



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Biochemical characterisation of the recombinant peroxiredoxin (FhePrx) of the liver fluke, *Fasciola hepatica*

Mary Sekiya^{a,*}, Grace Mulcahy^{a,b}, Jane A. Irwin^a, Colin M. Stack^c, Sheila M. Donnelly^c, Weibo Xu^c, Peter Collins^c, John P. Dalton^c

^a School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

^b Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

^c Institute for the Biotechnology of Infectious Diseases (IBID), University of Technology Sydney (UTS), Building 4, Corner, Harris and Thomas Street, Ultimo, Sydney NSW 2007, Australia

Received 16 May 2006; revised 31 July 2006; accepted 4 August 2006

Available online 22 August 2006

Edited by Judit Ovádi

Abstract The parasitic helminth *Fasciola hepatica* secretes a 2-Cys peroxiredoxin (Prx) that may play important functions in host–parasite interaction. Recombinant peroxiredoxin (FhePrx) prevented metal-catalyzed oxidative nicking of plasmid DNA and detoxified hydrogen peroxide when coupled with *Escherichia coli* thioredoxin and thioredoxin reductase ($k_{\text{cat}}/K_m = 5.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). Enzyme kinetic analysis revealed that the catalytic efficiency of FhePrx is similar to other 2-Cys peroxiredoxins; the enzyme displayed saturable enzyme Michaelis–Menten type kinetics with hydrogen peroxide, cumene hydroperoxide and *t*-butyl hydroperoxide, and is sensitive to concentrations of hydrogen peroxide above 0.5 mM. Like the 2-Cys peroxiredoxins from a related helminth, *Schistosoma mansoni*, steady-state kinetics indicate that FhePrx exhibits a saturable, single displacement-like reaction mechanism rather than non-saturable double displacement (ping–pong) enzyme substitution mechanism common to other peroxiredoxins. However, unlike the schistosome Prxs, FhePrx could not utilise reducing equivalents supplied by glutathione or glutathione reductase. © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Peroxiredoxin; Thioredoxin peroxidase; *Fasciola hepatica*

1. Introduction

Peroxiredoxins (Prx) are a ubiquitous family of peroxidases that reduce hydrogen peroxide to water and alkyl hydroperoxides to alcohols [4,5,17,24,33]. At least in bacteria (AhpC), yeast (thioredoxin peroxidase), and trypanosomatids (tryparedoxin peroxidase) Prxs are central in the defence against reactive oxygen species (ROS) and protect these cells from oxidative stress [17,33]. More recent studies indicate that these enzymes also function intracellularly [32] and extracellularly [12] in the regulation of H₂O₂-mediated cell-signalling events in eukaryotes.

The catalytic cycle begins with oxidation by the peroxide substrate of the essential peroxidatic cysteine, the N-terminal proximal active site Cys, forming a Cys-sulfenic acid derivative. The intermediate is resolved by a condensation reaction when Cys-sulfenic acid reacts with a Cys in the C-terminal distal domain of a second inverted Prx molecule, thus forming a dimer linked by two disulfide bridges [17,33]. Reduction of the Prx dimer regenerates the Prx monomer and in most cases the physiological hydrogen donor is thioredoxin, but this may also be performed by tryparedoxin or glutathione [17]. Thioredoxin itself is maintained in the reduced state by electrons transferred from NADPH by thioredoxin reductase [4,5,21,33].

The liver fluke, *Fasciola hepatica*, is an important helminth pathogen of humans and livestock [8,11,22]. Infective parasites (metacercariae) excyst from a dormant state following ingestion and penetrate the intestinal wall before migrating to the liver. In this nutrient and oxygen rich environment the parasites undergo rapid growth and development, and energy is supplied by aerobic respiration [9,31]. As part of its antioxidant defence, *F. hepatica* expresses glutathione-S-transferase and superoxide dismutase, which detoxify ROS to hydrogen peroxide [1,2,21]. The pathway of hydrogen peroxide detoxification is unclear, since the liver fluke lacks catalase and exhibits little glutathione peroxidase activity [1,2,24]. Our laboratory was the first to describe the presence of Prxs in helminth parasites when we reported the isolation of a cDNA encoding this enzyme in *F. hepatica* [23]. Since these enzymes can detoxify hydrogen peroxide we suggested that Prx performs this function in *F. hepatica*, and other helminths that lack catalase [23,24].

Here we report the biochemical characterisation of a functionally active recombinant *F. hepatica* 2-Cys peroxiredoxin (FhePrx). We show its protective effects against hydrogen peroxide in a mixed-function oxidation assay, and by coupling FhePrx with *Escherichia coli* thioredoxin and thioredoxin reductase determined the specific activity and reaction kinetics of the enzyme. FhePrx displays saturable, Michaelis–Menten type kinetics when assayed at low concentrations of substrate but is sensitive, rather than robust, to over-oxidation by hydrogen peroxide. The FhePrx does not accept reducing equivalents from glutathione or glutathione reductase, which is in contrast to Prx from the related parasite *Schistosoma mansoni*.

*Corresponding author. Fax: +353 1 716 6185.
E-mail address: Mary.Sekiya@ucd.ie (M. Sekiya).

