Identification and biochemical characterization of two novel peroxiredoxins in a liver fluke, *Clonorchis sinensis*

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(*Received 10 February 2011; revised 4 April and 22 April 2011; accepted 22 April 2011; first published online 22 July 2011*)

SUMMARY

We identified 2 novel genes encoding different 2-Cys peroxiredoxins (PRxs), designated CsPRx2 and CsPRx3, in *Clonorchis sinensis*, which invades the human hepatobiliary tracts. The *CsPRx2* gene expression was temporally increased along with the parasite's development and its protein product was detected in almost all parts of adult worms including subtegument, as well as excretory-secretory products. Conversely, *CsPRx3* expression was temporally maintained at a basal level and largely restricted within interior parts of various tissues/organs. The recombinant forms of CsPRx proteins exhibited reducing activity against various hydroperoxides in the presence of either thioredoxin or glutathione (GSH) as a reducing equivalent, although they preferred H_2O_2 and GSH as a catalytic substrate and electron donor, respectively. A steady-state kinetic study demonstrated that the CsPRx proteins followed a saturable, Michaelis-Menten-type equation with the catalytic efficiencies (k_{cat}/K_m) ranging from 10^3 to 10^4 M^{-1} s^{-1}, somewhat lower than those for other PRxs studied (10^4–10^5 M^{-1} s^{-1}). The expression patterns and histological distributions specific to CsPRx2 and CsPRx3 might suggest different physiological functions of the antioxidant enzymes in protecting the worms against oxidative damage.

Key words: *Clonorchis sinensis*, antioxidant enzyme, peroxiredoxin, thioredoxin-dependent peroxidase, glutathione-dependent peroxidase.

INTRODUCTION

Almost all organisms from bacteria to humans are subject to oxidative stress in their normal aerobic habitats. Accumulation of reactive oxygen species (ROS) during a variety of physiological and pathological processes might invoke the inevitable stress. The electrophilic ROS-mediated attack on DNA and other biological intermediates disturbs diverse biological processes. To prevent this adverse damage, the living organisms have evolved multiple defence mechanisms comprising enzymatic and non-enzymatic components (Sies, 1993). These oxidants and antioxidants also interact directly with signalling proteins to transduce the stress signal into the nucleus or to control their activity (Mieyal *et al*. 2008; Ahsan *et al*. 2009). Under normal physiological conditions, the balance between the generation and elimination of ROS is elaborately regulated by the action(s) of antioxidant defence molecules to ensure redox homeostasis and cell survival (Trachootham *et al*. 2008). Together with glutathione (GSH)-related molecules, the thioredoxin (Trx) system, which is mainly composed of Trx, peroxiredoxin (PRx) and Trx reductase (TR), plays a central role for the redox homeostasis in eukaryotic organisms including helminth parasites (Henkle-Dührsen and Kampkötter, 2001; Salinas *et al*. 2004; Kuntz *et al*. 2007). The PRx proteins, also known as Trx peroxidase, are a ubiquitous family of peroxidases that catalyse the reduction of H_2O_2, peroxynitrite and other organic hydroperoxides. The PRx family has recently received considerable attention as the primary thiol-specific antioxidant proteins across major taxa (Wood *et al*. 2003 and references therein).

Clonorchis sinensis is a trematode parasite that thrives in the hepatobiliary tract of definitive hosts including humans. Its enzootic infection is prevalent in several parts of the world, but is highly endemic in Asian countries such as Vietnam, China, Japan and Korea. Approximately 35 million people are at risk of and are infected with this food-borne trematode worldwide (Keiser and Utzinger, 2009). The traditional habits of eating raw or undercooked cyprinoid fish harbouring the infective metacercariae are the major factors for the high infection rate. A light infection with *C. sinensis* usually does not invoke severe clinical manifestations; however, chronic infection can cause biliary obstructions, cholangiectasis, biliary stones and jaundice (Lun *et al*. 2005). The most important complication of clonorchiasis is an

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doi:10.1017/S0031182011000813

increased risk of cholangiocarcinoma (Choi *et al*. 2004; Sripa *et al*. 2007; Bouvard *et al*. 2009), while detailed biological processes relevant to the carcinogenesis remain largely elusive. One of the major factors triggering the malignancy might be associated with inflammatory responses in addition to the mechanical irritation of the hepatobiliary epithelium by *C. sinensis* (Vennervald and Polman, 2009).

The *C. sinensis* metacercaria excysts in the duodenum and migrates into the hepatic bile ducts, where the juvenile matures into adult (Hong *et al*. 1993; Lun *et al*. 2005). During its lifespan, the fluke is continuously exposed to the cytopathic bile and interacts with epithelial cells in the ductal system, in which oxidizing and nitrosating molecules released by the host immune and inflammatory cells prevail. The parasite is also exposed to endogenous ROS generated by its own metabolic processes. These highly reactive molecules would seriously threaten the molecular and cellular homeostasis of the parasite (Mkoji *et al*. 1988; Sripa *et al*. 2007). *C. sinensis* faces the need to protect itself against ROS derived from both the host cells and the normal cellular metabolism of the parasite. A series of antioxidant enzymes, which appeared to participate in the removal of these detrimental oxidants, have been characterized in *C. sinensis* including GSH S-transferase (GST; Hong *et al*. 2001, 2002), GSH peroxidase (GPx; Cai *et al*. 2008) and superoxide dismutase (SOD; Li *et al*. 2005). In relation to the enzymes directly involved in the H_2O_2-detoxification pathway, GPx expression is largely restricted within a female-specific sexual organ/cell in *C. sinensis* (Cai *et al*. 2008). Accordingly, PRx activity might be contributable to the major defence against soluble hydroperoxide in catalase-deficient trematode species including *C. sinensis* (Callahan *et al*. 1988; McGonigle *et al*. 1998).

In this study, we isolated 2 novel 2-Cys PRx genes from *C. sinensis* (*CsPRx2* and *CsPRx3*), each of which exhibited similarity with the parenchymal and mitochondrial PRxs isolated from other trematode species (Sayed and Williams, 2004; Sekiya *et al*. 2006). The spatiotemporal expression patterns of these genes were examined at the RNA and protein levels. The GSH-dependent peroxidase activity of the *Clonorchis* proteins was also investigated against various hydroperoxide molecules employing respective recombinant proteins produced in bacterial cells.

MATERIALS AND METHODS

Parasite materials

C. sinensis metacercariae were isolated from naturally infected freshwater fish, *Pseudorasbora parva*, and were given orally to Sprague-Dawley rats (100 metacercariae/rat). The worms were harvested from the bile ducts of the experimental rats at 1, 2, 2·5, 3, 3·5 and 4 weeks post-infection (p.i.). The worms were washed >10 times with physiological saline at 4 °C to avoid any possible contamination from the host tissues and molecules. The collected parasites were stored at −80 °C or immersed in RNAlater (Ambion, Austin, TX, USA). Some of 4-week-old worms were carefully examined under a dissecting microscope and intact live worms were selected for *in vitro* incubation experiments and fixation in 4% paraformaldehyde in PBS (pH 7·4). All animals were housed in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Review Board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University.

