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Molecular Cloning, Expression and Functional Characterization of a Thioredoxin Peroxidase from the Silkworm *Bombyx mori*

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Objectives

The thioredoxin peroxidase (TPx) is an antioxidant member of the peroxiredoxin family of enzymes. The TPx enzyme system has been implicated in the elimination of hydrogen peroxide and hydroxyl radicals generated during cellular processes. Such reactive molecules have been shown to cause damage to all major classes of biological macromolecules, including lipid, protein and DNA.

Compared to mammalian peroxiredoxin genes, little is known about the insect TPx. Except for *Drosophila melanogaster*, genetic information on insect TPx is very limited to a few other species such as *Aedes aegypti* and *Apis mellifera*. Five peroxiredoxin genes have been identified in *D. melanogaster*: three of the genes fall into the 2-Cys subgroup, while the other two belong to the 1-Cys subgroup. These five proteins were shown to reduce H_2O_2 in the presence of dithiothreitol. The three 2-Cys peroxiredoxins were also shown to be active in the thioredoxin system and were, consequently, classified as TPx.

Until recently insect TPx genes had been cloned extensively from only *D. melanogaster*. Thus, our objective in initiating this study was to illustrate the cDNA cloning and molecular characterization of the TPx from the silkworm, *Bombyx mori*, which is one of the lepidopteran species. In this study, the molecular cloning, expression and functional characterization of the silkworm *B. mori* thioredoxin peroxidase (BmTPx) are described.

Materials and Methods

Materials - The silkworm *Bombyx mori*

Methods - cDNA library screening, nucleotide sequencing and data analysis, RNA isolation and Northern blot analysis, Construction of baculovirus transfer vector, Cell culture and construction of recombinant virus, SDS-polyacrylamide gel electrophoresis, Purification of recombinant TPx, Preparation of polyclonal antiserum and Western blot analysis, Determination of enzyme activity

Results and Discussion

A cDNA encoding the thioredoxin peroxidase (TPx) from the silkworm, *Bombyx mori*, was cloned and characterized. The *B. mori* TPx (BmTPx) cDNA contains an open reading frame of 585 bp encoding 195 amino acid residues (Fig. 1 and 2). The BmTPx belongs to the 2-Cys subgroup of peroxiredoxin family

(Fig. 2). The deduced amino acid sequence of the BmTPx cDNA showed 78% identity to *Drosophila melanogaster* (DmTPx-1), 73% to *Aedes aegypti* (AaTPx), and 54% - 48% to other insects TPx (Fig. 3). Phylogenetic analysis confirmed a closer relationship of the deduced amino acid sequences of the BmTPx gene to the DmTPx-1 and AaTPx within the 2-Cys PTx group (Fig. 3). The cDNA encoding BmTPx was expressed as a 24-kDa polypeptide in the baculovirus-infected insect Sf9 cells (Fig. 4) and the purified recombinant BmTPx was shown to reduce H_2O_2 in the presence of dithiothreitol (Fig. 5). Northern blot analysis revealed that BmTPx transcripts are present in all tissues examined, suggesting that BmTPx gene is expressed in most, if not all, body tissues (Fig. 6). Western blot analysis showed the presence of the BmTPx in the fat body and midgut, but not in the hemolymph, suggesting cytosolic TPx (Fig. 7). The induction of BmTPx to H_2O_2 by Northern blot analysis showed that the level of BmTPx mRNA significantly increased during the *in vivo* exposure (Fig. 8). Interestingly, the expression levels of BmTPx enzyme from fat body were particularly high when *B. mori* larva was exposed at low (4°C) and high (37°C) temperatures, suggesting that the BmTPx is responsive to temperature stimuli (Fig. 9).

