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Characterization of the antioxidant enzyme, thioredoxin peroxidase, from the carcinogenic human liver fluke, *Opisthorchis viverrini*

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Abstract

The human liver fluke, *Opisthorchis viverrini*, induces inflammation of the hepatobiliary system. Despite being constantly exposed to inimical oxygen radicals released from inflammatory cells, the parasite survives for many years. The mechanisms by which it avoids oxidative damage are unknown. In this study, thioredoxin peroxidase (TPx), a member of the peroxiredoxin superfamily, was cloned from an *O. viverrini* cDNA library. *O. viverrini* TPx cDNA encoded a polypeptide of 212 amino acid residues, of molecular mass 23.57 kDa. The putative amino acid sequence shared 60-70% identity with TPxs from other helminths and from mammals, and phylogenetic analysis revealed a close relationship between TPxs from *O. viverrini* and other trematodes. Recombinant *O. viverrini* TPx was expressed as soluble protein in *Escherichia coli*. The recombinant protein dimerized, and its antioxidant activity was deduced by observing protection of nicking of supercoiled plasmid DNA by hydroxyl radicals. Antiserum raised against *O. viverrini* TPx recognized native proteins from egg, metacercaria and adult developmental stages of the liver fluke and excretory-secretory products released by adult *O. viverrini*. Immunolocalization studies revealed ubiquitous expression of TPx in *O. viverrini* organs and tissues. TPx was also detected in bile fluid and bile duct epithelial cells surrounding the flukes two weeks after infection of hamsters with *O. viverrini*. In addition, TPx was observed in the secondary (small) bile ducts where flukes cannot reach due to their large size. These results suggested that *O. viverrini* TPx plays a significant role in protecting the parasite against damage induced by reactive oxygen species from inflammation.

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Keywords

liver fluke; *Opisthorchis viverrini*; peroxiredoxin; thioredoxin peroxidase; thiol-specific antioxidant; antioxidant enzyme

1. Introduction

Opisthorchis viverrini is a human liver fluke that is endemic in Thailand, Lao PDR, Cambodia and central Vietnam. In Thailand, an estimated 6 million people are infected by the fluke (calculated from an overall 9.6% prevalence within the population in 2001) [1]. Since the liver fluke inhabits the bile ducts, the infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis [2]. Moreover, both experimental and epidemiological studies strongly implicate liver fluke infection in the etiology of the bile duct cancer, cholangiocarcinoma [3-5].

A major pathological consequence of liver fluke infection is inflammation of the hepatobiliary system, described from both naturally infected humans and experimentally infected animals [6-8]. The inflammation has been demonstrated, at least in part, to be a response to the fluke's excretory/secretory (ES) products which are highly immunogenic [9]. The inflammatory infiltrate, including eosinophils, neutrophils and macrophages, are potent sources of oxidants (reactive oxygen species; ROS) targeting the fluke but also causing tissue damage. Despite this potent oxidative assault, *O. viverrini* can survive in infected hosts for many years [10], and the mechanism by which the fluke evades oxidative damage is not known.

To protect themselves from reactive oxygen species generated from host responses or even their own oxygen metabolism, parasites produce a number of antioxidant enzymes. Helminth parasites contain at least one of the three main antioxidant enzymes [11]. Superoxide dismutase (SOD) converts the superoxide anion to H₂O₂, while catalase, and glutathione peroxidase (GPx) are involved in H₂O₂ detoxification. In a number of parasites, SOD activity has been demonstrated in the absence of catalase or GPx activities [11]. Peroxiredoxins (Prx), now called thioredoxin peroxidases (TPx) [12], have antioxidant activity that reduces hydrogen peroxide and organic hydroperoxides to water or the corresponding alcohol, and are thought to be the principal enzyme for removal of hydrogen peroxide in these helminth parasites. Recently, we identified an *O. viverrini* expressed sequence tag (EST) which encoded a TPx protein [13]. Here we have determined the sequence of the entire cDNA encoding *Opv*-TPx, characterized its recombinant expression, functional activity in vitro, and tissue localization in fluke organs and nearby host cells, and address its putative role in opisthorchiasis.