Identification of C. sinensis *PRx (*CsPRx*) genes*

A degenerate primer pair was designed by comparing the nucleotide sequences of trematode and cestode *PRx* genes retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov). The primers (PRx-5F, 5′-CACTKGAYTTYACTTTYGTCTGTCC-3′ and PRx-3R, 5′-CCAYTTCGCTGGACAMAC-TTCRCCATG-3′) were used in pairs with each other or in combination with the universal T7 and T3 promoter primers, in PCR to amplify the homologous gene(s) from a *C. sinensis* cDNA library. Thermal cycling profile included pre-heating for 2 min at 94 °C, 35 cycles of 1 min at 48 °C, 1·5 min at 72 °C, 50 s at 94 °C and a final extension of 10 min at 72 °C. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced using the BigDye Terminator Cycle Sequencing Core Kit (ver. 3.0; Perkin Elmer, Foster City, CA, USA) and an ABI PRISM 377A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). All of the obtained sequences were aligned to make consensus contigs and the integrity of each contig was further confirmed by PCR using primers matched to both ends of the contig sequences.

Sequence analysis

The coding profiles and deduced amino acid (aa) sequences were determined using the Open Reading Frame (ORF) Finder program at the National Center for Biotechnology Information (NCBI). Similarity patterns of the *C. sinensis* proteins were examined with the aa sequences as queries in the BLAST and the Hidden Markov Model analyses (InterProScan; http://www.ebi.ac.uk/Inter-ProScan/). The isoelectric point (pI) and molecular weight were estimated using the Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). The PSORT (http://psort.nibb.ac.jp/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) programs were used for the prediction

https://doi.org/10.1017/S0031182011000813 Published online by Cambridge University Press

of putative signal peptides. Mitochondrial targeting signals were searched using the MITOPREP (http://bioinformatics.albany.edu/~mitopred/) and iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/). The tertiary structures were simulated with the ESyPred3D program in the ExPASy proteomics server (http://www.expasy.ch/), based on the homology model.

The aa sequences of trematode and cestode PRxs were aligned together with the *C. sinensis* PRxs with ClustalX and optimized with the GeneDoc program (http://www.psc.edu/biomed/genedoc/). The alignment was used as an input to investigate the phylogenetic relationships among the related proteins using the MEGA program (ver. 4.0; Tamura *et al.* 2007). The sequence divergences were calculated with the Jones-Taylor-Thornton (JTT) substitution model and indels between pairs of sequences were regarded as missing data. A phylogenetic tree was constructed using the neighbor-joining algorithm. The standard error of sequence divergence and statistical significance of each branching node were evaluated by the bootstrapping analysis of 1000 replicates.

Recombinant CsPRx proteins (rCsPRxs) and specific mouse antisera

ORF regions of *CsPRx* genes were amplified from the cDNA library using specific primers tagged with the restriction sequences for *Bam*H I and *Xho* I (CsPRx2-BamH-F, 5′-AAGGATCCATGGCACTTCTACCGAACAACCCG-3′; CsPRx2-Xho-R, 5′-GGCTCGAGCTAGTTAACGGAAGAGAAATACTCC-3′; CsPRx3-BamH-F, 5′-AAGGATCCATGTTTCTTCGACAACTCGCTCTGAC-3′; CsPRx3-Xho-R, 5′-GGCTCGAGTCACTGTTGTTTGCTGAAATATTCC-3′). PCR products and the pET-28a-c(+) vector (Novagen, Madison, WI, USA) were digested with the respective endonucleases and ligated. The resulting plasmids were transformed into competent *Escherichia coli* DH5a cells. The identity of the expression constructs was verified by sequencing. The plasmids were transformed into *E. coli* strain BL21 (DE3). Protein expression was induced with 0·5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and the recombinant proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography (Qiagen, Valencia, CA, USA). The purity of the rCsPRxs was monitored by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The purified proteins were dialysed against PBS (50 mM, pH 7·2) and used in both the generation of specific mouse antisera and the enzyme studies.

Specific pathogen-free 6-week-old BALB/*c* mice were subcutaneously immunized with the rCsPRx proteins (30 μg/mouse) mixed with Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA) 3 times at 2-week intervals, after which the mice were finally boosted by the intravenous route (10 μg in PBS). Ten days after the final boosting, blood was collected by heart puncture and centrifuged for 10 min at 3000 ***g***. The rCsPRx-specific mouse antisera were stored at −80 °C until use.

Western blot analysis

The whole bodies of *C. sinensis* adults (4 weeks old) were homogenized in PBS containing either a protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) or denaturants (8 M urea, 2% CHAPS and 0·4% dithiothreitol) using a Teflon pestle-homogenizer. The homogenates were centrifuged at 20 000 ***g*** for 1 h at 4 °C and the supernatants were taken as crude extracts. The viable 4-week-old worms were incubated in PBS at 37 °C for 1·5 h (30 worms in 3 ml). The incubation medium was centrifuged to remove eggs and cell debris and the resulting supernatant fraction was considered as the excretory-secretory products (ESP). The patency of the ESP was validated by light microscopic observation of possible degenerative changes of the tegument after incubation (Supplementary Fig. 1, Online version only). The protein samples (30 or 50 μg) and the rCsPRx2 and rCsPRx3 proteins (each 100 ng) were resolved by 12% SDS-PAGE gels under reducing conditions. The proteins were stained with Coomassie Blue G-250 or transferred onto nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). The membranes were probed with each specific mouse antiserum (1:1000 dilution) and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:2000 dilution; Cappel, West Chester, PA, USA). Positive signals were detected using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Buckinghamshire, UK).

Semi-quantitative reverse transcription-PCR (RT-PCR)

The live *C. sinensis* adults were stimulated by oxidative stress by *in vitro* incubation in PBS supplemented with the various concentrations of H_2O_2 (0·025–0·8 mM) for 1 h at 37 °C (10 worms/group/ml). Total RNA was extracted from the parasite materials using TriZol reagents (Invitrogen, Carlsbad, CA, USA). The RNA samples were treated with RNase-free DNase (GIBCO BRL, Rockville, MD, USA). The *CsPRx* transcripts in the total RNAs were converted into the first strand of respective cDNAs with the gene-specific reverse primers (CsPRx2/3-Xho-R) and the RNA PCR Kit (AMV, ver. 2.1; Takara, Shiga, Japan). The single-stranded cDNAs were amplified by adding each of the specific forward primers (CsPRx2/3-BamH-F) in

https://doi.org/10.1017/S0031182011000813 Published online by Cambridge University Press

subsequent PCR (thermal cycling profile: 94 °C for 4 min, 25 cycles of 50 s at 94 °C, 40 s at 60 °C, 1 min at 72 °C and 10 min at 72 °C). The cycling profile was empirically determined to ensure that the final products originated from the exponential phase of the amplifications. A tropomyosin gene was selected as a housekeeping control (CsTrop-F, 5′-TGAGTCTCGTCTAGAAGCTGCTG-3′ and CsTrop-R, 5′-GGTGAAATACGTAGGTTTGAACAC-3′). The PCR products were visualized by 1% agarose gel electrophoresis and following ethidium bromide (EtBr) staining. Induction profiles of the antioxidant genes were also investigated from total RNAs extracted from *C. sinensis* worms at the different developmental stages.

Immunohistochemical staining

Adult worms fixed with 4% paraformaldehyde were embedded in paraffin and 4 μm-thick slices were prepared according to the standard protocol. The sections were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0·05% Tween 20 (TBS/T) for 1 h. The slides were incubated overnight at 4 °C with the mouse antiserum specific to rCsPRxs (1:200 dilution in TBS/T supplemented with 3% BSA) and then incubated with the rhodamine-conjugated goat anti-mouse IgG antibody (1:1000 dilution; Abcam, Cambridge, UK). The signals were examined using a LSM510 Meta DuoScan confocal fluorescent microscope (Carl Zeiss, Jena, Germany).