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-60 AGTCATTCTCCGGTGCTTTCGGTTTTCGTCTATTATTCAAAAGTCTTTAGTTTCAACAAG
  1 ATGCCTCTGCAGATGACCAAACCCGCTCCCCAGTTCAAGGCCACGGCCGTCGTCAACGGA
  1 M  P  L  Q  M  T  K  P  A  P  Q  F  K  A  T  A  V  V  N  G
 61 GAGTTCAAGGACATTTCTCTGTCTGACTACAAGGGGAAATATGTTGTGCTGTTCTTCTAT
 21 E  F  K  D  I  S  L  S  D  Y  K  G  K  Y  V  V  L  F  F  Y
121 CCTTTGGACTTCACGTTCGTGTGCCCGACGGAGATCATCGCGTTCTCGGAGAAGGCGGAC
 41 P  L  D  F  T  F  V  C  P  T  E  I  I  A  F  S  E  K  A  D
181 GAGTTCCGCAAGATCGGCTGCGAGGTGCTCGGCGCCTCCACCGACTCGCACTTCACTCAC
 61 E  F  R  K  I  G  C  E  V  L  G  A  S  T  D  S  H  F  T  H
241 CTCGCCTGGATCAACACGCCGCGCAAGCAGGGCGGACTCGGCCCCATGAACATTCCTCTG
 81 L  A  W  I  N  T  P  R  K  Q  G  G  L  G  P  M  N  I  P  L
301 ATAAGCGACAAGTCGCACCGCATCTCCCGCGACTACGGAGTGCTGGACGAGGAGACGGGC
101 I  S  D  K  S  H  R  I  S  R  D  Y  G  V  L  D  E  E  T  G
361 ATTCCCTTCCGAGGACTCTTCATCATCGACGACAAGCAGAACCTCAGGCAGATCACGATC
121 I  P  F  R  G  L  F  I  I  D  D  K  Q  N  L  R  Q  I  T  I
421 AACGACCTGCCCGTGGGGAGGTCGGTGGAGGAGACCCTGCGGCTGGTGCAGGCCTTCCAG
141 N  D  L  P  V  G  R  S  V  E  E  T  L  R  L  V  Q  A  F  Q
481 TTCACGGACAAGCACGGCGAGGTGTGCCCCGCCAACTGGAGGCCCGGCGCCAAGACCATC
161 F  T  D  K  H  G  E  V  C  P  A  N  W  R  P  G  A  K  T  I
541 AAGCCCGACACCAAGGCCGCCCAGGAGTACTTCGGCGACGCCAACTAGACACGACACCAC
181 K  P  D  T  K  A  A  Q  E  Y  F  G  D  A  N  *
601 ACCAACAATCACTCTAATTGAATACACAATTTCGAAAAGGGCACATCTCTGAAAATGTAA
661 AATGTTCAGGAATTGAAAAAAAAACTGATTATTTTCTAAAGCAGTGTAAAATTTAAAATA
721 TTAACTTTTGAGATACGTCTTAGTCTTAATTAGCAATTTATGAGATATGCCCTTTTCGAA
781 ATTGCATATTCAATGAATACTTCTTATTTGGACTTCACTTGGTAAATCAGTATTGGATTA
841 ACTTAATGGAATCCAGCGGCTGCAATAAAGAAGCTGTGCTGGTTTGGAAAAAAAAAAAAA
901 AAAAAA
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Fig. 1. The nucleotide and deduced protein sequence of the BmTPx cDNA. The start codon of ATG is boxed and the termination codon is shown by asterisk. In the cDNA sequence, the polyadenylation sequence is underlined. This cDNA sequence has been deposited in GenBank.

Fig. 2. The amino acid sequence alignment of BmTPx gene. Residues are numbered according to the aligned insect TPx sequences, and invariant residues are shaded black. The conserved cysteine residues are shown by asterisk. The insect TPx sequences were taken from the following sources: DmTPx-1 (Q9V3P0), AaTPx (AAL37254), DmTPx-2 (AAF42986), AmlTPx-3 (AAP93584), and DmTPx-3 (AAG41976).

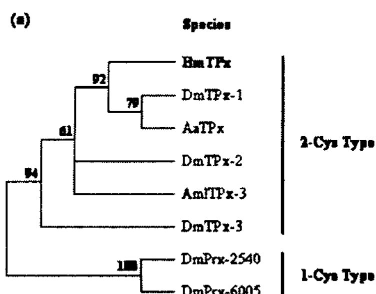

Fig. 3. Phylogenetic relationship among insect TPx sequences. (a) A maximum parsimony analysis for the BmTPx and the other known insect TPx sequences. The accession numbers of the sequences in the GenBank are as follows: DmTPx-1 (Q9V3P0), AaTPx (AAL37254), DmTPx-2 (AAF42986), AmlTPx-3 (AAP93584), DmTPx-3 (AAG41976), DmPrx-2540 (AAF58797), and DmPrx-6005 (NM_523463). The tree was obtained by bootstrap analysis with the option of heuristic search and the numbers on the branches represent bootstrap values for 1,000 replicates. (b) Pairwise identities and similarities of the deduced amino acid sequence of BmTPx among insect TPx sequences.