2. Materials and Methods

2.1 Parasites

O. viverrini metacercariae were obtained from naturally infected cyprinoid fish captured from a fresh water reservoir in the endemic area of Khon Kaen province, Thailand. The fish were digested by pepsin-HCl. After several washings with normal saline, the metacercariae were collected and identified under a dissecting microscope. Viable metacercariae were used to infect hamsters (*Mesocricetus auratus*), which were maintained at the animal facility of the Khon Kaen University Faculty of Medicine. Protocols approved by the Khon Kaen University Animal Ethics Committee were used for all animal research conducted in this study. After 2-3 months of infection, hamsters were euthanized and necropsied, and adult *O.*

viverrini flukes recovered from their bile ducts. Adult *O. viverrini* were washed several times with phosphate buffered saline (PBS, pH 7.4) containing antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) and cultured in serum free RPMI-1640 medium contain 1% glucose, antibiotics and protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 2 µM E-64 and 10 µM leupeptin) at 37°C with 5% CO₂. Culture medium was collected every 24 h and centrifuged at 1500 *g* to separate the ES products and eggs into supernatant and pellet, respectively. ES products were then concentrated and diafiltrated into PBS using Amicon centrifugal filter devices with a cut-off size of 10 kDa (Millipore, USA). Egg, metacercaria and adult worm extracts were prepared as described [14]. Protein concentrations of *O. viverrini* extracts were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA), after which parasite extracts and material were stored at -80°C until needed.

2.2 *O. viverrini* TPx identification and sequence analysis

The *O. viverrini* EST corresponding to TPx (OvAE54, dbEST accession number EL618736) was identified from an adult *O. viverrini* cDNA library [13]. The nucleotide and deduced amino acid sequences of *O. viverrini* TPx were analyzed using BioEdit 7.0.1 and Expasy (<http://au.expasy.org/tools/>). EST OvAE54 contained only a partial ORF. The complete cDNA sequence was obtained from an *O. viverrini* cDNA library by PCR amplification using a combination of gene-specific primers (OVTPxF; 5'-TGCCCAACAGAGTTGATT-3', OVTPxR; 5'-GACAGTTATCTGACGCAA-3') and vector-derived primers. The full length cDNA sequence was called *Ov-TPx-1* and was deposited in GenBank with the accession number EU376958. The sequences of TPx proteins from different species were compared by the NCBI BLAST search program. A multiple sequence alignment was assembled with ClustalW [15].

2.3 Phylogenetic analysis

Phylogenetic relationship of TPxs was inferred using PHYLIP (version 3.67) (<http://evolution.genetics.washington.edu/phylip.html>) [16]. Bootstrap values were obtained with the SEQBOOT program (1000 data sets were generated) and distance matrices were generated with the PRODIST program (Kimura formula; analysis of 1000 data sets). Neighbor-joining analysis was carried out with the NEIGHBOR program (Neighbor-Joining method; analysis of 1000 data sets). The phylogenetic tree was drawn with TreeView software (version 1.6.6) [17].

2.4 Expression and purification of recombinant *O. viverrini* TPx

The *Ov-tpx-1* coding sequence (minus the predicted signal peptide) was amplified from the full length cDNA using primers, OVTPx-EF (5'-ATGGCTCTCCTGCCGAAC-3') and OVTPx-ER (5'-GTTAACGGACGAGAAATA-3') and ligated into the expression vector pCR® T7/CT-TOPO® TA (Invitrogen, USA). *E. coli* BL21 (DE3) cells were transformed with the ligation products, and recombinant clones obtained used antibiotic selection of the transformed *E. coli*. Recombinant protein production was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). The expression of target recombinant protein was determined by SDS-PAGE (15% separating gel and 5% stacking gel) followed by staining with Coomassie Brilliant Blue. The expressed recombinant *O. viverrini* TPx (*Ov-TPx-1*) was affinity purified from *E. coli* lysates using TALON® metal affinity resin (Clontech Laboratories, Inc., USA). Purified *Ov-TPx-1* was dialysed into PBS, yields were quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA), and recombinant *Ov-TPx-1* stored at -80°C until used.