Enzyme assay

The specific activities and saturation kinetic constants of rCsPRx2 and rCsPRx3 were measured by a NADPH consumption assay. In brief, the reaction mixtures contained 5 mM potassium phosphate (pH 7·0), 1 mM ethylenediaminetetraacetic acid (EDTA), 0·1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and either 5 μM *E. coli* Trx and 0·1 unit *E. coli* TR, or 1 mM GSH and 0·1 unit yeast GSH reductase (GR). The NADPH oxidation was initiated by adding 5 μM hydroperoxide substrates (H_2O_2, cumene hydroperoxide and 13-hydroperoxy octadecadienoic acid (13-HpODE)). The thiol-related molecules and hydroperoxides were purchased from Sigma-Aldrich. The enzymatic reactions (200 μl) were prepared in a 96-well quartz microplate and monitored at A_{340} for 5 min using an Infinite M200 spectrophotometer (Tecan, Grödig, Austria). The assays were independently done in triplicate and reactions without CsPRxs were also prepared to estimate background NADPH consumption. The specific activity of CsPRxs was calculated by adapting an equation, units of PRx/mg of protein = $\{([\Delta A/\Delta T \times V_t]/[5{\cdot}3 \times V_s]) \times 1000\}$/[protein] (mg/ml) [5·3 is the extinction coefficient of NADPH (path length = 0·552 cm), where V_t is the total reaction volume, V_s is the sample volume and 1000 is used to convert to nanomoles. The hydroperoxide substrates were used in a range of 0·1–20 μM under identical conditions.

RESULTS

Isolation and in silico *analyses of* C. sinensis *PRx genes*

By screening a cDNA library of *C. sinensis* through degenerate followed by conventional PCR, we isolated complete nucleotide sequences of 2 cDNAs, which correspond to the *Clonorchis* PRx genes. The genes were comprised of 749 and 900 bp, and contained an ORF for 195- and 225-aa polypeptides, respectively. The deduced aa sequences of these genes revealed significant similarities to those of 2-Cys PRx proteins identified from diverse animal taxa including platyhelminths (BLAST *e*-values <5e-56). Compared to trematode orthologues, the similarity profile of one sequence was somewhat biased toward PRx2 of *Schistosoma mansoni* (SmPRx2) and its related proteins, while the other was more closely related to the SmPRx3-like proteins, as supported by the divergence values (Supplementary Table 1, Online version only). Based on the differential homology patterns, we designated these sequences as *CsPRx2* and *CsPRx3*. Their sequence information has been deposited in the GenBank database under Accession numbers HQ896672 and HQ896673.

The molecular weights (kDa)/pIs of CsPRx2 and CsPRx3 were estimated as 21·97/7·01 and 24·93/8·75, respectively. The larger molecular weight of CsPRx3 was attributable to an aa stretch found in the N-terminal region. In addition to CsPRx3, the extra N-terminal sequence was detected in proteins isolated from *Opisthorchis viverrini* (ACB13822, OvPRx2), *Fasciola gigantica* (ABY85786, FgPRx2), *S. japonicum* (AAW25625, SjPRx2 and AAW25436, SjPRx3) and *S. mansoni* (XP_002571905, SmPRx3) (Fig. 1a). Similar to the cases in schistosome orthologues (Sayed and Williams, 2004; Kumagai *et al.* 2006), the sequence information of the CsPRx3 further revealed that it might harbour a target signal to export the protein to mitochondria (confidence value 99% by MITOPRED program; dotted blue box in Fig. 1a). The trematode 2-Cys PRxs tightly conserved the N-terminal Cys, which is oxidized into sulfenic acid by peroxides, and C-terminal Cys that forms an intermolecular disulfide with the active N-terminal Cys of another subunit (red letters marked by red circles). Residues involved in the stabilization of the Cys sulfenic acid (Hirotsu *et al.* 1999) were also recognized at each of the corresponding positions (green letters denoted by green circles). The tertiary structures of CsPRx2 and CsPRx3 were simulated on the basis of a homology model using human erythrocyte 2-Cys PRx (Protein Data Bank id. 1QMV; 55·6% identity) and human heme-binding

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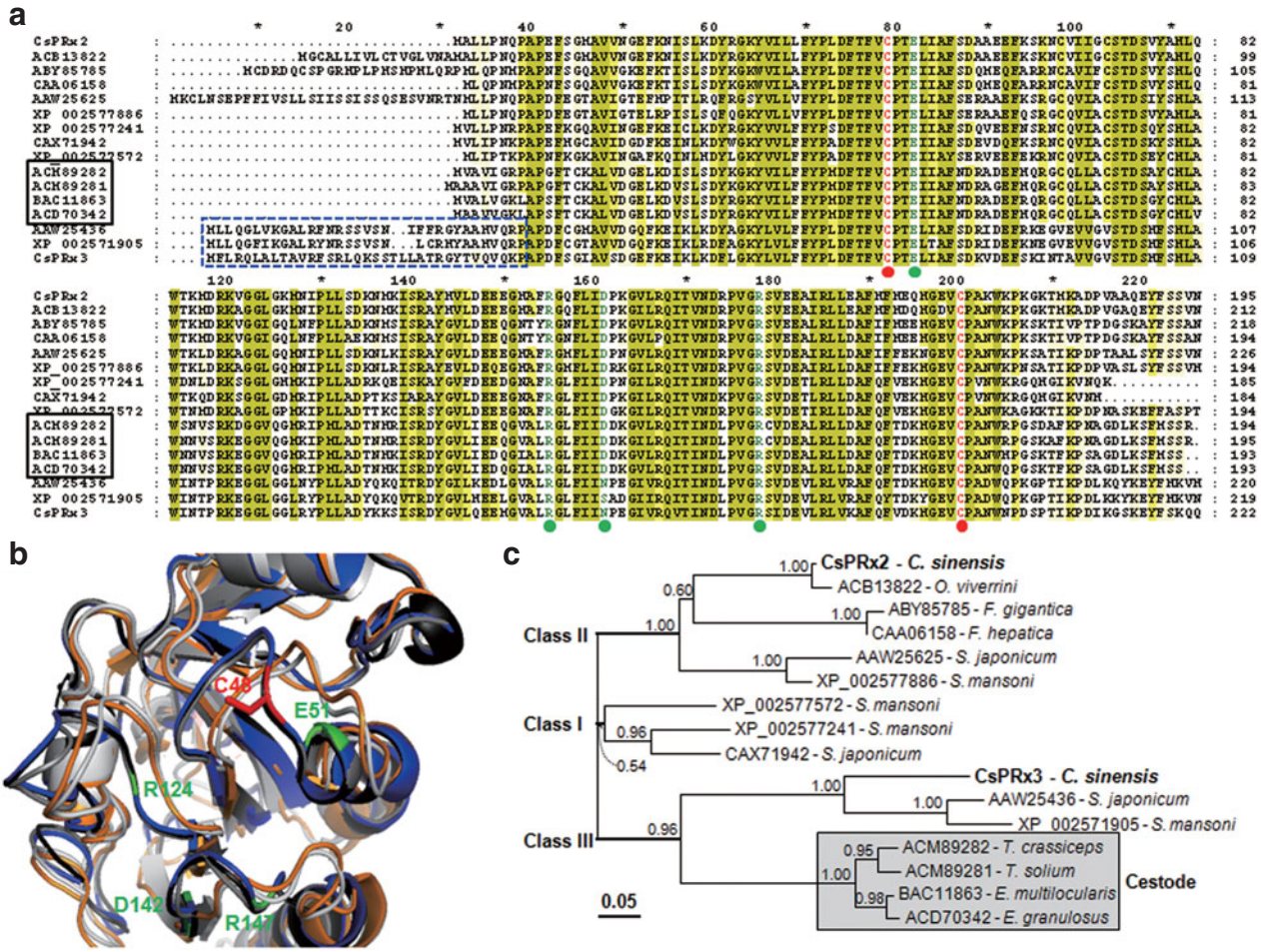