Abbreviations: Prx, peroxiredoxin; ES, excretory–secretory products

2. Materials and methods

2.1. Parasite materials, proteins and RNA extraction

Adult *F. hepatica* were recovered from the bile ducts of sheep experimentally challenged for 14 weeks with 200 infective metacercariae. Soluble extracts were prepared by three cycles of freeze-thaw at -20°C and sonication for 30 s in a soni-bath. Extracts were centrifuged at $14000 \times g$ for 30 min, the supernatant decanted and stored at -80°C . Excretory–secretory (ES) products were prepared by culturing parasites in RPMI-1640, containing 30 mM HEPES, 1% glucose and gentamycin (25 $\mu\text{g}/\text{ml}$) for 6 h. The culture medium was concentrated to 1 mg/ml on a Centricon (Amicon) with a 3000 Da molecular size cutoff. The cysteine protease inhibitor E-64 (final concentration 1 mM) was added to parasite extracts and ES products to prevent degradation by parasite cysteine proteases. Juvenile parasites were excysted from infective metacercaria as previously described [3,8]. Following a single freeze-thaw cycle at -80°C , the parasites were homogenized in Tris-reagent (Sigma) and total RNA extracted according to the manufacturers' specifications. For reverse transcription-PCR (RT-PCR), first strand cDNA was produced with oligo (dT) primers from 2 μg of total RNA by using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) at 42°C for 60 min. A 5- μl aliquot of the resultant cDNA was amplified by using primers specific for β -actin (mouse, Stratagene, La Jolla, CA) or Prx (FhePrx fwd and FhePrx rev, see Section 2.3) under the following conditions: 30 s denaturation at 95°C , 5 s annealing of primers at 55°C , and 12 s elongation at 72°C for 40 cycles [13].

2.2. Preparation of anti-peroxiredoxin serum

Sheep were vaccinated three times with 100 μg of purified recombinant FhePrx at three week intervals using Quil A (100 μg per sheep) as an adjuvant. Antigen and adjuvant were formulated in 2 ml sterile phosphate buffered saline (PBS), pH 7.3, and injected sub-cutaneously in the neck region.

2.3. Cloning and functional expression of *F. hepatica* peroxiredoxin (FhePrx)

The FhePrx gene was PCR amplified from cDNA isolated from a $\lambda\text{gt}11$ library immunoscreened with sera from cattle protectively vaccinated with a high molecular mass fraction from adult liver fluke (GenBank Accession No. U88577) [8,23]. The primers used to amplify the gene were FhePrx Forward: 5'CCGGAATTCATGTTGCAGCC T3', which inserted an *EcoRI* site (underlined) proximal to the ATG start codon of the Prx open reading frame and FhePrx Reverse: 5'CGGCTCGAGTCACTAGTTGGCTGA3' which inserted an *XhoI* site (underlined) proximal to a double stop codon. The full length cDNA corresponds to the 580 nucleotide open reading frame described in McGonigle et al., [23], initiating with the amino acid residues, MLQPN – and terminating with the residues – FSSAN, resulting in a polypeptide of 194 amino acid residues. The full length recombinant protein has an additional N-terminal 6X Histidine tag and a recombinant tobacco etch virus protease cleavage site.

Sequence accuracy was checked (GATC Biotech, Konstanz, Germany) and the cDNA was inserted into the pPRO Ex HtA vector (Life Sciences) and used to transform *E. coli* BL21 (DE3). Recombinant FhePrx protein was induced with IPTG (1 mM) and extracted using a method modified from that described by Frangioni and Neel [15]; bacterial pellets were resuspended in 10 ml STE (150 mM NaCl, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) with lysozyme (10 $\mu\text{g}/\text{ml}$) and kept on ice for 15 min. DTT (10 $\mu\text{g}/\text{ml}$) and *N*-laurylsarcosine (0.5%) were added and solution was sonicated for 1 min in a water bath then centrifuged for 5 min at 4000 rpm to clarify. Triton X-100 (2%), DNase (75 $\mu\text{g}/\text{ml}$) and RNase (10 $\mu\text{g}/\text{ml}$) were added, and the solution was kept on ice for 15 min. Five volumes of buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl and 10 mM imidazole) were added and the solution was passed through a 0.8 μm filter (Sartorius). Recombinant FhePrx was purified on Ni²⁺ NTA resin using the non-denaturing protocol detailed by the manufacturer (Qiagen). Protein concentration was determined by BCA (Pierce) and purity was determined by SDS–PAGE.

2.4. Protection of plasmid DNA from nicking

Plasmid pGEM 3zf (Promega) was prepared using a miniprep kit (Qiagen). DNA protection assays were performed by pre-incubating recombinant FhePrx with dithiothreitol (DTT) (8 mM) and FeCl₃

(0.8 mM) for 10 min prior to adding 300 ng of plasmid DNA. The reactions were incubated for 2.5 h at 37°C and then analysed on a 0.8% agarose gel [20].

2.5. Kinetic analysis of recombinant *F. hepatica* peroxiredoxin

The specific activity of FhePrx was determined by the reduction of H₂O₂ in a reaction containing *E. coli* thioredoxin and thioredoxin reductase (Sigma–Aldrich). The reaction mix contained 5 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.25 mM NADPH, 10 μM *E. coli* thioredoxin and 0.15 μM *E. coli* thioredoxin reductase in a total volume of 500 μl . Consumption of NADPH at 22°C was monitored at A_{340} for 1 min in a spectrophotometer after adding hydrogen peroxide. The specific activity of the FhePrx was determined by the following equation: units of Prx/mg of protein = $\{[(\Delta A/\Delta T \times V_1)/(6.22 \times V_2)] \times 1000\}/[\text{protein}]$ in mg/ml, where 6.22 = extinction coefficient of NADPH ($\text{mM}^{-1} \text{cm}^{-1}$), V_1 is the total reaction volume, V_2 is the sample volume and 1000 is used to convert to nanomoles [4,5,19]. Steady-state kinetic analysis was performed using the same assay; however, concentrations of thioredoxin ranged from 5 μM to 20 μM and hydrogen peroxide ranged from 25 μM to 100 μM . K_m and k_{cat} values were determined using Enzpack for Windows, version 1.4 (Biosoft Software for Science).