2.5 Antioxidant activity assay

Antioxidant activity was measured with a mixed-function oxidation (MFO) system containing transition metals (Fe^{3+}), O_2 , and DTT as the electron donor. Assays were performed as described previously [18, 19] with the following modifications: reaction mixtures contained 16.5 μM FeCl_3 , 1.0 mM DTT and concentrations of *Ov*-TPx-1 ranging from 6-200 $\mu\text{g/ml}$ were incubated at 37°C. After 30 min, 300 ng of pUC19 supercoiled plasmid was added and the reaction tubes incubated at 37°C for 2.5 h. The mixtures were then examined by agarose gel electrophoresis for evidence of DNA nicking. *O. viverrini* recombinant thioredoxin (expressed in the same manner as *Ov*-TPx-1 described herein - unpublished) and bovine serum albumin were included as control proteins.

2.6 Preparation of anti -*Ov*-TPx-1 antibody

Mice were vaccinated subcutaneously with purified *Ov*-TPx-1 (25 μg per immunization). The first immunization was carried out with recombinant protein formulated with Freund's complete adjuvant; the second and third immunizations were carried out with recombinant protein formulated with Freund's incomplete adjuvant. Immunizations were conducted on days 1, 15 and 29. Antisera were obtained two weeks after the third immunization.

2.7 Western blot analysis

Ten micrograms of egg, metacercaria and adult worm extracts, ES products and 1 μg of recombinant *Ov*-TPx-1 were separated by SDS-PAGE (15% separating gel and 5% stacking gel) and transferred onto nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS tween-20 (0.05% tween-20 in PBS) (PBS-T) for 2 h. The mouse anti - *Ov*-TPx-1 serum diluted 1:10000(v/v) in 2% skim milk in PBS-T was then applied to the membrane and incubated for 2 h at room temperature. After washing 3×5 mins with PBS-T the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody for 1 h. The membrane was then washed again 3×5 mins with PBS-T followed by a final 10 min rinse with PBS. Reactive bands were then visualized with chemiluminescent detection methods following the manufacturer's instructions (ECL Plus™, GE healthcare, USA).

2.8 Immunohistochemistry

O. viverrini adult worms or liver tissue from hamsters infected with *O. viverrini* (weeks 1-24) were fixed and cut into sections of 4 μm with a microtome as described elsewhere [9]. The sections were deparaffinized in xylene, hydrated in a series of ethanol and distilled water, respectively. The endogenous peroxidase was eliminated by treating sectioned tissues with absolute methanol containing 5% H_2O_2 for 30 min. The sections were then washed in water and PBS. Non-specific staining was blocked by treating slides with 5% normal serum in PBS for 30 min. Mouse anti - *Ov*-TPx-1 serum diluted 1:1000 (v/v) in PBS was applied to the sections and incubated for 2 h at room temperature. After rinsing 3×5 mins with PBS the sections were then incubated with HRP-conjugated goat anti - mouse IgG for 1 h. Sections were rinsed with PBS 2×10 mins then bound antibody was detected using diaminobenzidine (DAB) reagent. The sections were then counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene and mounted in Permount®. Mounted sections were examined under a light microscope fitted with a digital camera.

3. Results

3.1 Sequence analysis

The 746 bp *O. viverrini* TPx cDNA contained a 5 bp 5' untranslated region followed by an open reading frame of 639 bp and a 102 bp 3' untranslated region including a polyA tail.