Fig. 1. Structural comparison between *Clonorchis sinensis* peroxiredoxins (CsPRxs) and their trematode/cestode orthologues. (a) The deduced amino acid sequences of CsPRxs were aligned with those of the related proteins, which were retrieved from the GenBank database by BLAST searches. The degrees of amino acid conservation are highlighted by differently graded yellow boxes. The red and green letters/circles indicate active cysteine (C) and other amino acid residues involved in the stabilization of the cysteine sulfenic acid, respectively. The C-terminal region cysteine, which formed an intermolecular disulfide bond with the active cysteine, is also marked in red. Dotted blue box in N-terminal region represents mitochondrial target signal predicted by the MITOPROT program. (b) Tertiary structures of CsPRxs were simulated by the ESyPred3D program and compared with those of *Schistosoma mansoni* proteins. Regions around the active site are shown with the positions of critical amino acids as marked in panel (a). The position numbers were based on CsPRx2 sequence. Blue, CsPRx2; orange, CsPRx3; light grey, SmPRx1 (XP_002577241); half grey, SmPRx2 (XP_002577886); black, SmPRx3 (XP_002571905). (c) The phylogenetic relationships among the platyhelminth proteins were examined with MEGA program using the sequence alignment (panel a) as an input. Divergence rates were calculated with the Jones-Taylor-Thornton substitution model and the tree was constructed using the neighbor-joining algorithm. Numbers at branching nodes indicate the likelihood of their appearance in the bootstrapping analysis with 1000 replicates.

protein 23 kDa (HBP23) (1QQ2; 57·8% identity) as a template. Comparison around the active site is shown in Fig. 1b. Similar three-dimensional structures were predicted with the sequence information of schistosome proteins. Consistent with the differentiated aa conservations, trematode PRxs were largely clustered into 3 distinct clades (Classes I, II and III) in a phylogenetic analysis (Fig. 1c). CsPRx2 and CsPRx3 were separately positioned into Class II and Class III.

Expression profiles of CsPRx *genes*

In a preliminary observation, we detected native CsPRx proteins at approximately 20 kDa in the soluble worm extracts by Western blotting employing antibody specific to rCsPRx2. However, we were not able to identify CsPRx proteins reactive to antibody against rCsPRx3 (data not shown). This observation suggested either that the expression level of CsPRx3 was below the detection range of the assay, or that CsPRx3 had a poor solubility in the extraction buffer used. Since the major target site for CsPRx3 seemed to be mitochondria (Fig. 1a), we extracted the parenchymal proteins with a denaturing buffer containing urea (8 M) and CHAPS (2%). This fraction (50 μg) was resolved by SDS-PAGE and probed with each of the specific antibodies, concomitantly with ESP proteins (30 μg) obtained by incubating live

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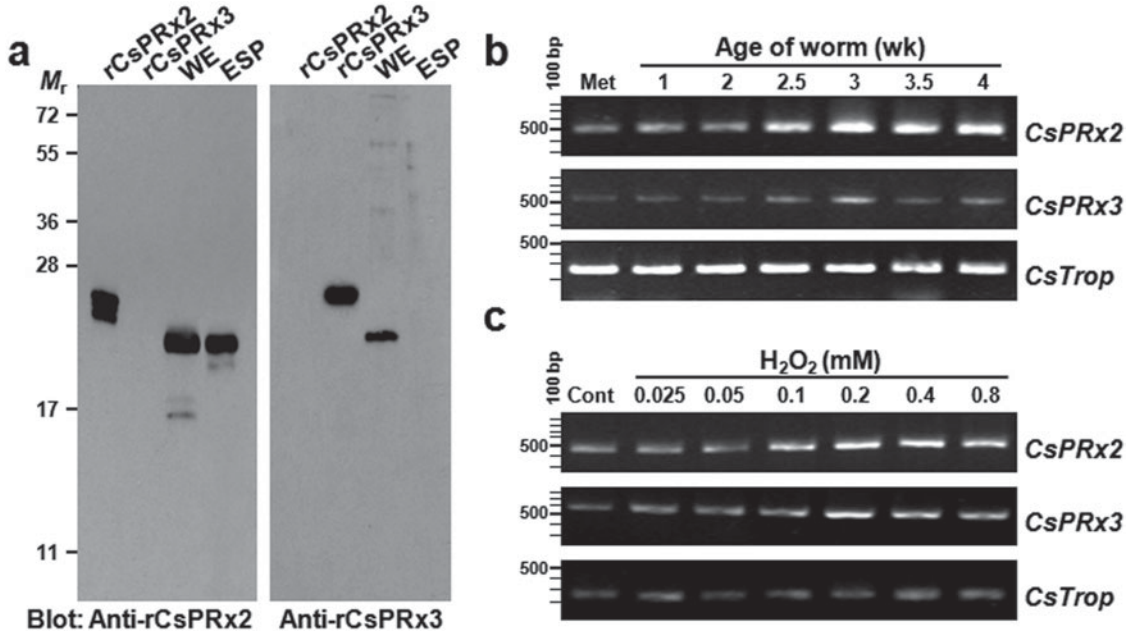

Fig. 2. Expression profiles of *Clonorchis sinensis* peroxiredoxins (CsPRxs). (a) Adult soluble proteins extracted in a denaturing buffer (WE; 50 μg) and excretory-secretory products (ESP; 30 μg in PBS) of *C. sinensis* were resolved by 12% reducing SDS-PAGE, and transferred onto nitrocellulose membranes. The recombinant forms of CsPRxs (rCsPRx2 and rCsPRx3; 100 ng) were also included. The membranes were probed with each of the mouse antisera specific to rCsPRx2 and rCsPRx3, and the positive signals were developed with the ECL detection system. M_r, molecular weights in kDa. (b) Total RNAs were extracted from *C. sinensis* worms at various development/maturation stages as indicated at the top. The mRNA transcripts corresponding to *CsPRx2* and *CsPRx3* were amplified by a semi-quantitative RT-PCR. The amplified products were separated on 1·2% agarose gels and visualized by ethidium bromide staining. Reactions to amplify tropomyosin gene transcript (*CsTrop*) were included as a standard control for each of the RNA samples. The absence of any contaminated genomic DNA was confirmed by preparing reactions without reverse transcriptase during the first reverse transcription step (data not shown). Met, metacercaria; 100 bp, DNA size standards. (c) The live *C. sinensis* adults were stimulated for 1 h in PBS supplemented with various concentrations of H_2O_2, as indicated at the top. Induction profiles of the *CsPRx* genes under the oxidative-stress condition were similarly examined by the RT-PCR method. Cont, RNA sample from the incubation in PBS only.

adult worms in PBS (Fig. 2a). Both of the antibodies detected a specific immunoreactive signal with molecular weights similar to each of the predicted ones in the parenchymal extracts. Antibody against rCsPRx2, but not anti-rCsPRx3 antibody, further reacted with a homologous protein(s) in ESP. The relatively weaker signal of anti-rCsPRx3 antibody against the parenchymal proteins appeared to suggest the substantially low-level expression of CsPRx3 compared to that of CsPRx2.

