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Molecular expression and enzymatic characterization of thioredoxin 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 years. Defense against oxidative damage can be mediated through glutathione and/or thioredoxin utilising systems. Here, we report the molecular expression and biochemical characterization of a thioredoxin (Trx) from *O. viverrini*. *O. viverrini* Trx cDNA encoded a polypeptide of 105 amino acid residues, of molecular mass 11.63 kDa. The predicted protein has similarity to previously characterized thioredoxins with 26–51% identity. Recombinant *O. viverrini* Trx (*Ov*-Trx-1) was expressed as soluble protein in *E. coli*. The recombinant protein showed insulin reduction activity and supported the enzymatic function of *O. viverrini* thioredoxin peroxidase. Expression of *Ov*-Trx-1 at mRNA and protein levels were observed in all obtainable developmental stages of the liver fluke. *Ov*-Trx-1 was also detected in excretory-secretory products released by adult *O. viverrini*. Immunohistochemistry, *Ov*-Trx-1 was expressed in nearly all parasite tissue excepted ovary and mature sperms. Interestingly, *Ov*-Trx-1 was observed in the infected biliary epithelium but not normal bile ducts. These results suggest that *Ov*-Trx-1 is essential for the parasite throughout the life cycle. In the host-parasite interaction aspect, *Ov*-Trx-1 may support thioredoxin peroxidase in protecting the parasite against damage induced by reactive oxygen species from inflammation.

Keywords

liver fluke; *Opisthorchis viverrini*; thioredoxin; redox system; antioxidant

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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 [1]. People become infected by eating uncooked cyprinoid fish harboring infective metacercariae of the fluke. After infection, the juvenile parasite develops to adult worms and resides in the bile ducts. Infection with this liver fluke causes opisthorchiasis, which is associated with a number of hepatobiliary abnormalities including cholangitis, hepatomegaly and portal fibrosis. Experimental and epidemiological evidence strongly implicates *O. viverrini* infection in the etiology of cholangiocarcinoma, cancer of the bile ducts. [2,3].

Inflammation of the hepatobiliary system is a major pathological consequence of liver fluke infection which responds to the highly immunogenic fluke's excretory/secretory (ES) products [4]. Reactive oxygen species (ROS) produced from the inflammatory cells is toxic to the fluke and host tissue. Despite this potent oxidative assault, *O. viverrini* can survive in infected hosts for many years [5]. However, mechanism by which the fluke evades oxidative damage is not known. Recently, thioredoxin peroxidase (*Ov*-TPx-1), an antioxidant enzyme has been identified and characterized from *O. viverrini* and it was suggested to be the main enzyme that protects the parasite from reactive oxygen species produced by host effectors' cells [6]. Thioredoxin peroxidase is a member of a recently identified family of antioxidants, peroxiredoxins, which is involved in the detoxification of hydrogen peroxide and other hydroperoxides [7]. Thioredoxin peroxidase requires reducing equivalents from thioredoxin to catalyse the reduction of hydroperoxides. Thioredoxin is in turn maintained in the reduced state by thioredoxin reductase and nicotinamide adenine dinucleotide phosphate (NADPH).

Thioredoxin (Trx) is a 12 kDa intracellular protein ubiquitously found in all living cells from prokaryotes to eukaryotes. Trx possesses a highly conserved redox active Cys-Gly-Pro-Cys (CGPC) motif, called Trx-motif. They are involved in a wide variety of fundamental biological functions including protein disulfide reduction, protein repair, molecular chaperons, growth factor, structural component of enzymes, redox regulation of transcription factor, immune modulation, and regulation of apoptosis [8-10].

In this study, we characterized a thioredoxin from the liver fluke *O. viverrini*. Recombinant active proteins were produced from *E. coli* and its oxidoreductase activity was tested in vitro. Expression of *O. viverrini* thioredoxin in different stages were detected at both mRNA and protein levels. Antibodies against the recombinant protein were used to determine the localization of the protein in *O. viverrini*. Putative roles of thioredoxin in opisthorchiasis were also addressed.