The deduced polypeptide sequence contained 212 amino acid residues including a predicted signal peptide of 17 amino acids, and a calculated molecular mass of 23.57 kDa and an isoelectric point of 6.59. Two VCP motifs of the 2-Cys peroxiredoxin active sites were conserved. Figure 1 shows a multiple sequence alignment of *Ov*-TPx-1 with 2-Cys peroxiredoxins from a range of phylogenetically distinct organisms. Two highly conserved domains (the N-terminal FYPLDFTFVCPTSLIA and C-terminal VCPA motifs) which contain the VCP motifs of TPx active sites were present in *Ov*-TPx-1. A putative *N*-linked glycosylation site at Asn-41 was observed. Two homologs from blood feeding trematodes (*Schistosoma japonicum* and *S. mansoni*) showed highest identity to *Ov*-TPx-1 (74% identity). TPx from the related liver fluke, *F. hepatica*, was 70% identical to *Ov*-TPx-1. Mammalian (*Homo sapiens*) and other helminths (the free-living *Caenorhabditis elegans* and the tapeworm *Taenia solium*) peroxiredoxins were ~60% identical to *Ov*-TPx-1.

3.2 Phylogenetic analysis

Phylogenetic analysis (Fig. 2) showed that TPx proteins grouped according to the phyla of the organisms from which they were derived. *Ov*-TPx-1 was most similar to TPx proteins from other flatworms. *Ov*-TPx-1 formed a robust clade with TPx proteins from other trematodes but a separate clade to TPx sequences from cestodes.

3.3 Expression and purification of recombinant *Ov*-TPx-1

Recombinant *Ov*-TPx-1 was predominantly expressed as insoluble protein, but about 10% of the protein remained soluble in the cytoplasm of *E. coli*. The vector derived 6-histidine tag allowed purification of soluble recombinant protein from *E. coli* lysate with TALON® metal affinity chromatography under native conditions. The purified protein migrated as predicted with a monomeric molecular weight of 25 kDa under reducing and denaturing conditions and 50 kDa in non-reducing and non-denaturing SDS-PAGE gels, implying that the recombinant protein, like other TPx proteins, formed disulfide-bonded dimers [20]. A closely migrating doublet was observed at 50 kDa (and to a much lesser degree at 25 kDa) under native conditions (Fig. 3).

3.4 Antioxidant activity of recombinant *Ov*-TPx-1

Hydroxyl radicals produced by the MFO system caused nicking of pUC19 supercoiled plasmid DNA. The plasmid remained in supercoiled form in the absence of DTT, indicating that conversion of supercoiled plasmid to nicked form was dependent on oxidative stress induced by the MFO system. Nicking of the plasmid could be protected in the presence of recombinant *Ov*-TPx-1 but not a control recombinant protein containing a free thiol group (thioredoxin) expressed under the same conditions (Fig. 4), or with bovine serum albumin (not shown). Protective activity of *Ov*-TPx-1 was dose-dependent since reduction of *Ov*-TPx-1 in the reaction decreased the protection of plasmid from oxidative cleavage.

3.5 Expression of *Ov*-TPx-1 in different developmental stages of *O. viverrini*

Serum from mice immunized with recombinant *Ov*-TPx-1 was used to detect TPx in each stage of the parasite (egg metacercariae and adult) and the ES products of adult worms. Under denaturing and reducing conditions, anti- *Ov*-TPx-1 antibodies bound to a protein of the expected monomeric size of 25 kDa, as well as a larger protein with an apparent molecular mass of 50 kDa in extracts of all developmental stages of the parasite (Fig. 5). The larger protein might reflect an inefficiently reduced dimer of native, parasite-derived TPx, also seen with the recombinant *Ov*-TPx-1 (lane 1). Anti- *Ov*-TPx-1 antibody also bound to a protein that migrated slightly ahead of the major band in metacercariae, adult extract and ES products. Due to the high level of sequence conservation of TPx proteins, this band might reflect cross-reactivity of the antibody with other related TPx proteins from *O.*

viverrini. The lower molecular mass bands might also reflect partially degraded forms of *Ov*-TPx-1, especially seeing as the amount of these smaller proteins increased after long-term storage of recombinant *Ov*-TPx-1 (lane 2).