The temporal expression of *CsPRx* genes was investigated at the different developmental stages by semi-quantitative RT-PCR. The *CsPRx2* and *CsPRx3* transcripts were efficiently amplified from total RNAs extracted from all of the stages examined. As shown in Fig. 2b, however, the amplification profiles were found to be different between them. The amount of *CsPRx*2 amplicon increased gradually in accordance with the development/maturation of the worm from metacercaria to adult, whereas that of *CsPRx3* remained constant at the residual level throughout all the stages. PCR product of tropomyosin gene (*CsTrop*), which was employed as a control housekeeping gene, exhibited no significant change among the RNA samples. We further analysed *in vitro* stress-related induction profiles of these antioxidant genes of *C. sinensis* adults. The intact live worms were incubated in PBS supplemented with H_2O_2 at various concentrations (0·025–0·8 mM) for 1 h at 37 °C, after which the expression levels of the *CsPRx*s were examined in the differentially stimulated worms by RT-PCR. As shown in Fig. 2c, the amount of *CsPRx2* transcripts was significantly increased in the RNA samples stimulated by H_2O_2 at the concentration of more than 0·1 mM. Expression of the *CsPRx3* transcripts was also augmented upon stimulation, although the effect was not so prominent compared to that of *CsPRx2*.

Tissue and organ distributions of the CsPRx proteins were determined by staining *C. sinensis* adult worm sections with the rCsPRx-specific antibodies. The anti-rCsPRx2 antibody reacted with protein(s) in almost all parasite tissues including the vitelline gland, uterine eggs, testis, ovary, gut epithelium and subtegumental region. The anti-rCsPRx3 antibody showed a similar recognition pattern, but the signal intensity was relatively weak compared to that against anti-rCsPRx2 antibody (data not shown).

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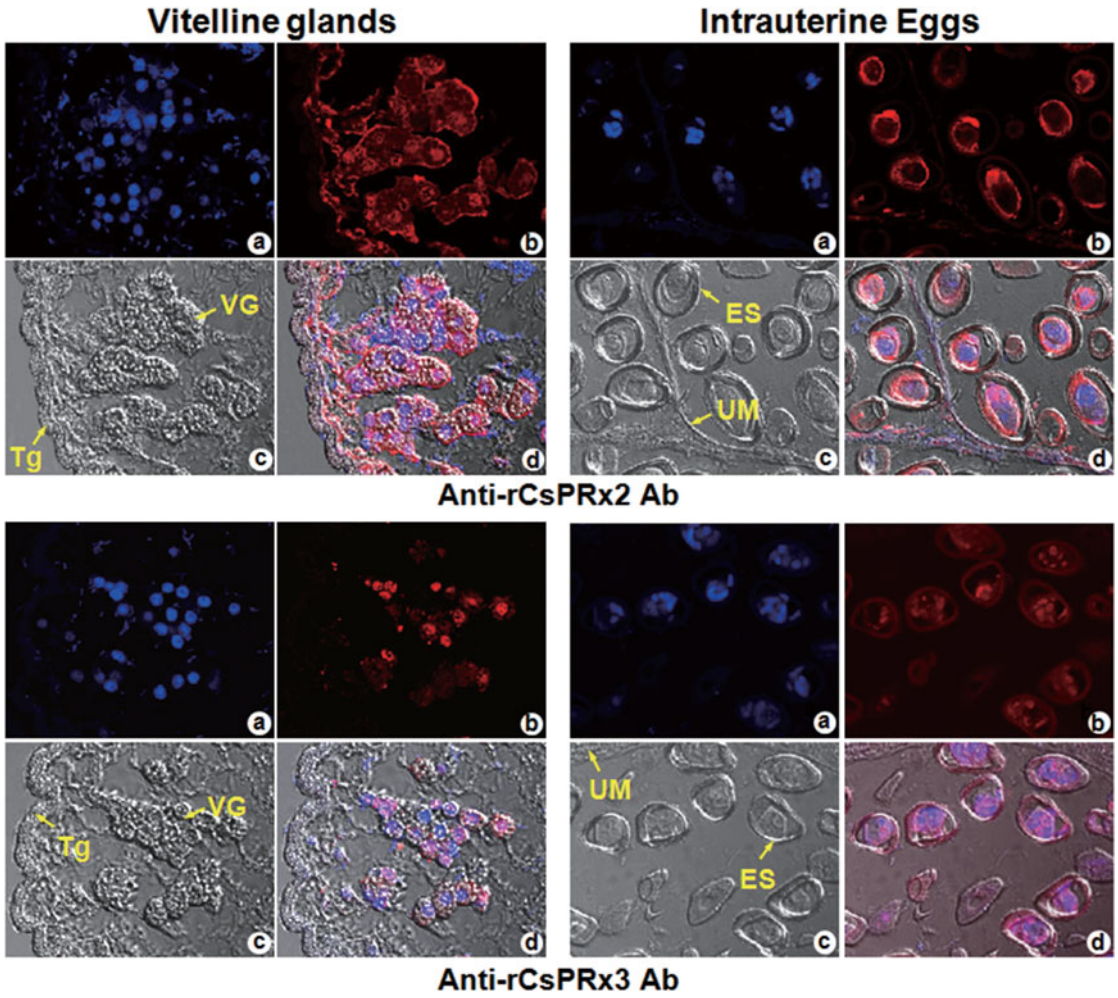

Fig. 3. Spatial expressions of native CsPRx proteins in *Clonorchis sinensis* adult sections. The worm sections (4 μm thick) were incubated with the rCsPRx-specific antibodies (1:200 dilution) and subsequently with rhodamine-conjugated goat anti-mouse IgG antibody (1:1000 dilution, red colour). The chromatins in the worm sections were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue colour in panel a). The immunohistochemical staining patterns observed under a fluorescent microscope (panel b) present images for rhodamine staining). The sections were also observed under the laser-scanning microscope (panel c) and the images were merged with those from fluorescent microscope observations (panel d). Results from only around the vitelline glands, tegument, and uterine eggs are presented in this figure. Tg, tegument; VG, vitelline glands; ES, eggshell; UM, uterine membrane.

Interestingly, the focal distributions of these proteins appeared to be different in certain organs such as vitelline glands and eggs. The CsPRx2 was localized largely in the peripheral region of vitelline glands and surface of embryonated ovum in intrauterine eggs. In contrast, CsPRx3 was mainly distributed in the inner region of vitelline gland and cellular compartment of the egg. There was also no reaction in the subtegumental region against the anti-rCsPRx3 antibody (Fig. 3).

Antioxidant activities of rCsPRx proteins

The rCsPRx2 and rCsPRx3 proteins expressed in *E. coli* were used to assay the specific activity against various peroxide substrates such as H_2O_2, cumene hydroperoxide and 13-HpODE in the presence of reducing thiol system (Trx/TR or GSH/GR). The rCsPRx2 protein was highly reactive with H_2O_2 when GSH was provided for the reducing electron (Fig. 4). The enzyme also reduced the soluble hydroperoxide with Trx although the activity reached approximately 40% of the GSH-dependent activity. The specific activities of rCsPRx2 against cumene and lipid hydroperoxides appeared to be comparable in these reactions, regardless of electron donors used. The reduction levels were low but similar to that for the Trx-dependent hydroxylation of H_2O_2. The rCsPRx3 protein exhibited enzymatic activities toward the selected substrates analogous to rCsPRx2, while the activity against H_2O_2 was slightly elevated (125%). The overall reaction profiles of rCsPRx2 and rCsPRx3 against the hydroperoxides were equivalent to those of SmPRx2 and SmPRx3 in terms of GSH dependency and the substrate preference toward H_2O_2 (Sayed and Williams, 2004).