2. Materials and methods

2.1 Parasites

O. viverrini metacercariae were obtained from naturally infected cyprinoid fish captured from a fresh water reservoir in 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 cultured to collect eggs and excretory-secretory (ES)

products as previously described [6]. Egg, metacercaria and adult worm extracts were prepared by following the protocol described by Sripa and Kaewkes [4]. 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* Trx identification and sequence analysis

The *O. viverrini* EST corresponding to Trx (OvAE1717, dbEST accession number EL620399) was identified from an adult *O. viverrini* cDNA library [11]. The nucleotide and deduced amino acid sequences of *O. viverrini* Trx were analyzed using BioEdit 7.0.1 and Expasy (<http://au.expasy.org/tools/>). The sequences of Trx proteins from different species were compared by the NCBI BLAST search program. A multiple sequence alignment was assembled with ClustalW [12].

2.3 Phylogenetic analysis

Phylogenetic relationship of Trx proteins was inferred using PHYLIP (version 3.67) (<http://evolution.genetics.washington.edu/phylip.html>) [13]. 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) [14].

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

The *Ov-trx-1* coding sequence was amplified from the *O. viverrini* cDNA library using a proof-reading DNA polymerase enzyme reaction mix (AccuPrime™ *Pfx* DNA polymerase, Invitrogen, USA) and primers, OVTrxF (5'-GCGCGCCTCGAGGTAAAACAGATCTGCACAAAA-3') and OVTrxR (5'-GCGCGCCTCGAGTCATTTGTGCTTTTTCACCATTTCA-3'). Amplicon was then verified by DNA sequencing and ligated into the expression vector pET-15b (Novagen, CA, 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 (20% separating gel and 5% stacking gel) followed by staining with Coomassie Brilliant Blue. The expressed recombinant *O. viverrini* Trx (*Ov*-Trx-1) was affinity purified using Ni NTA affinity chromatography according to the manufacturer's instructions (QIAGEN Inc., CA, USA). Purified *Ov*-Trx-1 was dialyzed into PBS, yields were quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA), and recombinant *Ov*-Trx-1 stored at -80°C until required.

2.5 Enzymatic activity assays

The oxidoreductase activity of recombinant *Ov*-Trx-1 was determined by the dithiothreitol (DTT)/insulin reduction method described by Holmgren [15] with following modifications. Reactions were carried out in 100mM Tris buffer pH 7.0 containing 1mg/ml insulin, 2mM EDTA and a given concentrations of recombinant *Ov*-Trx-1 in a final volume of 250 μ l. The reaction was started by adding 0.33 mM final concentration of DTT into all wells, except the blank. The reaction then was thoroughly mixed and placed in the spectrophotometer. The measurements were performed at 650nm using 1min recordings. Recombinant *Ov*-Trx-1 activity in supporting the activity of *Ov*-TPx-1 was determined in a mixture containing 100mM Tris buffer pH 7.4, recombinant *Ov*-TPx-1 (10 μ g/ml), DTT (250 μ M), recombinant

Ov-Trx-1 at the given concentrations. Reactions were started by adding hydrogen peroxide (200 μ M) and were stopped at time intervals by the addition of hydrochloric acid (final concentration of 0.2M). Remaining hydrogen peroxide was determined using the ferrithiocyanate method [16].

2.6 RNA extraction and RT-PCR

Total RNA from each stage of *O. viverrini* was extracted with TRIzol (Invitrogen, USA) according to the manufacturer's instruction. Unexpected contaminated genomic DNA was destroyed by treating with DNase I (Promega, Madison, Wis.) followed manufacturer's instruction. For reverse transcription-PCR (RT-PCR), first-strand cDNA was produced with an oligo (dT) primer from 1.0 μ g of total RNA using avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) at 42°C for 60 min. A 1.0 μ l aliquot of the resultant cDNA was amplified by using primers specific for β -actin (5', 5'-CGAGGTATCCTCACCTCAA-3'; 3', 5'-GCGACTCGCAACTCATTGTA-3') and *Ov*-Trx-1 (5', 5'-GCGGAAATTCAGGCAAATA-3'; 3', 5'-CCTTAAACTCCTCCGCCATA-3') based on the following conditions: 30 sec denaturation at 94°C, 30 sec annealing at 55°C, and 30 sec extension at 72°C for 30 cycles. PCR reactions without reverse transcription step were performed as negative control to ensure that amplified products were from reverse transcribed cDNA. All PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

2.7 Preparation of anti - *Ov*-Trx-1 antibody

Mice were vaccinated subcutaneously with purified *Ov*-Trx-1 (25 μ g 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.