3. Results and discussion

3.1. Purification of recombinant *F. hepatica* peroxiredoxin

Purified recombinant FhePrx migrated at an approximate molecular weight of 26 kDa when analysed by reducing SDS–PAGE, which agrees with the molecular weight of the FhePrx predicted from the deduced amino acid sequence, 21.6 kDa [23] plus the 6X His-tag (Fig. 1). When examined by SDS–PAGE under non-reducing conditions, the FhePrx enzyme migrated close to the 50 kDa molecular size marker indicating that the recombinant protein forms a dimeric enzyme containing a reactive center (Fig. 1B, lane 1). This was confirmed firstly by reduction of the inter-subunit disulfide bridges, and thereby splitting the two monomers, by addition of a small dithiol, DTT, to the sample prior to electrophoresis (Fig. 1B, lane 2). Addition of hydrogen peroxide to the recombinant FhePrx caused the dimer to migrate as two bands, one slightly slower than that seen in lane 1, presumably because either one or two covalent disulfide linkages were formed between the monomers with corresponding conformation rearrangements (Fig. 1B, lane 5) [16]. However, somewhat surprisingly, the addition of DTT before or after the addition of hydrogen peroxide to the recombinant FhePrx (Fig. 1B, lanes 3 and 4) did not increase the amount of monomeric form. It was expected that the reduced active site residues, Cys-SH, would react with the hydrogen peroxide to form the sulfenic acid derivative, Cys-SOH, which is resolved by dimerisation, and that addition of DTT should reduce the disulfide bonds to give rise to a monomeric form; however, this did not occur and the protein migrated similarly to that observed when hydrogen peroxide was added alone (Fig. 1B, compared lanes 3 and 4 with 5). It is possible that under the experimental conditions hydrogen peroxide reacted directly with DTT and prevented its capacity to reduce the active site cysteines. Dimer formation was also confirmed by gel permeation chromatography which showed that recombinant FhePrx eluted at a molecular weight of approximately 50 kDa (data not shown). Gel permeation chromatography also detected high molecular sized forms of the FhePrx (not shown) and some material is retained on the top of the gels lanes in the non-reducing SDS–PAGE analysis that is reducible by DTT (Fig. 1B, see lanes

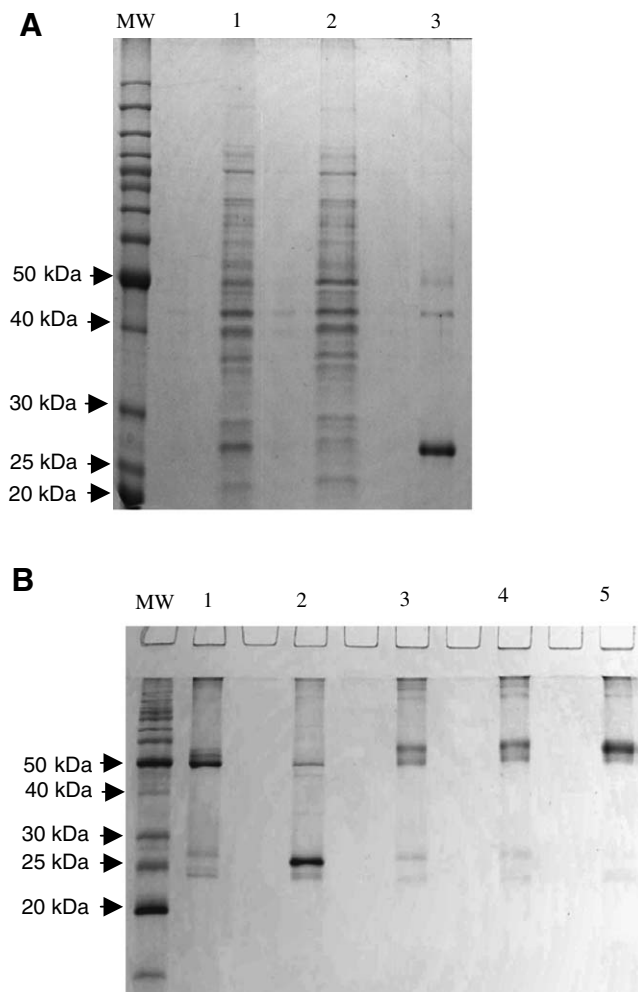


Fig. 1. Production and purification of FhePrx under reducing (A) and non-reducing (B) conditions. (A) Total soluble bacterial extract from induced FhePrx culture (1); run-through from Ni-NTA resin column (2), and eluted recombinant FhePrx (3). (B) Recombinant FhePrx (1), recombinant FhePrx incubated with 0.5 mM DTT for 20 min before loading (2), recombinant FhePrx incubated with 0.5 mM DTT for 10 min before addition of 0.5 mM hydrogen peroxide for a further 10 min (3), recombinant FhePrx incubated with 0.5 mM hydrogen peroxide for 10 min before addition of 0.5 mM DTT for a further 10 min (4), and recombinant FhePrx incubated with 0.5 mM hydrogen peroxide for 20 min. MW, molecular weight markers.

1 and 2); this likely corresponds to a decameric form of the FhePrx which is known to exist for other Prxs, and is important in regulating the activity of the enzymes [17,33].

Using antibodies prepared against recombinant FhePrx we identified a single band migrating at an apparent molecular size of approximately 26 kDa in immunoblots of soluble extracts of *F. hepatica* that co-migrated with a major band in the Coomassie-stained extract (Fig. 2A and B, compare lane 1). A band of similar size was also detected in parasite ES products of *F. hepatica* (Fig. 2A and B, lane 2) but is not a major component of this preparation; the two major bands observed in the ES products following Coomassie staining are two previously characterised cathepsin L cysteine proteases that have similar molecular sizes to FhePrx [7,8]. The native FhePrx proteins detected in the parasite extracts and ES products co-migrated with the recombinant FhePrx protein (Fig. 2A and B, lane 3) and also formed dimers under non-

reducing conditions (not shown). Pre-immune sheep sera did not detect any bands (data not shown). Due to the paucity of material that can be obtained from metacercariae we could not detect FhePrx by immunoblotting; however, RT-PCR using total mRNA isolated from these showed that the Prx is expressed by this infective parasite stage (Fig. 2C).

