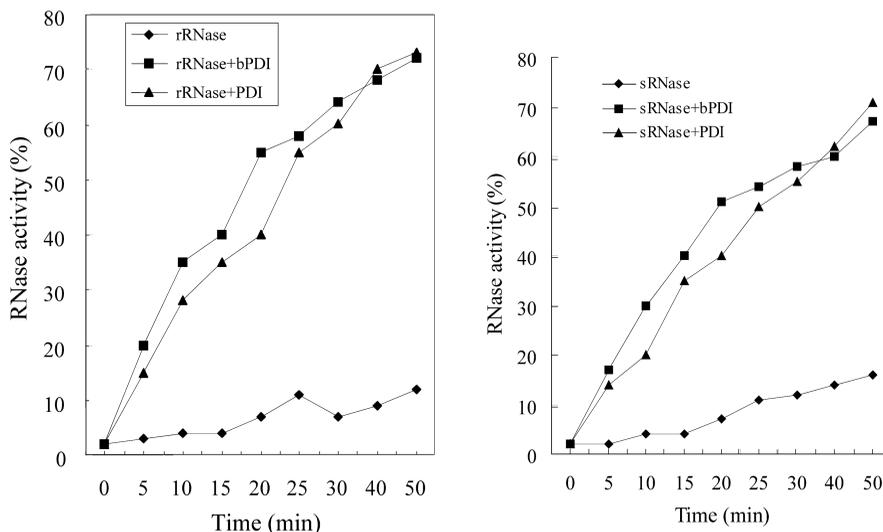


Fig. 2. Enzymatic activity of the recombinant bPDI protein. The assay mixture contained 10 μ M DTT, 0.5 mg/mL reduced (rRNase) and scrambled (sRNase) RNase with 5 g/mL bovine liver PDI, purified recombinant bPDI, or nothing else in 50 mM phosphate buffer (pH 7.5). RNase activity was measured at the indicated time points. The graph shows the percentage of refolding mediated by recombinant bPDI and bovine liver PDI. All experiments were performed 3 times and the graphs indicate the averages.

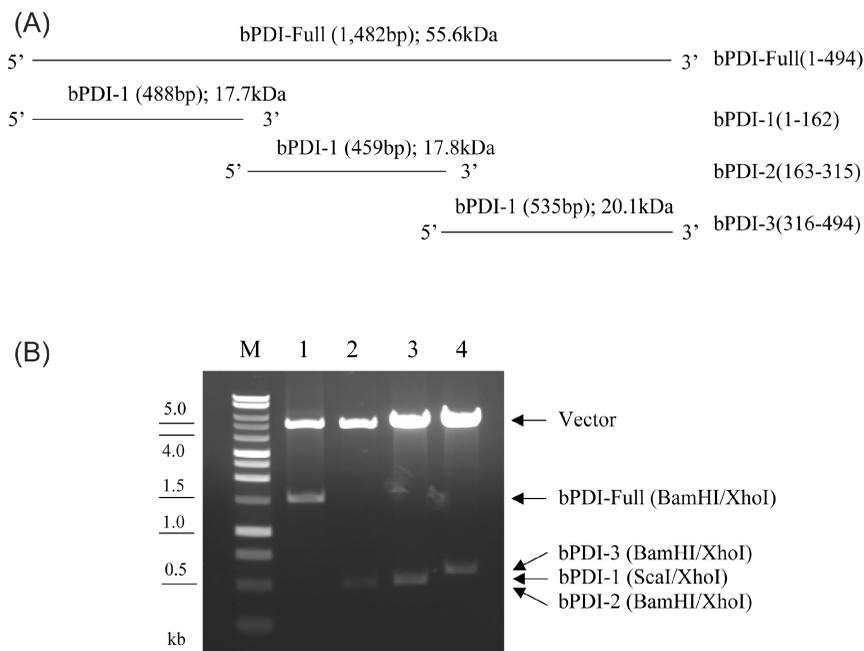


Fig. 3. (A) Construction of the *bPDI* cDNA variants. The cDNA encoding bPDI was divided as follows: bPDI-Full (amino acids [aa] 1–494), bPDI-1 (aa 1–162), bPDI-2 (aa 163–315), and bPDI-3 (aa 316–494). (B) The PCR-amplified bPDI variants.

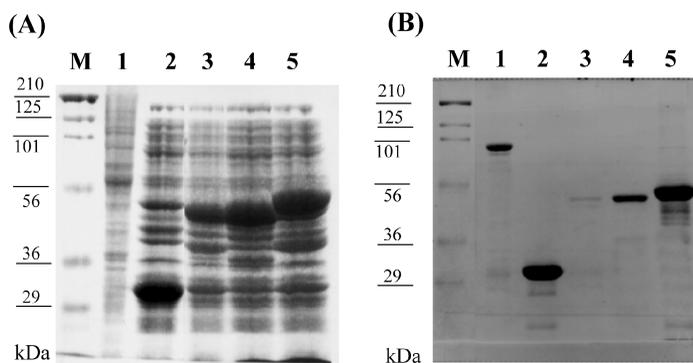


Fig. 4. Expression and purification of the bPDI variants. (A) SDS-PAGE and (B) column purification of the bPDI variants. Lane 1, bPDI-Full; lane 2, Mock; lane 3, bPDI-1; lane 4, bPDI-2; lane 5, bPDI-3; M, protein size marker.

in the ER of insect cells. Understanding the enzymatic activity of bPDI will facilitate the production of heterologous proteins in eukaryotic environments, since heterologous proteins frequently form insoluble aggregates or are improperly folded in the ER. In addition, a better understanding of the *in vivo* protein folding will benefit the field of protein engineering and help to unravel

the molecular basis of diseases associated with protein misfolding.

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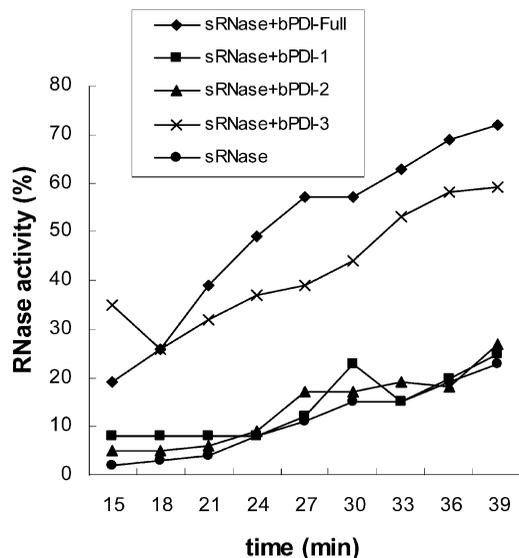


Fig. 5. Chaperone activity of the bPDI variants (RNase activity assay using the bPDI variants). rRNase or sRNase was incubated with bovine liver PDI or purified recombinant bPDI as described in the Materials and Methods. RNase activity was monitored at the indicated time points. The graph shows the percentage of refolding (as in Fig. 2) by the bPDI variants and bovine liver PDI. All experiments were performed 3 times and the graphs indicate the averages.

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