3.6 Immunohistochemistry

Immunolocalization of *Ov*-TPx-1 in the fluke and infected liver tissue sections is shown in Figure 6. Anti- *Ov*-TPx-1 antibody bound specifically to all tissues and organs of *O. viverrini* adults. Juvenile flukes (days 7, 14, and 21) were also positive for TPx. Interestingly, *Ov*-TPx-1 was also detected on biliary epithelial cells surrounding the liver fluke after two weeks of infection. In addition, *Ov*-TPx-1 was observed inside mononuclear cells surrounding the fluke and in epithelia of small bile ducts distant from where the liver fluke cannot reach due to its large size (Fig. 6F). *Ov*-TPx-1 was not seen in the bile ducts that were distant from the flukes and normal uninfected hamster biliary epithelium.

4. Discussion

Like other parasitic infections, opisthorchiasis induces a vigorous host immune response. The host effector cells release oxidative species that can cause damage and kill the parasite, so antioxidant enzymes have evolved to protect them from oxidative stress. Here we characterized an antioxidant enzyme, thioredoxin peroxidase, from the carcinogenic liver fluke, *O. viverrini*.

Ov-TPx-1 exhibited hallmark features of the 2-Cys Prx family. Structural and mechanistic data support the further division of the 2-Cys Prxs into two classes called the 'typical' and 'atypical' 2-Cys Prxs [21]. Typical 2-Cys Prxs are obligate homodimers containing two identical active sites, while atypical 2-Cys Prxs are functionally monomeric. Recombinant *Ov*-TPx-1 formed dimers under native conditions, supporting its classification as a typical 2-Cys Prx.

The mixed-function oxidation (MFO) system generates hydroxyl radicals that inflict damage on proteins and lipids. Hydroxyl radicals also attack DNA to yield modified bases and produce strand breaks. *Saccharomyces cerevisiae* thioredoxin peroxidase (previously named thiol-specific antioxidant, TSA) has been shown to protect proteins, DNA and lipids from ROS-induced damage [18, 19, 22]. Antioxidant activity of TPx proteins from other helminths has been described [23-26] and those TPxs showed similar activity in protection of supercoiled plasmid DNA. In this study, recombinant *Ov*-TPx-1 showed antioxidant activity by protecting pUC19 supercoiled DNA from oxidative-nicking by hydroxyl radicals generated from the MFO system. This suggests that *Ov*-TPx-1 may play a similar role to yeast TPx in protection of fluke macromolecules from oxidative damage.

Western blot analysis showed consistent expression of *Ov*-TPx-1 in all developmental stages of *O. viverrini* that we examined. This suggested that *Ov*-TPx-1 might serve multiple antioxidant functions throughout the life cycle of the parasite. Moreover, *Ov*-TPx-1 was detected in ES products of adult worms, implying that the protein is secreted into host tissues where it protects the parasite in the bile ducts from reactive oxygen species released by host inflammatory cells.

TPx proteins from other helminth parasites exhibit different expression patterns. *Brugia malayi* TPx-1 was localized in the cells of the hypodermis-lateral chord in adult parasites but not at the surface or in excretory-secretory products, prompting the authors to suggest that *Bm*-TPx-1 plays its major role in countering radicals produced within the parasite's own cells [24]. In contrast, *Onchocerca volvulus* TPX-2 protein was predominantly localized to the hypodermis and cuticle, suggesting that *Ov*-TPX-2 may protect the parasite from being

damaged by host-generated oxidative stress [25]. In *S. japonicum*, three types of Prx have been reported: Prx-1, Prx-2 and Prx-3 [26]. In adult flukes, Prx-1 was expressed in the tegument and excretory/secretory products. Prx-2 was detected in the sub-tegumental tissues, parenchyma, vitelline glands and gut epithelium, but was not detected in the tegument. The authors suggested that Prx-1 acts to protect the parasite against the ROS produced by host immune cells, but Prx-2 plays important roles in intracellular redox signaling and/or in the reduction of ROS generated through the hemoglobolytic process in the digestive tract.