These data suggest that FhePrx is abundant in *F. hepatica* but whether the protein is actively secreted into the medium by the parasite is not known. Prxs do not possess signal sequences and are not normally secreted by mammalian cells [17]; however, in certain infectious situations, such as severe acute respiratory syndrome [6], HIV infection [16], and in non-small cell lung cancer [5] extracellular human Prx has been detected in serum samples. Moreover, FhePrx may appear in the ES products due to the continual sloughing and replacement of the parasite tegumental and/or intestinal cells [9]. We investigated the possibility that FhePrx is a non-classically secreted protein using the SecretomeP 2.0 server [www.cbs.dtu.dk/services/SecretomeP/, Ref. 14]. The analysis of FhePrx returned a value of 0.527, compared to a threshold value of 0.5, which indicates it is possible that the enzyme is secreted without a signal peptide, but the probability is not high. In contrast, a similar analysis of the schistosome egg Prx (Genbank Accession No. AF121199) which is known to be secreted returned a value of 0.622, indicating a higher probability of secretion. FhePrx is expressed by all stages of the developing parasite, including the earliest infectious larvae, and antibodies are detected in serum both during acute infection and at low levels in chronic disease (not shown).

3.2. Antioxidant activity of recombinant *F. hepatica* peroxiredoxin

In the mixed-function oxidation assay, reactive oxygen species are produced in the presence of FeCl_3 , leading to strand breaks in super-coiled plasmid DNA that can be visualised by the formation of slower migrating nicked, open circular plasmid in agarose gels [20] (Fig. 3A, compare lanes 2 and 3). Addition of recombinant FhePrx with DTT to the reaction tube protected the plasmid from nicking (Fig. 3A, lane 4), since this did not occur when DTT was substituted by ascorbic acid (Fig. 3A, lane 5) the activity of FhePrx is, therefore, thiol-dependent. The anti-nicking activity was FhePrx-specific, as another *F. hepatica* recombinant protein, cathepsin L1 [7], did not protect plasmid DNA from nicking (Fig. 3A, lane 6). Although cathepsin L1 was expressed in the yeast *Pichia pastoris*, the active enzyme is His-tagged and was purified in an identical manner to FhePrx. Also, incubation of the plasmid DNA with another *E. coli*-expressed recombinant protein, the surface antigen of *Toxoplasma gondii*, gave the same result as the cathepsin L1 negative control. Our data supports that of Salazar-Calderon and coworkers [28] who demonstrated that a recombinant *F. hepatica* thioredoxin peroxidase fused to GST was able to protect inactivation of glutamine synthetase and enolase when subjected to FeCl_3 -induced oxidative stress.

To determine the specific activity of recombinant FhePrx, the enzyme was coupled with *E. coli* thioredoxin and thioredoxin reductase in an NADPH oxidation assay. In this assay recombinant FhePrx exhibited a specific activity of 1200 nmole/min/mg. Exclusion of any of the three proteins from the assay resulted in no significant consumption of NADPH (Fig. 3B) although a low level of activity was

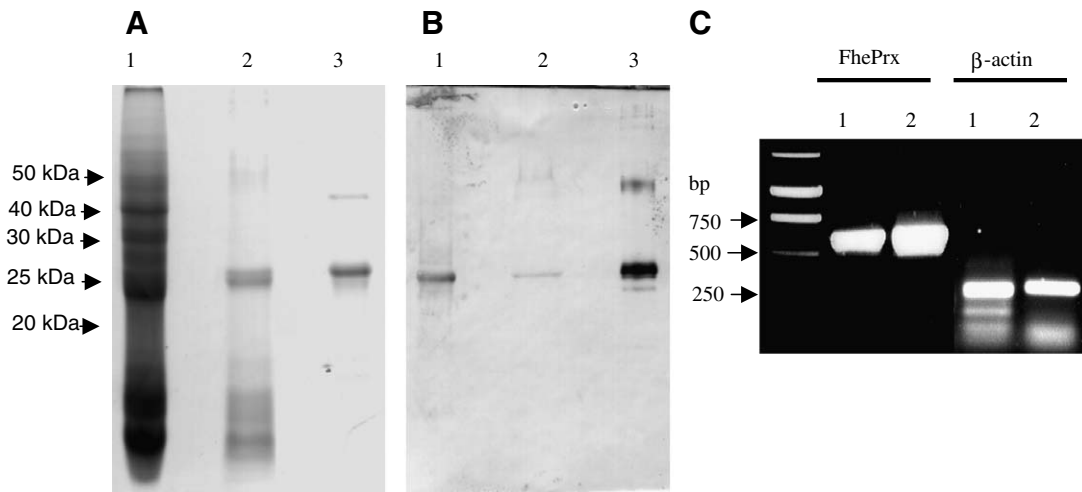


Fig. 2. Expression of FhePrx by parasitic stages in SDS-PAGE (A), immunoblotting (B) and RT-PCR (C). (A) Soluble extract of adult *F. hepatica* (1), adult *F. hepatica* ES products (2), and recombinant FhePrx (3). (B) Arrangement of lanes as for (A), anti-Prx sera was raised in sheep. (C) Expression of FhePrx and actin (control) in infective larvae (1) and adult *F. hepatica* (2) was analysed by RT-PCR with gene-specific primers.