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Table 1. Enzymatic kinetics of recombinant CsPRxs with various hydroperoxides and electron donors

(The kinetic constants were estimated from reactions with different concentrations of hydroperoxides at a saturating level of reducing thiols, by the kinetic NADPH consumption assay.)

		Substrate								
		H_2O_2			Cumene hydroperoxide			13-HpODE[a]		
Enzyme	Thiol system	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
CsPRx2	GSH/GR	0·5	12·5	$2·5\times10^4$	0·9	4·0	$4·6\times10^3$	0·8	4·2	$5·3\times10^3$
	Trx/TR	10·0	29·0	$2·9\times10^3$	6·0	7·6	$1·3\times10^3$	5·0	5·8	$1·2\times10^3$
CsPRx3	GSH/GR	2·3	32·1	$1·4\times10^4$	3·3	17·5	$5·3\times10^3$	3·7	14·6	$3·9\times10^3$
	Trx/TR	14·2	54·2	$3·8\times10^3$	23·5	35·9	$1·5\times10^3$	1·2	3·0	$2·5\times10^3$

[a] 13-hydroperoxy octadecadienoic acid.

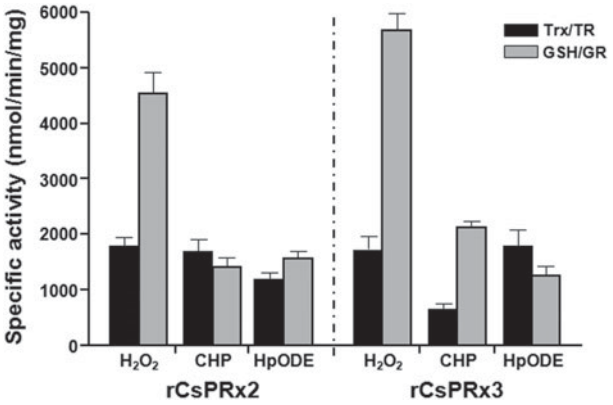

Fig. 4. Relative enzyme activities of recombinant CsPRx2 and CsPRx3 proteins. The antioxidant activities were examined by NADPH consumption assay against H_2O_2, cumene hydroperoxide (CHP) and 13-hydroperoxy octadecadienoic acid (HpODE) under the thioredoxin (Trx)/Trx reductase (TR) or glutathione (GSH)/GSH reductase (GR) system. The specific activity was determined using the hydroperoxide substrates at a concentration of 5 μM and expressed as the concentration of consumed NADPH in nmol/min/mg protein. The measurements were repeated with 3 separate preparations and a representative result is shown.

The enzymatic reactions followed Michaelis-Menten kinetics with the increased concentrations of peroxide substrates assayed, when the reactions were initially saturated with the reducing thiols (data not shown). Table 1 summarizes the saturating kinetic constants including Michaelis constant (K_m), catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m) for the rCsPRx2 and rCsPRx3 proteins. The K_m values for the hydroperoxides were highly dependent on the thiol molecules added in the reactions. The recombinant proteins had higher affinities for all of these substrates in the presence of GSH/GR than those with Trx/TR (6–7 times by K_m values), except for that of rCsPRx3 toward 13-HpODE (3 times greater K_m value). The ratio between K_m values of rCsPRx2 for H_2O_2 was especially prominent in association with each of the thiol systems (20 times). Consistent with the specific activities, rCsPRxs showed the highest catalytic efficiency against H_2O_2, when supplemented with the GSH-thiol system ($2·5\times10^4$ and $1·4\times10^4$). The values were similar and remained in a range of $1·2–5·3\times10^3$ for the enzyme reactions with other combinations of substrate-reducing equivalent.

DISCUSSION

In the present study, we screened the *C. sinensis* cDNA library by degenerate PCR and isolated 2 novel genes putatively responsible for the PRx activity. The encoded proteins contained aa sequences and catalytic motifs highly conserved in the eukaryotic PRx proteins. Their cladistic affinities were found to be much higher toward PRx2- (CsPRx2) and PRx3-like (CsPRx3) members of trematode orthologues. The native CsPRx3 protein, which might be targeted into mitochondria, was constantly expressed at a residual level throughout the developmental stages of *C. sinensis* in the definitive host. CsPRx2 expression appeared to be highly inducible along with the parasite's maturation and in response to exogenously introduced H_2O_2. The protein was localized in the marginal basement and subtegument, as well as the parenchymal portions. It was also apparent that CsPRx2 or its closely related parologue(s) were secreted into the surrounding environment. The recombinant CsPRx2 and CsPRx3 generated in *E. coli* preferred H_2O_2 and GSH as a catalytic substrate and electron donor, respectively.

In general, the defensive response against ROS is initiated with SOD to change superoxide anions to H_2O_2, which is intimately associated with the generation of more deleterious hydroxyl radicals. The H_2O_2 level is appropriately regulated by the action of a series of enzymes such as catalase, GPx and PRx. Since trematode parasites express no catalase activity and the GPx proteins have been largely detected in the vitelline glands and intrauterine eggs, PRx might

https://doi.org/10.1017/S0031182011000813 Published online by Cambridge University Press

comprise the major antioxidant enzyme protecting the worms from the ROS-related chemical attacks (Mkoji *et al*. 1988; Kumagai *et al*. 2006; Cai *et al*. 2008; Kim *et al*. 2009; Chaithirayanon and Sobhon, 2010).

It has been reported that 3 isoforms of schistosome PRxs have undergone structural and functional diversification after duplication to acquire different biochemical properties (Sayed and Williams, 2004; Kumagai *et al*. 2006). CsPRx2 displays a strong phylogenetic affinity toward multiple PRx proteins characterized in other trematodes including *F. gigantica* (Chaithirayanon and Sobhon, 2010), *O. viverrini* (Suttiprapa *et al*. 2008) and *F. hepatica* (Sekiya *et al*. 2006), in addition to the *Schistosoma* spp. isoform 2 (Class II). CsPRx3 presently appeared to be an isoform 3 orthologue (Class III), according to the degrees of aa conservation and clustering pattern in the phylogenetic tree (Supplementary Table 1 and Fig. 1c, Online version only). A recent study revealed the transcriptomes of *C. sinensis* and *O. viverrini* by analysing nucleotide information included in the expressed sequence tag (EST) datasets of 50,769 and 61,417 contigs (http://research.vet.unimelb.edu.au/gasserlab/index.html; Young *et al*. 2010). *In silico* examination of the *C. sinensis* ESTs based on the BLAST algorithm retrieved 2 contig sequences identical to the *CsPRx2* (Cs_Contig10436) and *CsPRx3* (Cs_Contig9931) genes. The similarity analysis further detected a third sequence (CS1_c35731), whose aa sequence showed the highest matching score to the *S. japonicum* Class III PRx (AAW25436) among the trematode PRx proteins including CsPRx3. The *Opisthorchis* database also displayed a coding profile similar to that of *C. sinensis*: Ov_Contig10547 (ACB13822; Cs_Contig10436 orthologue), Ov_Contig48391 (Cs_Contig9931 orthologue) and Ov_Contig6289 (CS1_c35731 orthologue). BLAST searches using the aa sequences of SmPRx1 and SjPRx1 as queries demonstrated similar results and provided no molecular evidence that both of the trematode genomes encode gene(s) homologous to the Class I genes of schistosomes. Currently, it is not clear whether the evolutionary pathway to generate the 3 *PRx* gene classes is universal in trematode species and awaits future studies.