2.8 Western blot analysis

Twenty micrograms of egg, metacercaria and adult worm extracts, ES products and 1 μ g of recombinant *Ov*-Trx-1 were separated by SDS-PAGE (20% separating gel and 5% stacking gel) and transferred onto nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS/0.05% tween-20 (PBS-T) for 2 h. The mouse anti - *Ov*-Trx-1 serum diluted 1:100 (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 $\times$ 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 $\times$ 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).

2.9 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 μ m with a microtome as described elsewhere [4]. 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% H₂O₂ 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*-Trx-1 serum diluted 1:100 (v/v) in PBS was applied to the sections and incubated for 2 h at room temperature. After rinsing 3 $\times$ 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 $\times$ 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 552 bp *O. viverrini* Trx cDNA contained a 50 bp 5' untranslated region followed by an open reading frame of 318 bp and a 184 bp 3' untranslated region including a polyA tail. The deduced polypeptide sequence contained 105 amino acid residues and a calculated molecular mass of 11.63 kDa and an isoelectric point of 7.57. Figure 1 shows a multiple sequence alignment of *Ov*-Trx-1 with thioredoxins from a range of phylogenetically distinct organisms. The active site WGCP residues were completely conserved. A thioredoxin from the related liver fluke, *F. hepatica* showed highest identity to *Ov*-Trx-1 (51% identity). Homologs from blood feeding trematodes (*S. mansoni*), Mammalian (*Homo sapiens* and *Mus musculus*) and protozoa (*Entamoeba histolytica*) were ~40% identical to *Ov*-Trx-1. Thioredoxins from bacteria *E. coli* and the tape worm *Echinococcus granulosus* were 33% and 26% identical to *Ov*-Trx-1, respectively.

3.2 Phylogenetic analysis

Phylogenetic analysis (Fig. 2) showed that Trx proteins grouped according to the phyla of the organisms from which they were derived, suggesting that the phylogeny presented here reflects taxonomic relationships. *Ov*-Trx-1 formed the same clad to Trx protein from trematode *F. hepatica*, but a separate clad to TPx sequences from cestodes.

3.2 Expression, purification and enzymatic activity assays of *Ov*-Trx-1

Recombinant *Ov*-Trx-1 was predominantly expressed as insoluble protein, but about 30% 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 condition. The purified protein migrated as predicted with a molecular weight of 14 kDa (11.6 kDa of *Ov*-Trx-1 plus 2.4 kDa of expression vector) under reducing SDS-PAGE gel (Fig. 3). Activity of the recombinant *Ov*-Trx-1 was determined by the insulin reduction method in the presence of a reducing agent (DTT). In the reaction containing recombinant *Ov*-Trx-1, reduction of insulin was increased in dose dependent manner (Fig. 4). Insulin reduction was very slow in the absence of recombinant *Ov*-Trx-1, indicated that reduction of insulin was dependent on the activity of recombinant *Ov*-Trx-1. The ability of recombinant *Ov*-Trx-1 to support recombinant *Ov*-TPx-1 activity in hydrogen peroxide consumption was determined by using DTT to maintain reduced state of recombinant *Ov*-Trx-1. The hydrogen peroxide reducing activity of recombinant *Ov*-TPx-1 showed dose dependency of recombinant *Ov*-Trx-1 (Fig. 5).