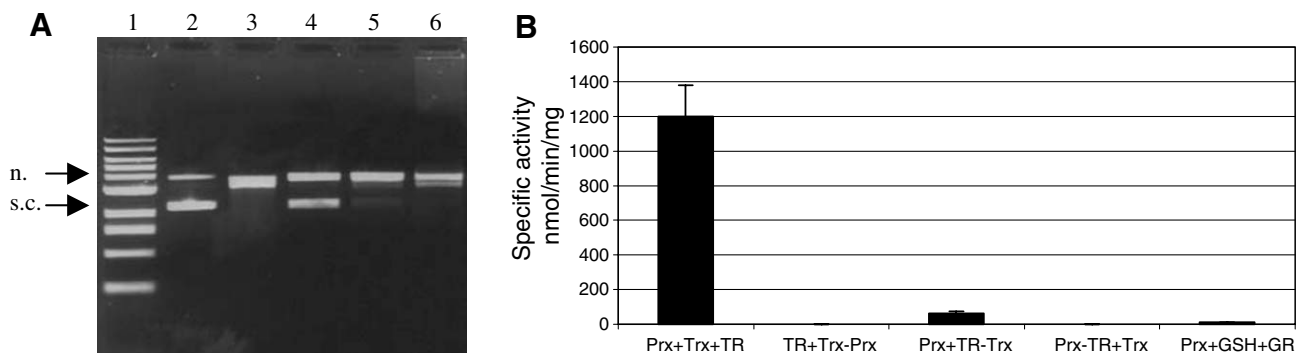


Fig. 3. Enzymatic activity of recombinant FhePrx, FhePrx protects plasmid DNA from oxidative nicking (A) and specific activity determined by NADPH consumption assay (B). (A) Molecular weight markers (1), plasmid pGEM 3zf only (2), pGEM 3zf with Fe^{3+} and DTT (3), pGEM 3zf, Fe^{3+} , DTT and recombinant FhePrx [880 ng] (4), pGEM 3zf, Fe^{3+} , Ascorbic acid and recombinant FhePrx [880 ng] (5), pGEM 3zf, Fe^{3+} , DTT, and recombinant *F. hepatica* cathepsin L [1.4 μg] (6). The nicked (n) and supercoiled (s.c) form of plasmid are indicated by arrows. (B) Specific activity of FhePrx determined by NADPH (0.25 mM) consumption. Hydrogen peroxide at a concentration of 0.25 mM was provided as substrate. FhePrx, recombinant FhePrx, TR, *E. coli* thioredoxin and Trx, *E. coli* thioredoxin reductase. Values are the means of three independent experiments.

detectable with thioredoxin reductase and peroxiredoxin in the absence of thioredoxin.

Peroxioredoxins of the parasitic helminth *S. mansoni* (SmPrx 2 and SmPrx 3) can utilise glutathione and glutathione reductase in addition to the thioredoxin system to provide reducing equivalents [19,29]. Therefore FhePrx, which is 67% identical to SmPrx 2 on the amino acid level, was tested for glutathione affinity. We found that recombinant FhePrx was not active with glutathione (concentration ranging from 0.1 mM to 1 mM) and glutathione reductase (0.2 U) (Fig. 3B) as the addition of FhePrx failed to increase activity over the background rate of NADPH consumption. By preparing truncated recombinant versions of SmPrx 2 and SmPrx 3, Sayed and Williams [29] showed that the affinity for glutathione was dependent on the 22 amino acid C-terminal tail. In addition, another schistosome Prx, SmPrx 1, that lacks this C-terminal tail exhibited no affinity for glutathione [29]. In the Prx II group of peroxiredoxins, which includes SmPrx2 and FhePrx, the C-terminus is known to form a well-ordered helix, which stabilizes the C-terminal arm and reduces mobility [29,33]. The FhePrx C-termi-

nal tail contains the conserved Tyr-Phe motif at residues 89 and 90, and is predicted to form a helical structure from residues 183–194 by the PredictProtein server [www.predictprotein.org, Ref. 27]. However, the C-terminal tails of SmPrx 2 and FhePrx are only 55% identical on the amino acid level and this sequence variation between the *S. mansoni* and *F. hepatica* enzymes may account for the difference in C-terminal arm mobility, resulting in differences in glutathione affinity.

3.3. Steady-state enzyme kinetics and sensitivity to hydrogen peroxide

Prxs from various species can react with different hydroperoxides, although their efficiencies may differ between substrates [17]. FhePrx displayed saturable Michaelis–Menten type kinetics with the substrates hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide (Table 1). The $k_{\text{cat}}/K_{\text{m}}$ values for the substrates hydrogen peroxide and cumene hydroperoxide were similar, whereas the value for *t*-butyl hydroperoxide was slightly lower. The kinetic measurements for all three substrates were consistent with previously

Table 1
Enzyme kinetics for recombinant FhePrx with various hydroperoxides

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
H_2O_2	30.0	15.8	5.2×10^5
Cumene hydroperoxide	12.7	6.3	5.2×10^5
<i>t</i> -Butyl hydroperoxide	18.6	7.6	4.1×10^5

reported values for peroxiredoxins of mammals and other helminth parasites [17].

The steady-state kinetics of FhePrx were analysed by measuring initial rates of FhePrx activity using varying concentrations of substrates hydrogen peroxide and reduced thioredoxin. Two substrate enzyme reactions can be described by the equation of Dalziel (1) [10].

$$E/v_0 = \phi_0 + \phi_1/[A] + \phi_2/[B] + \phi_{1,2}/[A][B] \quad (1)$$

where E is total enzyme molarity, v_0 is initial rate, A is concentration of hydrogen peroxide, B is concentration of thioredoxin, and ϕ_0 , ϕ_1 , ϕ_2 and $\phi_{1,2}$ are constants associated with the enzyme–substrate affinity. The results were plotted as total enzyme molarity divided by initial rate versus the reciprocal of the hydrogen peroxide concentration (Fig. 4A). With typical peroxidase kinetics, regression lines of the primary data are parallel, however in Fig. 4A the lines are convergent. In secondary plots, the slopes and intercepts of the curves from the primary data were plotted against the reciprocal of the reduced thioredoxin concentration and values for constants ϕ_0 , ϕ_1 , ϕ_2 , and $\phi_{1,2}$ were derived [10]. The first order rate constant ϕ_0 is the reciprocal of k_{cat} , in non-saturable kinetics, k_{cat} approaches infinity, and the value of the reciprocal is zero. The ϕ_0 value obtained for FhePrx is 0.0538 s and therefore finite, supporting previous conclusions that FhePrx is saturable and has Michaelis–Menten type kinetics. The constant ϕ_1 refers to the affinity of the enzyme for the hydrogen peroxide substrate and ϕ_2 refers to affinity of the oxidized enzyme for reduced thioredoxin. The value for ϕ_1 for FhePrx is $0.105 \mu\text{M s}^{-1}$ and is relatively low compared to that reported for Prx enzymes of schistosomes [28], bacteria and protozoa [16]. The significance of this relative low rate of association of reduced FhePrx for hydrogen peroxide is unknown, but these rates can differ by several orders of magnitude between Prxs of various species and may reflect differences in the levels of substrate in the particular microenvironment in which the enzymes function. Alternatively, the reported rates of association for hydrogen peroxide for Prxs of various sources may also be an artifact of recombinant protein production and/or the capacity of these proteins to form dimers or decamers. However, the ϕ_2 constant of $0.173 \mu\text{M s}^{-1}$ for the *F. hepatica* enzyme is similar to values for other peroxiredoxins, indicating that regeneration of the reduced enzyme likely occurs at a similar rate [17]. The third order rate constant $\phi_{1,2}$ was calculated as $12.42 \mu\text{M}^2 \text{s}^{-1}$, it is surprising that our analysis yields a term for a ternary complex as nearly all kinetic evidence for reaction order in peroxiredoxins suggest that the detoxification of hydrogen peroxide occurs as two half reactions [17]. There are two possible explanations for deviation from a typical double displacement mechanism: (1) thioredoxin interacts with Prx before hydrogen peroxide can be attacked, in which case a type of ternary complex is formed. Thioredoxin certainly does interact with Prx, but all experimental evidence to date indicates that the function of thioredoxin is to provide reducing power