Ov-TPx-1 was detected in all tissues of the adult parasites. Two weeks after hamsters were infected with *O. viverrini* metacercariae, *Ov*-TPx-1 was detected in the bile duct epithelium surrounding the liver fluke and in small bile ducts where liver flukes cannot physically reside due to their size. Moreover, intensity of TPx staining in epithelial cells increased with the duration of infection. *Ov*-TPx-1 was also found inside mononuclear cells surrounding the fluke. These findings are similar to our previous study using anti-ES antibody [9], where *O. viverrini* antigens were detected in the fluke and the biliary epithelium of intrahepatic and extrahepatic bile ducts of infected hamster liver. Our data here showed that *Ov*-TPx-1 is at least one of these antigens that binds to the host cells. The mechanism by which TPx adheres to the biliary epithelium is unknown, but simple diffusion or endocytosis has been suggested [9]. The presence of *Ov*-TPx-1 in the biliary epithelium in late infection may be due to the larger size of adult flukes (and the larger amount of *Ov*-TPx-1) compared with juvenile worms, and also the pathology of the biliary epithelium due to chronic inflammation that may in turn increase the permeability of some other mechanisms by which the cells become more permissive for TPx uptake. However, it is not due to cross-reactivity with host tissue, since in the same tissue section, the bile duct epithelium that is distant from the fluke is negative for anti-TPx staining, even if it is inflamed.

The presence of *Ov*-TPx-1 as well as other fluke antigens within host cells implicates these parasite enzymes in modulations of normal cells processes in the contaminated host cells. ES products of *O. viverrini* have been demonstrated to induce NIH3T3 fibroblast cells to proliferate [27]. We also observed that biliary cells co-cultured with *O. viverrini* showed increase cell proliferation in a dose dependent manner and also induced anti-apoptosis (Sripa et al., unpublished). Similarly, the Cag A protein of *Helicobacter pylori* can drive gastric cells to proliferate and induces anti-apoptosis that leads to malignant transformation in gastric cancer [28]. Our preliminary data on *Ov*-TPx-1 cocultured with biliary cells did not result in cell proliferation but, interestingly, did induce anti-apoptosis under a H₂O₂ oxidative stress model (Suttiaprapa et al., unpublished). *Ov*-TPx-1 may therefore be involved in stimulating pathogenic pathways of infected host biliary cells in a similar fashion to that seen in *H. pylori* infection.

Other functions for helminth TPx have been reported. TPx in *F. hepatica* ES products induced the recruitment and alternative activation of macrophages, associated with the development of a polarized T helper type 2 immune response [29]. We performed similar experiments to those described by Donnelly et al. but with recombinant *Ov*-TPx-1, and did not detect any bias towards a particular T helper cell response (not shown), despite the fact that our recombinant protein was properly folded and that *O. viverrini* induces a predominantly Th2 response, at least in hamsters [30]. Absence of glycosylation seems not to affect the Th2 response since recombinant *F. hepatica* TPx produced in *E. coli* (without glycosylation) did induce a Th2 bias [29]. *O. viverrini* molecules other than TPx may therefore be involved in immunomodulation.

In summary, we have characterized a novel and functional antioxidant enzyme from *O. viverrini*. Expression in all life cycle stages and localization in most parasite tissues suggests

that *Ov*-TPx-1 may play multiple roles in *O. viverrini* throughout its development. Furthermore, *Ov*-TPx-1 was detected in ES products of adult worms, suggesting that it has additional roles outside of the parasite in protection against reactive oxygen radicals generated from host defensive mechanisms. Positive staining for *Ov*-TPx-1 in the bile duct epithelia implies a role in host-parasite interactions, as supported by our preliminary data on its anti-apoptotic activity *in vitro*, an area of particular relevance given that *O. viverrini* ES products can stimulate cell growth [27] and this might then accelerate tumorigenesis [5].

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Fig. 1. Multiple alignment of deduced amino acid sequences of *Opisthorchis viverrini* TPx with 2-Cys Prx from other species: *Schistosoma mansoni* (TPx, AAD40685), *Schistosoma japonicum* (TPx, BAD90102), *Fasciola hepatica* (TPx, P91883), *Caenorhabditis elegans* (Prx2, AAN63412), *Taenia solium* (Prx, AAV91322) and *Homo sapiens* (Prx1, NP_002565). Two conserved domains of 2-Cys Prx (FYPLDFTFVCPTELIA and VCPA) are boxed and VCP motifs are indicated by letters above the sequences. Predicted signal peptide of *Ov*-TPx-1 has been removed from the sequence in the alignment.