3.3 Expression profile of *O. viverrini* thioredoxin

Conventional RT-PCR and western blot detection were used to determine the expression of *O. viverrini* thioredoxin. Both techniques showed similar *O. viverrini* thioredoxin expression profile. The expression was detected in all obtainable developmental stages of the liver fluke (Fig. 6, 7). The lowest expression was observed in egg stage, but the expression was gradually increased in more developed stages of *O. viverrini*. The thioredoxin was also detected in ES products of the fluke.

3.4 Localization of *O. viverrini* thioredoxin

Expression of *O. viverrini* thioredoxin was assessed by immunostaining of adult worm and infected livers using anti-*Ov*-Trx-1. Immunohistochemically, *Ov*-Trx-1 was expressed in nearly all tissue of the fluke including parenchymal cells, gut epithelium, vitelline glands, and eggs in the uterus (Fig. 8). The testis but not in the mature sperms in the seminal receptacle exhibited thioredoxin expression in only the immature spermatogonia. Interestingly, nearby infected biliary epithelium also showed *Ov*-Trx-1 staining while those far from the fluke and normal bile ducts did not stain for *Ov*-Trx-1 (Fig. 8).

4. Discussion

Opisthorchiasis induced by the liver fluke *O. viverrini* causes the inflammation of infected tissues. The host effector cells release oxidative species that can cause damage and kill the parasite, so antioxidant enzymes are evolved to protect them from lethal radicals. Recently, we have characterized an antioxidant enzyme, thioredoxin peroxidase, from *O. viverrini* [6]. Its activity in protection of supercoiled plasmid DNA from hydroxyl radical and the detection in all developmental stages, suggesting its 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.

In antioxidant mechanism of *Ov*-TPx-1, its redox-active cysteines are used to reduce peroxides. The catalytic reaction results in the formation of a stable intersubunit disulfide bond, which is then reduced by disulfide oxidoreductases [17]. In this study, we have characterized the putative disulfide reducing protein, thioredoxin, from *O. viverrini*.

The *O. viverrini* thioredoxin cDNA contained 552 bp which encoded a poly peptide of 105 amino acid residues with a calculated molecular mass of 11.63 kDa. The active site WGCPG residues were completely conserved and there is 26-51% amino acid identity to thioredoxin from other organisms. *Ov*-Trx-1 was expressed as the soluble protein and purified in native condition from *E. coli*. The recombinant *Ov*-Trx-1 showed insulin reduction activity and exhibited the reducing equivalent property to support *Ov*-TPx-1 on scavenging hydrogen peroxide. Similar to *Ov*-TPx-1, *Ov*-Trx-1 was expressed in all developmental stages and secreted into ES products of *O. viverrini*. These results may support the hypothesis that *Ov*-Trx-1 is the physiological hydrogen donor for *Ov*-TPx-1.

It is interesting that *Ov*-Trx-1 which absence of predicted signal peptide could be secreted into ES products. But, thioredoxin secretion by a unique leaderless secretory pathway has been shown in a number of normal and neoplastic mammalian cells [18]. Thioredoxins from related liver fluke, *F. hepatica* and blood feeding trematode, *S. mansoni* are also detected in ES products [19-21]. This secretion mechanism may be equipped in these parasites and needed to be more characterized.

In human, secreted thioredoxin in infected and inflamed tissues has been shown to be a chemotactic for monocytes, polymorphonuclear leukocytes, and T lymphocytes, in both in vitro and in vivo models [22]. Secreted thioredoxin also exhibits several cytokine-like activities [23]. Tryparedoxin, a special class thioredoxin from protozoan parasite, *Leishmania infantum*, was proposed to participate in immunopathological processes by targeting B-cell effector's function, leading to IL-10 secretion and specific antibodies production [24]. The secreted thioredoxin from *O. viverrini* may play such roles in infected liver tissue.

In summary, we have characterized a putative reducing equivalent, thioredoxin, from *O. viverrini*. Its activity to support *Opv*-TPx-1 may suggest that *Opv*-Trx-1 play a role in antioxidant mechanism. Expression in all life cycle stages and localization in most parasite tissues suggests that *Opv*-Trx-1 may play house-keeping roles in *O. viverrini* throughout its development. Moreover, *Opv*-Trx-1 was detected in ES products of adult worms, suggesting that it has additional roles outside of the parasite. Since, thioredoxin has been shown to be involved in cellular growth and anti-apoptosis mechanism[25,26], positive staining for *Opv*-Trx-1 in the infected bile duct epithelia implies that *O. viverrini* thioredoxin may stimulate cell growth and/or inhibit cell apoptosis, this might then accelerate carcinogenetic processes in liver fluke associated CCA [2].