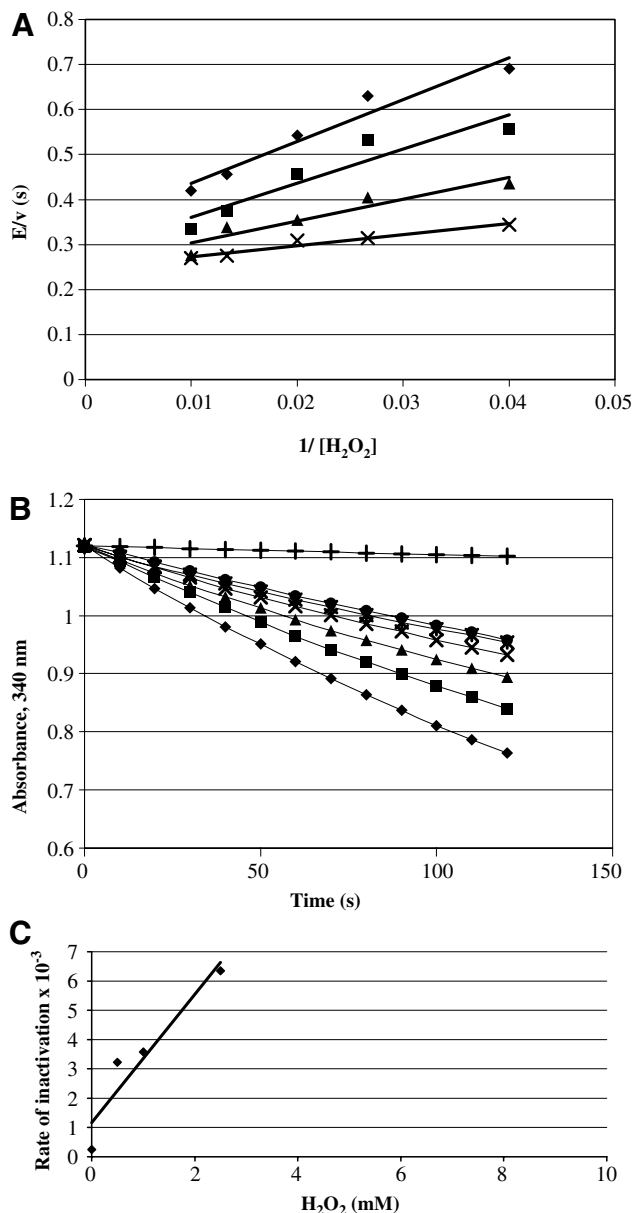


Fig. 4. Steady-state kinetics for FhePrx at varying concentrations of thioredoxin (A) and susceptibility of FhePrx to inactivation by H_2O_2 (B). (A) The enzyme-normalized reciprocal initial velocities are plotted against the reciprocal molarities of hydrogen peroxide. Thioredoxin concentrations are 5 μM (diamond), 7.5 μM (square), 10 μM (triangle) and 20 μM (X). (B) The time course of NADPH (0.25 mM) oxidation by recombinant FhePrx coupled with *E. coli* thioredoxin (10 μM) and thioredoxin reductase (2 U) is shown for H_2O_2 concentrations of 0 mM (plus sign), 0.5 mM (diamond), 1.0 mM (square), 2.5 mM (triangle), 5.0 mM (X), 7.5 mM (X with bar), and 10 mM (closed circle). (C) FhePrx is sensitive to over-oxidation. The reaction rates from (B) were plotted over time at the varying hydrogen peroxide concentrations, then slopes of this data were plotted against reciprocal substrate (hydrogen peroxide) concentrations.

in the regeneration of Prx [4] and no evidence of an intermediate in which Prx is interacting with both substrates simultaneously has been reported (2) Prx has an apparent single-displacement mechanism, which deviates from double displacement mechanism for a biologically significant reason. There is precedence for deviation from typical peroxidase

kinetics, the kinetics of *S. mansoni* Prx 2 and 3 [29] and the mitochondrial tnxp of *Leishmania infantum* [17] do not conform to a double displacement mechanism. Reasons for a change in kinetic pattern could be (1) enzymatic activity is dependent on decameric versus dimeric conformation [17,33] and conformational changes of recombinant Prx versus native molecules are responsible, (2) sensitivity to hydrogen peroxide substrate, especially at higher concentrations of thioredoxin, alters the rates or (3) as the coupled enzyme reaction relies on the *E. coli*-derived thioredoxin and thioredoxin peroxidase, the use of the *F. hepatica* enzymes could result in a different pattern.

