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Molecular Cloning and Characterization of a Thioredoxin Gene from *Echinococcus granulosus*

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The insert of a clone from a λgt11 *Echinococcus granulosus* (Platyhelminth, Cestoda) protoscolex cDNA library, showed an open reading frame whose deduced protein sequence presents a high homology with all described thioredoxins (TRX). The TRX active site (Trp-Cys-Gly-Pro-Cys) is completely conserved. With a monospecific antibody, selected from a total anti-protoscolex sera by the isolated clone, a 12 kDa polypeptide was immunoprecipitated from a protoscolex total protein extract. Furthermore, an antiserum raised against a recombinant EgTRX also recognizes a 12 kDa band in these extracts. The recombinant protein presents TRX activity, using the insulin reduction assay. Finally, a TRX activity was characterized in protoscolex extracts. In all organisms where TRXs were studied, they participate in a cascade of redox exchanges, contributing to the maintaining of cell homeostasis. Considering that the parasitic flatworm *E. granulosus* is probably submitted to an important oxidative stress due to host defences, EgTRX protein could be involved in the survival strategies of this parasite.

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Nucleotide sequence data reported in this paper have been submitted to the GenBank data base with the accession number AF034637.

Abbreviations: SSC, sodium saline citrate; BSA, bovine serum albumin; PBS, phosphate buffered saline; DTT, dithiothreitol; bp, base pairs; nt, nucleotide; NADPH, nicotinamide dinucleotide phosphate; NBT, nitro blue tetrazolium; BCIP, 5-bromo-1-chloro-3-indolyl phosphate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; kDa, kilodalton; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; MOPS, 3-N-morpholinopropanesulfonic acid; AcONa, sodium acetate; Tris, tris-(hydroxymethyl)-aminomethane; MW, molecular weight; IPTG, isopropyl β-D-thiogalactopyranoside.

The cestode *Echinococcus granulosus* is the agent of hydatid disease, one of the major zoonoses, affecting man as well as domestic and wild animals. This parasite requires two mammalian hosts for the completion of its life cycle. The hermaphroditic adult commonly develops into the dog intestine, whereas the metacestode larva (hydatid cyst containing protoscoleces produced by asexual multiplication) develops in the viscera of many ungulates and man (1).

Complex life cycles as those exhibited by parasitic Platyhelminths imply that different sets of genes have to be activated in each stage of the cycle and that each stage must convey the genetic program enabling survival and development to the next stage (2). In addition, growth and development of a parasite requires the triggering of an adaptive response consecutive to changes in environmental conditions and, in particular, to host defence mechanisms. This requires the biosynthesis of a number of new molecules, particularly stress proteins and molecular chaperones and also proteins involved in the metabolism of reactive oxygen species (ROS), enabling cell adaptation processes at different levels (1, 3).

Thioredoxins (TRXs) are small (typically 12 kDa), ubiquitous, heat-stable inducible proteins, containing a redox active disulphide (4–6). They participate in many intracellular electron transfer pathways through the reversible oxidation of two neighbouring cysteines in a biochemical cycle that involves also TRX reductase and NADPH (7). Thus, TRXs have been involved in a wide variety of cellular events through their participation as coenzymes in several redox reactions (5). Moreover, they have been described as associated to a number of regulatory processes including enzyme activation and modulation of transcription factors (8, 9). Finally, TRXs provide cytoprotection against oxidative stress, either reactivating denatured proteins that contain mispaired disulphide bonds (10), or scavenging ROS (in particular H_2O_2 and hydroxyl radicals).

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TRXs are widely distributed in all kingdoms. Their structure and function have been well characterized in plants and micro-organisms. In plants, TRXs constitute one of the key elements in adapting the metabolic switch from "light" to "dark" reactions (11). In recent years, studies on TRXs have started in a number of animal systems including mammals (12).

In this paper we report the cloning and characterization of a TRX from *E. granulosus* (EgTRX), and we describe its expression and activity in the protoscolex stage. Furthermore, we show that the recombinant protein (rEgTRX) presents thioredoxin activity, and that an antiserum raised against this recombinant protein recognizes a 12 kDa band in protoscolex extracts.

MATERIALS AND METHODS

Parasite materials. *E. granulosus* protoscoleces were obtained from hydatid cysts of infected animals from local abattoirs. Parasite material was washed in Hanks' solution and frozen at 80°C until used.

Cloning and sequencing of EgTRX. A protoscolex cDNA expression library constructed in λgt11 was immunoscreened with a rabbit antiserum raised against a homeoprotein (EgHbx1) (13), following standard protocols (14). The insert of the selected clones was amplified by PCR using forward and reverse λ primers and Taq DNA polymerase, and subcloned into a Bluescript KS^+ plasmid (Stratagene). Both strands of the insert were sequenced using the dideoxy chain termination method (15) with standard oligonucleotide primers. DNA sequences were analyzed using the FASTA and ClustalV computer software packages (16).

Southern blotting. Parasite DNA was prepared as previously described (17). 10 μg of genomic DNA were digested either with *Eco*RI or *Bgl*II, resolved on 0.8% agarose gels and transferred to Hybond N membranes (Amersham). The insert of the isolated λgt11 clone (λ*EgTRX*) used as hybridization probe was radiolabelled by the random priming method using Klenow polymerase. High stringency hybridizations were performed at 65°C in 6× SSC, 5× Denhardt's solution, 0.1% SDS. The filters were washed twice at 65°C with 2× SSC, 0.1% SDS and twice with 0.1× SSC, 0.1% SDS (1× SSC: 0.15 M NaCl, 17 mM sodium citrate, pH 7).