In schistosomes, PRx proteins show unique physiological implications with differential tissue distribution patterns: Class I members might protect worms in the external regions against exogenous ROS produced by host immune cells, while Class II members might be involved in the regulation of endogenous ROS generated during the parasite's metabolic reactions. Proteins belonging to Class III might be targeted into mitochondria to remove harmful species in the cellular power plants (Kumagai *et al*. 2006). In contrast to the schistosome proteins, however, PRx proteins categorized into Class II were detected in the ESPs of *C. sinensis*, *F. hepatica* (CAA06158; Sekiya *et al*. 2006) and *O. viverrini* (ACB13822; Suttiprapa *et al*. 2008). A previous proteomic analysis of *C. sinensis* ESP has also identified a protein spot, whose peptide mass fingerprint was similar to that of a SjPRx2 isoform (BAD90102; Ju *et al*. 2009). In order to collect the ESP sample used in this study, we incubated *C. sinensis* worms in PBS for a relatively short period (1·5 h). Microscopic examination of the worms ensured that the tegument was not physically injured by the culture medium. Therefore, the proteins contained in the EPS, if not all, might be secreted by the worms rather than extracted from detached tegumental tissues. The taeniid proteins characterized to date exhibited divergence values similar to those among trematode members, and appear to be more closely related to the members of Class III (bootstrapping value 0·96; grey box in Fig. 1c). Further information on the cestode PRxs will be necessary to establish an accurate phylogenetic relationship between trematode and cestode proteins, since the mitochondrial-target signal is not currently available in the cestode proteins.

The schistosome PRx2 and PRx3 proteins exhibited higher enzyme activities in reactions coupled with the GSH system than those with Trx equivalent, as an electron source for the reduction of their oxidized forms (Sayed and Williams, 2004). Conversely, a study with the *Fasciola* enzyme (FhPRx2) demonstrated that the protein was not reactive when the reducing hydrogen atom is provided by GSH/GR system (Sekiya *et al*. 2006). The aa residues in the C-terminal tail have been shown to be involved in the GSH-dependent peroxidase activity of trematode PRxs (Sayed and Williams, 2004). The FhPRx2/FgPRx2 also possessed the tail, but certain aa residues, especially with charged R groups such as Lys, Asp and Glu, were substituted by other aa residues with uncharged groups. The conformational alteration induced by the aa changes might reduce the accessibility and/or binding affinity of GSH against the *Fasciola* proteins. The aa stretch was not found in corresponding regions of the Trx-dependent PRx1 of *S. mansoni* and *S. japonicum*. Consistent with the GSH-sensitive activity, CsPRxs had the C-terminal tails with aa conservation shared with the schistosome orthologues (PRx2 and PRx3). Further investigations with the *Fasciola* proteins, where the critical aa residues are replaced by those in the GSH-dependent proteins, might be beneficial to understand the detailed molecular mechanism(s) relevant to the reducing equivalent dependency. Biochemical properties of the *S. mansoni* protein (XP_002577572) would also be informative, since the protein contains the C-terminal tail and shows a tight relationship with the Class I members.

Expression of *C. sinensis* GPx proteins is largely restricted within vitellocytes (Cai *et al*. 2008), which proliferate and mature in the female-specific vitelline glands and then migrate into ootype to comprise egg

https://doi.org/10.1017/S0031182011000813 Published online by Cambridge University Press

with a fertilized ovum (Smyth and Halton, 1983; Mei *et al*. 1996; Kim *et al*. 2009). Therefore, the systemic PRx proteins might play a central antioxidant role in the catalase-lacking trematode parasites including *C. sinensis* against ROS attacks from both endogenous and exogenous origins. The PRx proteins characterized from *C. sinensis* showed flexible reaction profiles, in terms of both hydroperoxide substrate and reducing-equivalent system. This biochemical flexibility might be advantageous for the compensatory and harmonized removal of oxidative molecules with the relatively small numbers of antioxidant genes encoded in the parasite genome. In addition to the fundamental role as an H_2O_2 scavenger, the PRx proteins are suggested to be involved in the intracellular signal transduction. Redox signalling is likely to be mediated by the YF motif found in the C-terminal regions of homologous molecules (Wood *et al*. 2003; Rhee *et al*. 2005). The histological distribution of CsPRx2 and its orthologues in the subtegumental region seemed to support its role as the signal transducer, together with the tightly conserved YF motif (Sayed and Williams, 2004). Interestingly, the CsPRx3-like proteins of trematodes, which contain the mitochondrial target signal, also display the conventional YF motif (Liu *et al*. 2006). Studies on the molecular events relevant to the functional diversification and the balance between the signal transduction and hydroperoxide scavenging will be required to give a detailed understanding of the unique antioxidant system evolved in trematode parasites.

ACKNOWLEDGEMENTS

We are grateful to T-S Kim for her technical assistance during the work.

FINANCIAL SUPPORT

This work was supported by the grant from the Samsung Biomedical Research Center (#SBRI BA70131).

REFERENCES

Ahsan, M. K., Lekli, I., Ray, D., Yodoi, J. and Das, D. K. (2009). Redox regulation of cell survival by the thioredoxin superfamily: an implication of redox gene therapy in the heart. *Antioxidants – Redox Signaling* **11**, 2741–2758.

Bouvard, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F., Benbrahim-Tallaa, L., Guha, N., Freeman, C., Galichet, L. and Cogliano, V.; **WHO International Agency for Research on Cancer Monograph Working Group** (2009). A review of human carcinogens – Part B: biological agents. *Lancet Oncology* **10**, 321–322.

Cai, G. B., Bae, Y. A., Kim, S. H., Sohn, W. M., Lee, Y. S., Jiang, M. S., Kim, T. S. and Kong, Y. (2008). Vitellocyte-specific expression of phospholipid hydroperoxide glutathione peroxidases in *Clonorchis sinensis*. *International Journal for Parasitology* **38**, 1613–1623.

Callahan, H. L., Crouch, R. K. and James, E. R. (1988). Helminth anti-oxidant enzymes: a protective mechanism against host oxidants? *Parasitology Today* **4**, 218–225.

Chaithirayanon, K. and Sobhon, P. (2010). Molecular cloning and characterization of two genes encoding 2-Cys peroxiredoxins from *Fasciola gigantica*. *Experimental Parasitology* **125**, 106–113.

Choi, B. I., Han, J. K., Hong, S. T. and Lee, K. H. (2004). Clonorchiasis and cholangiocarcinoma: etiologic relationship and imaging diagnosis. *Clinical Microbiology Review* **17**, 540–552.

Henkle-Dührsen, K. and Kampkötter, A. (2001). Antioxidant enzyme families in parasitic nematodes. *Molecular and Biochemical Parasitology* **114**, 129–142.

Hirotsu, S., Abe, Y., Okada, K., Nagahara, N., Hori, H., Nishino, T. and Hakoshima, T. (1999). Crystal structure of a multifunctional 2-Cys peroxiredoxin heme-binding protein 23 kDa/proliferation-associated gene product. *Proceedings of the National Academy of Sciences, USA* **96**, 12333–12338.