Many eukaryotic 2-Cys Prxs are sensitive to high levels of hydrogen peroxide due to over-oxidation of the peroxidatic cysteine to sulfinic acid, which cannot be rapidly reduced again, resulting in inactivation of the enzyme [17,32,33]. In the presence of high concentrations of hydrogen peroxide the effective concentration of the enzyme is reduced. We observed that FhePrx activity is subject to over-oxidation at hydrogen peroxide concentrations of 500 μM and greater (Fig. 4B). We further investigated the sensitivity of FhePrx to over-oxidation by determining the reaction rates of the enzyme at increasing hydrogen peroxide concentration. The rates of inactivation, determined by the ratio of the rate at each given time to the initial rate, were plotted against the concentration of hydrogen peroxide (Fig. 4C). The steep slope of the curve is consistent with an enzyme that is rapidly inactivated and is comparable to data reported for sensitive Prxs such as the schistosome Prx 2 and Prx 3 and human Prx 1 [29,32].

The data shows that, like other peroxiredoxins, the capacity of the FhePrx to scavenge hydrogen peroxide ($k_{\text{cat}}/K_m = 5.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) is one or two orders of magnitude lower than other peroxidases. However, given that *F. hepatica* parasites lack catalase activity and express minimal levels of glutathione peroxidase [1,2,23] the Prx system must be a requirement for detoxification of metabolically-produced hydrogen peroxide in this parasite. The cells of the liver fluke may be under particular oxidative stress during migration in the host at which time (approximately 8 weeks) metabolic processes (catabolism of host proteins and anabolism of parasite proteins) result in growth from <1 mm to >1.5 cm in length [11]. The high affinity of FhePrx for hydrogen peroxide, the concentration required to obtain half maximal rate (K_m) is 30 μM , coupled with the high abundance of enzyme in parasite cells, is sufficient to maintain peroxide levels below toxic levels, 120–150 μM [25]. Recently, Sayed and Williams [30] demonstrated that knocking down Prx activity by interference RNA techniques in larvae of *S. mansoni* resulted in an increase in parasite mortality by seven-fold; although Prx 1 dsRNA was used for silencing, reduction in both Prx 1 and Prx 2 expression was observed.

Parasite Prxs are of further interest because of their possible function in maintaining the host–parasite relationship. The FhePrx is abundant in the parasite tissues, and is also secreted by the parasite [this study and references 13, 18 and 26], despite lacking a signal sequence, which suggests that the anti-oxidant functions outside the parasite. We and others have speculated that this function may include the inactivation of ROS released by immune effector cells such as eosinophils, neutrophils and macrophages, although there is no supporting evidence for this function [2,17,26]. However, we have recently shown that injection of partially purified native FhePrx or recombinant

FhePrx into mice induced the recruitment of alternatively activated host macrophages [13]. The mechanism by which FhePrx exerts its function on macrophages needs to be elucidated but this may be via binding to specific surface receptors and/or drawing on emerging new functions of Prx in signal transduction [12], altering hydrogen peroxide levels on the extracellular surface of the cells.

Enzymes associated with antioxidant defence in parasites are considered targets at which new drug treatments could be developed, hence, it is important that we understand the biochemistry of these molecules. However, the high level of structural conservation between the Prx of *F. hepatica* and its hosts (approximately 65% at the overall amino acid levels and almost 100% in the catalytic motifs) and biochemical similarities do not augur well for selective chemotherapy. The alternative approach of disrupting the function of these molecules *via* vaccine-induced immune responses may prove more successful, particularly given the potential functions in host–parasite interaction.

Acknowledgements: Dr. Mary Sekiya was supported by Enterprise Ireland Research Innovation Fund Grant 2002/17. Dr. Colin Stack was supported by grants obtained from Enterprise Ireland, Republic of Ireland, and the Australian Research Council, Australia. Dr. Sheila Donnelly was funded by the National Health and Medical Research Council, Australia. Peter Collins and John Dalton were recipients of a Australian Department of Science, Education and Training (DEST) Endeavour Award. Prof. John Dalton is a recipient of a NSW Government BioFirst Award in Biotechnology which also supports Weibo Xu.

References

- [1] Barrett, J. (1980) Peroxide metabolism in the liver fluke. *J. Parasitol.* 66, 697.
- [2] Callahan, H.L., Crouch, R.K. and James, E.R. (1988) Helminth anti-oxidant enzymes – a protective mechanism against host oxidants. *Parasitol. Today* 4, 218–225.
- [3] Carmona, C., Dowd, A., Smith, A. and Dalton, J.P. (1993) Cathepsin L proteinase secreted by *Fasciola hepatica* in vitro prevents the antibody-mediated eosinophil attachment to newly excysted juveniles. *Mol. Biochem. Parasitol.* 62, 9–18.
- [4] Chae, H.Z., Chung, S.J. and Rhee, S.G. (1994) Thioredoxin-dependent peroxide reductase from yeast. *J. Biol. Chem.* 269, 27670–27678.
- [5] Chang, J.W., Lee, S.H., Jeong, J.Y., Chae, H.Z., Kim, Y.C., Park, Z.Y. and Yoo, Y.J. (2005) Peroxiredoxin-I is an autoimmunogenic tumor antigen in non-small cell lung cancer. *FEBS Lett.* 579, 2873–2877.
- [6] Chen, J.H., Chang, Y.W., Yao, C.W., Chiueh, T.S., Huang, S.C., Chien, K.Y., Chen, A., Chang, F.Y., Wong, C.H. and Chen, Y.J. (2004) Plasma proteome of severe acute respiratory syndrome analyzed by two-dimensional gel electrophoresis and mass spectrometry. *Proc. Natl. Acad. Sci. USA* 101, 17039–17044.
- [7] Collins, P.R., Stack, C.M., O'Neill, S.M., Doyle, S., Ryan, T., Brennan, G.P., Mousley, A., Stewart, M., Maule, A.G., Dalton, J.P. and Donnelly, S. (2004) Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence. *J. Biol. Chem.* 279, 17038–17046.
- [8] Dalton, J.P., McGonigle, S., Rolph, T.R. and Andrews, S.J. (1996) Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. *Infect. Immun.* 64, 5066–5074.
- [9] Dalton, J.P., Skelly, P. and Halton, D.W. (2004) The role of the tegument and gut in nutrient uptake by parasitic plathyhelminths. *Can. J. Zool.* 82, 211–232.
- [10] Dalziel, K. (1957) Initial steady state velocities in the evaluation of enzyme-coenzyme-substrate reaction mechanisms. *Acta Chem. Scand.* 11, 27670–27678.