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Research highlight

The human liver fluke, *Opisthorchis viverrini*, induces inflammation> the parasite produces thioredoxin (Trx) for detoxification> *O. viverrini* Trx (Ov-Trx-1) were expressed in all tissue components except ovary and mature sperms and in excretory-secretory products> protecting the parasite against damage induced by reactive oxygen species from inflammation.

Fig. 1.

Multiple alignment of deduced amino acid sequences of *O. viverrini* thioredoxin with thioredoxin from other species: *S. mansoni* (AAL79841), *F. hepatica* (AAF14217), *E. granulosus* (AAC14584), *H. sapiens* (NP_003320), *M. musculus* (NP_035790) and *E. coli* (AAA24696). The conserved active site WCGPC residues are boxed and two cysteines of active site are indicated by arrow head.

Fig. 2.

Phylogenetic tree of Trx family members, including *Ov*-Trx-1 from *O. viverrini* (EL620399) (in the box); flatworm, *F. hepatica* (AAF14217); roundworm, *Ascaris suum* (AAS78778), *Brugia malayi* (XP_001900802); plant, *Eucalyptus grandis* (ABB53600), *Medicago truncatula* (AAZ98843), *Brassica juncea* (ABM54166); archaeae, *Sulfolobus solfataricus* (NP_343612), *Archaeoglobus fulgidus* (NP_070969); bacteria, *E. coli* (AAA24696), *Clostridium perfringens* (NP_563271); fungi, *Aspergillus fumigatus* (XP_751916), *Pichia stipitis* (XP_001387891); and vertebrates, *H. sapiens* (NP_003320), *Bos Taurus* (NP_776393), *M. musculus* (NP_035790). 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*-Trx-1: Proteins were separated under SDS-PAGE; lane 1, *E. coli* lysate; lane 2, *E. coli* lysate after affinity purification with Ni-NTA affinity chromatography and lane 3, purified *Ov*-Trx-1.

Fig. 4. Insulin reduction by recombinant *Ov*-Trx-1. Reactions with 1.5 μM, 0.3 μM and without (0 μM) of recombinant *Ov*-Trx-1 are indicated. All reactions contain 0.33 mM DTT.

Fig. 5. Hydrogen peroxide reduction by recombinant *Opv*-TPx-1 supported by recombinant *Opv*-Trx-1. The rate of hydrogen peroxide consumption (μmol/min) by recombinant recombinant *Opv*-TPx-1 in the presence of variable amounts of recombinant *Opv*-Trx-1 (in μM).

Fig. 6.

Expression profile of *Ov-Trx-1* in different developmental stages of *O. viverrini* by conventional RT-PCR: lane 1, egg; lane 2, metacercaria; lane 3, 2 weeks juvenile worm; lane 4, 1 month adult worm and lane 5, 2 months adult worm. *Ov- β -actin* and *Ov-TPx-1* were used as control.

Fig. 7.

Western blot analysis of *Ov*-Trx-1 in different developmental stages of *O. viverrini*: lane 1, recombinant *Ov*-Trx-1; lane 2, egg extract; lane 3, metacercaria extract; lane 4, adult worm extract; lane 5, excretory-secretory products.

Fig. 8.

Immunohistochemistry of *Ov-Trx-1* in adult *O. viverrini* and infected bile ducts. The thioredoxin is moderately to highly expressed in tegumental cells (T, arrow) and parenchyma, gut epithelium (G), and eggs (E) in the uterus but not ovary (O) (B). The infected biliary epithelium that lining close to the fluke shows intense thioredoxin staining. Negative control using preimmune mouse serum exhibits no staining at all (A). (Immunoperoxidase staining, original magnification 100X).