Hong, S. J., Lee, J. Y., Lee, D. H., Sohn, W. M. and Cho, S. Y. (2001). Molecular cloning and characterization of a mu-class glutathione S-transferase from *Clonorchis sinensis*. *Molecular and Biochemical Parasitology* **115**, 69–75.

Hong, S. J., Yu, J. R. and Kang, S. Y. (2002). Ultrastructural localization of 28 kDa glutathione S-transferase in adult *Clonorchis sinensis*. *Korean Journal of Parasitology* **40**, 173–176.

Hong, S. T., Kho, W. G., Kim, W. H., Chai, J. Y. and Lee, S. H. (1993). Turnover of biliary epithelial cells in *Clonorchis sinensis* infected rats. *Korean Journal of Parasitology* **31**, 83–89.

Ju, J. W., Joo, H. N., Lee, M. R., Cho, S. H., Cheun, H. I., Kim, J. Y., Lee, Y. H., Lee, K. J., Sohn, W. M., Kim, D. M., Kim, I. C., Park, B. C. and Kim, T. S. (2009). Identification of a serodiagnostic antigen, legumain, by immunoproteomic analysis of excretory-secretory products of *Clonorchis sinensis* adult worms. *Proteomics* **9**, 3066–3078.

Keiser, J. and Utzinger, J. (2009). Food-borne trematodiases. *Clinical Microbiology Reviews* **22**, 466–483.

Kim, S. H., Cai, G. B., Bae, Y. A., Lee, E. G., Lee, Y. S. and Kong, Y. (2009). Two novel phospholipid hydroperoxide glutathione peroxidase genes of *Paragonimus westermani* induced by oxidative stress. *Parasitology* **136**, 553–565.

Kumagai, T., Osada, Y. and Kanazawa, T. (2006). 2-Cys peroxiredoxins from *Schistosoma japonicum*: the expression profile and localization in the life cycle. *Molecular and Biochemical Parasitology* **149**, 135–143.

Kuntz, A. N., Davioud-Charvet, E., Sayed, A. A., Califf, L. L., Dessolin, J., Arnér, E. S. and Williams, D. L. (2007). Thioredoxin glutathione reductase from *Schistosoma mansoni*: an essential parasite enzyme and a key drug target. *PLoS Medicine* **4**, e206.

Li, A. H., Kong, Y., Cho, S. H., Lee, H. W., Na, B. K., Park, J. K. and Kim, T. S. (2005). Molecular cloning and characterization of the copper/zinc and manganese superoxide dismutase genes from the human parasite *Clonorchis sinensis*. *Parasitology* **130**, 687–697.

Liu, F., Lu, J., Hu, W., Wang, S. Y., Cui, S. J., Chi, M., Yan, Q., Wang, X. R., Song, H. D., Xu, X. N., Wang, J. J., Zhang, X. L., Zhang, X., Wang, Z. Q., Xue, C. L., Brindley, P. J., McManus, D. P., Yang, P. Y., Feng, Z., Chen, Z. and Han, Z. G. (2006). New perspectives on host-parasite interplay by comparative transcriptomic and proteomic analyses of *Schistosoma japonicum*. *PLoS Pathogen* **2**, e29.

Lun, Z. R., Gasser, R. B., Lai, D. H., Li, A. X., Zhu, X. Q., Yu, X. B. and Fang, Y. Y. (2005). Clonorchiasis: a key foodborne zoonosis in China. *Lancet Infectious Diseases* **5**, 31–41.

McGonigle, S., Dalton, J. P. and James, E. R. (1998). Peroxiredoxins: a new antioxidant family. *Parasitology Today* **14**, 139–145.

Mei, H., Thakur, A., Schwartz, J. and LoVerde, P. T. (1996). Expression and characterization of glutathione peroxidase activity in the human blood fluke *Schistosoma mansoni*. *Infection and Immunity* **64**, 4299–4306.

Mieyal, J. J., Gallogly, M. M., Qanungo, S., Sabens, E. A. and Shelton, M. D. (2008). Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxidants & Redox Signaling* **10**, 1941–1988.

Mkoji, G. M., Smith, J. M. and Prichard, R. K. (1988). Antioxidant systems in *Schistosoma mansoni*: correlation between susceptibility to oxidant killing and the levels of scavengers of hydrogen peroxide and oxygen free radicals. *International Journal for Parasitology* **18**, 661–666.

Rhee, S. G., Kang, S. W., Jeong, W., Chang, T. S., Yang, K. S. and Woo, H. A. (2005). Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Current Opinion in Cell Biology* **17**, 183–189.

Roche, C., Liu, J. L., LePresle, T., Capron, A. and Pierce, R. J. (1996). Tissue localization and stage-specific expression of the phospholipid hydroperoxide glutathione peroxidase of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **75**, 187–195.

Salinas, G., Selkirk, M. E., Chalar, C., Maizels, R. M. and Fernández, C. (2004). Linked thioredoxin-glutathione systems in platyhelminths. *Trends in Parasitology* **20**, 340–346.

Sayed, A. A. and Williams, D. L. (2004). Biochemical characterization of 2-Cys peroxiredoxins from *Schistosoma mansoni*. *Journal of Biological Chemistry* **279**, 26159–26166.

Sekiya, M., Mulcahy, G., Irwin, J. A., Stack, C. M., Donnelly, S. M., Xu, W., Collins, P. and Dalton, J. P. (2006). Biochemical characterization of the recombinant peroxiredoxin (FhePrx) of the liver fluke, *Fasciola hepatica*. *FEBS Letters* **580**, 5016–5022.

Sies, H. (1993). Strategies of antioxidant defense. *European Journal of Biochemistry* **215**, 213–219.

Smyth, J. D. and Halton, D. W. (1983). *The Physiology of Trematodes*. Cambridge University Press, Cambridge, UK.

Sripa, B., Kaewkes, S., Sithithaworn, P., Mairiang, E., Laha, T., Smout, M., Pairojkul, C., Bhudhisawasdi, V., Tesana, S., Thinkamrop, B., Bethony, J. M., Loukas, A. and Brindley, P. J. (2007). Liver fluke induces cholangiocarcinoma. *PLoS Medicine* **4**, e201.

Suttiprapa, S., Loukas, A., Laha, T., Wongkham, S., Kaewkes, S., Gaze, S., Brindley, P. J. and Sripa, B. (2008). Characterization of the antioxidant enzyme, thioredoxin peroxidase, from the carcinogenic human liver fluke, *Opisthorchis viverrini*. *Molecular and Biochemical Parasitology* **160**, 116–122.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.

Trachootham, D., Lu, W., Ogasawara, M. A., Nilsa, R. D. and Huang, P. (2008). Redox regulation of cell survival. *Antioxidants & Redox Signaling* **10**, 1343–1374.

Vennervald, B. J. and Polman, K. (2009). Helminths and malignancy. *Parasite Immunology* **31**, 686–696.

Wood, Z. A., Poole, L. B. and Karplus, P. A. (2003). Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* **300**, 650–653.

Young, N. D., Campbell, B. E., Hall, R. S., Jex, A. R., Cantacessi, C., Laha, T., Sohn, W. M., Sripa, B., Loukas, A., Brindley, P. J. and Gasser, R. B. (2010). Unlocking the transcriptomes of two carcinogenic parasites, *Clonorchis sinensis* and *Opisthorchis viverrini*. *PLoS Neglected Tropical Diseases* **4**, e719.