- [11] Daniel, R. and Mitchell, S. (2002) Fasciolosis in cattle and sheep. *Vet. Rec.* 151, 219.
- [12] DeYulia, G.J., Carcamo, J.M., Borquez-Ojeda, O., Shelton, C.C. and Golde, D.W. (2005) Hydrogen peroxide generated extracellularly by receptor-ligand interaction facilitates cell signaling. *Proc. Natl. Acad. Sci. USA* 102, 5044–5049.
- [13] Donnelly, S., O'Neill, S.M., Sekiya, M., Mulcahy, G. and Dalton, J.P. (2005) Thioredoxin peroxidase secreted by *Fasciola hepatica* induces alternative activation of macrophages. *Infect. Immun.* 73, 166–173.
- [14] Dyrlov Bendtsen, J., Juhl Jensen, L., Blom, N., von Heijne, G. and Brunak, S. (2004) Feature based prediction of non-classical and leaderless protein secretion. *Protein Eng. Des. Sel.* 17, 349–356.
- [15] Frangioni, J.V. and Neel, B.G. (1993) Solubilization and purification of enzymatically active glutathione-S-transferase (pGEX) fusion proteins. *Anal. Biochem.* 210, 179–187.
- [16] Geiben-Lynn, R., Kursar, M., Brown, N.V., Addo, M.M., Shau, H., Lieberman, J., Luster, A.D. and Walker, B.D. (2003) HIV-1 antiviral activity of recombinant natural killer cell enhancing factors, NKEF-A and NKEF-B, members of the peroxiredoxin family. *J. Biol. Chem.* 278, 1569–1574.
- [17] Hofmann, B., Hecht, H.-J. and Flohe, L. (2002) Peroxiredoxins. *Biol. Chem.* 383, 347–364.
- [18] Jefferies, J.R., Campbell, A.M., van Rossum, A.J., Barrett, J. and Brophy, P.M. (2001) Proteomic analysis of *Fasciola hepatica* excretory–secretory products. *Proteomics* 1, 1128–1132.
- [19] Kwatia, M.A., Botkin, D.J. and Williams, D.L. (2000) Molecular and enzymatic characterization of *Schistosoma mansoni* thioredoxin peroxidase. *J. Parasitol.* 86, 908–915.
- [20] Kwon, S.J., Kim, K., Kim, I.H., Yoon, I.K. and Park, J.-W. (1993) Strand breaks in DNA induced by a thiol/Fe[III]/O₂ mixed-function oxidase system and its protection by a yeast antioxidant protein. *Biochem. Biophys. Res. Commun.* 192, 772–777.
- [21] Maggioli, G., Piacenza, L., Carambula, B. and Carmona, C. (2004) Purification, characterization, and immunolocalization of a thioredoxin reductase from adult *Fasciola hepatica*. *J. Parasitol.* 90, 205–211.
- [22] Mas-Coma, S., Bargues, M.D. and Esteban, J.G. (1999) in: *Human Fasciolosis* in: *Fasciolosis* (Dalton, J.P., Ed.), pp. 411–429, CAB International Publishing, Wallingford, Oxon.
- [23] McGonigle, S., Curley, G.P. and Dalton, J.P. (1997) Cloning of peroxiredoxin, a novel antioxidant enzyme from the helminth parasite *Fasciola hepatica*. *Parasitology* 115, 101–104.
- [24] McGonigle, S., Dalton, J.P. and James, E. (1998) Peroxiredoxins: a new antioxidant family. *Parasitol. Today* 14, 139–145.
- [25] Pryor, W.A., Houk, K.N., Foote, C.S., Fukuto, J.M., Ignaroo, L.J., Squadrito, G.L. and Davies, K.J.A. (2006) Free Radical Biology and Medicine: it's a gas man! *Am. J. Physiol. Reg. I.*, in press.
- [26] Rahlfs, S., Schirmer, R.H. and Becker, K. (2002) The thioredoxin system of *Plasmodium falciparum* and other parasites. *Cell. Mol. Life. Sci.* 59, 1024–1041.
- [27] Rost, B., Yachdav, G. and Liu, J. (2003) The PredictProtein Server. *Nucleic Acids Res.* 32 (Web Server issue), W321–W326.
- [28] Salazar-Calderon, M., Martin-Alonso, J.M., Ruiz de Eguino, A.D., Casais, R., Soledad Marin, M. and Parra, F. (2000) *Fasciola hepatica*: heterologous expression and functional characterization of a thioredoxin peroxidase. *Exp. Parasitol.* 95, 63–70.
- [29] Sayed, A.A. and Williams, D.L. (2004) Biochemical characterization of 2-Cys peroxiredoxins from *Schistosoma mansoni*. *J. Biol. Chem.* 279, 26159–26166.
- [30] Sayed, A.A., Cook, S.K. and Williams, D.L. (2006) Redox balance mechanisms in *Schistosoma mansoni* rely on peroxiredoxins and albumin and implicate peroxiredoxins as novel drug targets. *J. Biol. Chem.* 281, 17001–17010.
- [31] Tielens, A.G.M. (1999) in: *Metabolism in: Fasciolosis* (Dalton, J.P., Ed.), pp. 277–305, CAB International Publishing, Wallingford, Oxon, UK.
- [32] Wood, Z.A., Poole, L.B. and Karplus, P.A. (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300, 650–653.
- [33] Wood, Z.A., Schroder, E., Harris, J.R. and Poole, L.B. (2003) Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci.* 28, 32–40.