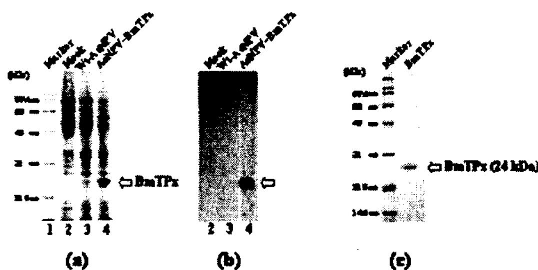

Fig. 4. Expression of the BmTPx in recombinant baculovirus-infected insect Sf9 cells and purification of its recombinant BmTPx. Sf9 cells were mock-infected (lane 2) or infected with the wild-type AcNPV (lane 3) and the recombinant AcNPV (lane 4) at an MOI of 5 PFU per cell. Cells were collected at 2 (lane 3) and 3 (lane 4) days p.i. Total cellular lysates were subjected to 12% SDS-PAGE (a), electroblotted and incubated with antiserum to recombinant BmTPx (b). The arrow on the right of the panel indicates the 24 kDa recombinant BmTPx polypeptide. Molecular weight standards were used as size marker (lane 1). (c) Purification of the recombinant BmTPx expressed in baculovirus-infected insect cells. The recombinant BmTPx purified by using FPLC techniques was analyzed by 12% SDS-PAGE. The arrow on the right of the panel indicates the purified recombinant BmTPx polypeptide of 24 kDa.

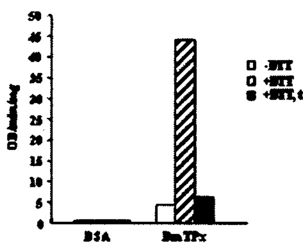

Fig. 5. Hydrogen peroxide elimination by the BmTPx expressed in recombinant baculovirus-infected insect Sf9 cells. H_2O_2 degradation was monitored at 240 nm and the differences in OD readings were plotted on the Y axis. The reactions were carried out with 10 μg of each purified BmTPx or 1.25 mg BSA in the absence (-DTT) or presence (+DTT) of 10 mM DTT or in the presence of DTT and the same amount of thermo-inactivated BmTPx [boiled for 5 min in 0.5% SDS (+DTT, t)]. Heat-inactivated BmTPx and BSA were used as negative controls.

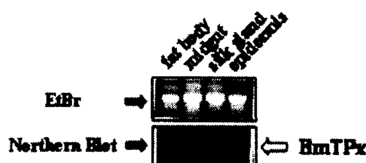

Fig. 6. Northern blot analysis of BmTPx. Total RNA was isolated from the fat body (lane 1), midgut (lane 2), silk gland (lane 3), and epidermis (lane 4), respectively (upper panel). The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis, transferred on to a nylon membrane, and hybridized with radiolabelled 585 bp BmTPx cDNA (lower panel). Transcripts are indicated on the right side of the panel by arrow.

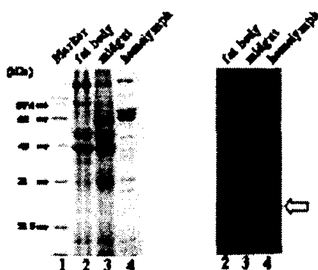

Fig. 7. Expression of BmTPx in the fat body and midgut of B. mori larva. Total cellular lysates were prepared from the fat body (lane 2) and midgut (lane 3), respectively. The hemolymph (lane 4) was collected on the 3rd day of 5th instar larva. The samples were subjected to 12% SDS-PAGE (left panel), electroblotted and incubated with antiserum to recombinant B mTPx (right panel). The arrow on the right of the panel indicates the 24 kDa BmTPx polypeptide. Molecular weight standards were used as size marker (lane 1).

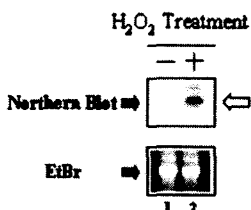

Fig. 8. Induction of BmTPx by in vivo injection of H_2O_2. The 3 day-old 5th instar silkworm was injected without (lane 1) or with (lane 2) 20 μM H_2O_2 per each larva. After 1 h-treatment, total RNA was isolated from the fat body, respectively. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (lower panel), transferred on to a nylon membrane, and hybridized with radiolabelled 585 bp BmTPx cDNA (Upper panel). Transcripts are indicated on the right side of the panel by arrow.

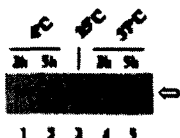

Fig. 9. Induction of BmTPx by external temperature stimulus. Western blot analysis of the BmTPx induced by external temperature stimulus. The 5th instar silkworm larva was incubated at 4°C (lanes 1 and 2) or 37°C (lanes 4 and 5) for 3 h (lanes 1 and 4) or 5 h (lanes 2 and 5), respectively. Control was indoor-reared at 25°C (lane 3). The protein samples were prepared from the fat body of B. mori larva of each temperature treatment. The samples were subjected to 12% SDS-PAGE, electroblotted and incubated with antiserum to recombinant BmTPx. The arrow on the right of the panel indicates the 24 kDa BmTPx polypeptide.

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