Fig. 2.

Phylogenetic tree of TPx family members, including *Ov*-TPx-1 from *Opisthorchis viverrini* (in the box); archaeae, *Thermoplasma volcanium* (TPx, BAB59365); bacteria, *Prochlorococcus marinus* (TPx, CAE20929), *Leptospira interrogans* (TPx, AAN50008), *Helicobacter pylori* (TPx, AAV33156), *Rickettsia typhi* (TPx, AAU03798); fungi, *Candida albicans* (TPx, XP_716082), *Phanerochaete chrysosporium* (Prx, AAV53576); flatworm, *Echinococcus granulosus* (TPx, AAD02002), *Taenia solium* (Prx, AAV91322), *Fasciola hepatica* (TPx, P91883), *Schistosoma japonicum* (TPx, BAD90102), *Schistosoma mansoni* (TPx, AAD40685); roundworm, *Caenorhabditis elegans* (Prx2, AAN63412), *Dirofilaria immitis* (TPx, AAC38831), *Onchocerca volvulus* (TPx, AAC48312); and vertebrates, *Homo sapiens* (Prx1, NP_002565), *Canis familiaris* (Prx4, XP_859371), *Mus musculus* (Prx4, NP_058044), *Bos taurus* (Prx4, AAG53660). The number on each branch represents the bootstrap value from 1000 replicates. Bootstrap values under 50% are omitted.

Fig. 3.

Expression and purification of recombinant *Ov*-TPx-1: Proteins were separated under native polyacrylamide gel electrophoresis; lane 1, molecular weight markers; lanes 2, 3 and 4, *E. coli* lysate, *E. coli* lysate after affinity purification with TALON® resin, and, purified *Ov*-TPx-1 treated with denaturing sample buffer, respectively; lane 5, purified *Ov*-TPx-1 treated with non-denaturing, non-reducing sample buffer.

Fig. 4. Antioxidant activity of *Ov*-TPx-1. Lane 1, pUC19 plasmid; lane 2, pUC19 plasmid + FeCl₃; lane 3, pUC19 plasmid + FeCl₃ + DTT; lanes 4-9, pUC19 plasmid + FeCl₃ + DTT + recombinant *Ov*-TPx-1 (200, 100, 50, 25, 12.5, 6.125 μ g/ml, respectively); lane 10, pUC19 plasmid + FeCl₃ + DTT + 200 μ g/ml recombinant *O. viverrini* thioredoxin. Supercoiled (SF) and nicked (NF) forms of pUC19 plasmid are indicated on the right.

Fig. 5. Western blot analysis of *Ov*-TPx-1 in different developmental stages of *O. viverrini*: lane 1, recombinant *Ov*-TPx-1 stored at 4°C for 1 day; lane 2, recombinant *Ov*-TPx-1 stored at -80°C for one year; lane 3, egg extract; lane 4, metacercaria extract; lane 5, adult worm extract; lane 6, excretory-secretory products.

Fig. 6.

Immunolocalization of *Ov*-TPx-1 in adult *O. viverrini* and in infected hamster livers. *Ov*-TPx-1 is observed in all tissues of the fluke including stromal parenchyma, vitelline glands, testis, ovary, eggs, miracidium, gut epithelium. During early infection (1 week), *Ov*-TPx-1 is observed mainly in the worm but not in the biliary epithelium (A). From 2 weeks post-infection, *Ov*-TPx-1 is also seen in the biliary epithelium, particularly that lying in close contact with the flukes (B-E). *Ov*-TPx-1 is also detected in small bile duct epithelia where the fluke cannot inhabit (F). A = 1 week, B = 2 weeks, C = 3 weeks, D = 4 weeks, E-F = 6 months post-infection. Immunoperoxidase staining, original magnification, 200 X (A-D, F) and 100X (E)