Northern blotting. Parasite RNA was purified using standard procedures (18). The sample was prepared by heating 10 μl (30 μg) of total RNA during 15 min at 65°C in 15 μl deionized formamide, 1.8 μl formaldehyde, 3 μl RB (Running Buffer (10×): 0.2 M MOPS, 40 mM AcONa, 10 mM EDTA). The gel was made of 1.5% Agarose, 4 μl EtBr (1 mg/ml), 8% (w/v) formaldehyde in 1× Running Buffer. The sample was resolved at 5 V/cm, with recirculating buffer during 4 h. The blotting was performed with 20× SSC during two days. The probe was the same as for the Southern blotting. Hybridizations were performed overnight at 42°C in hybridization buffer (14). High stringency washes were made as follows: from 2× SSC, 0.1% SDS, 42°C, 30 min., up to 0.5× SSC, 0.1% SDS, 60°C, 30 minutes. The membrane was exposed for autoradiography during two days.

Protein analysis and Western blotting. Total protein extracts from protoscoleces were prepared using a motorized tissue homogenizer in a 1:1 volume mixture with the lysis buffer (1 mM EDTA, 1% Triton X-100, 10 mM PMSF, 150 mM NaCl, 10 mM phosphate buffer pH 7.4). SDS-PAGE and Western blots were carried out using standard conditions (19).

E. granulosus TRX was purified by immunoprecipitation using antibodies coupled to Protein A Sepharose (Sigma). 50 μl of protoscoleces were homogenized in 500 μl extraction buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% Nonidet P-40, 1 mg/ml BSA, 10 mM PMSF) and incubated with 100 μl of appropriate antibody fraction (monospecific affinity purified antibody or preimmune serum) at 4°C with gentle agitation during two hours. After this, 50 μl Protein A-Sepharose (equilibrated in PBS) were added to the samples and the mixture was incubated overnight at 4°C with gentle agitation. The samples were then centrifuged for 20 min at 15 000 g and the pellets washed with extraction buffer, washing buffer A (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% Triton X-100), washing buffer B (650 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% Triton X-100), and PBS, twice with 1 ml each wash step. Finally, the pellets were resuspended in sample buffer, shaken for 5 min and the resin allowed to settle. Supernatants were boiled in sample buffer (17), centrifuged and analyzed by SDS-PAGE and Western blotting.

Nitro-cellulose filters were assayed with: 1) a rabbit serum raised against a recombinant peptide containing the homeodomain of the EgHbx1 protein fused to a His tag (anti-EgHbx1), 2) an anti-EgHbx1 fraction depleted with the λ*EgTRX* clone (used as a control), 3) a monospecific fraction prepared using the isolated λ*EgTRX* clone according to (20). Preimmune serum was also used as a control. Filters were developed with an anti-rabbit alkaline phosphatase-conjugated antibody, and the reaction visualized by standard BCIP and NBT reactions (17).

Detection of thioredoxin activity in E. granulosus protoscoleces. Proteins extracts were assayed for TRX activity according to Holmgren (21). The method is based in the turbidity increase due to insulin reduction in the presence of DTT. The assay (700 μl total volume) contained PBS pH 7.0, 0.75 μg/ml bovine insulin (Sigma) prepared as in (21), and 0.2 mg of protoscolex protein extract. The reaction was initiated by the addition of 10 μl 0.1 M DTT. The insolubilization of insulin reduced chains was monitored at 650 nm.

Expression, purification and thioredoxin activity of a recombinant EgTRX (rEgTRX). The *EgTRX* cDNA insert was subcloned into pMAL-c2 (New England Biolabs). Conservation of the appropriate reading frame was checked by sequencing the construction. Expression and purification of the fusion protein (EgTRX-maltose binding protein) were performed according to the manufacturer's current protocols. Briefly, expression of the fusion protein in *E. coli* was induced by IPTG, and the fusion protein purified from bacterial lysates by affinity chromatography on an amylose resin. The fusion protein was then cleaved with factor Xa and the rEgTRX separated from the maltose binding protein by anion exchange on a MonoQ column (start buffer: 20 mM Tris-HCl, 25 mM NaCl, pH 8.0; elution gradient to 500 mM NaCl). The activity of rEgTRX was assayed as in 2.6, using 0.3 μM of rEgTRX protein instead of protoscolex total protein extract.

Preparation of antiserum. A rabbit was immunized with rEgTRX (day 0: priming with 200 μg in complete Freund's adjuvant; days 30 and 45: boosters with 100 μg incomplete Freund's adjuvant) following standard protocols. The rabbit was blooded 10 days after the third injection. The serum was diluted at 1:250 for use on Western blots.

RESULTS

Cloning of an Echinococcus granulosus TRX Gene

As part of an ongoing study to identify cDNA clones encoding homeodomain proteins, a cDNA library prepared from *E. granulosus* protoscoleces was screened with a rabbit antiserum raised against a fusion homeoprotein (EgHbx1, EMBL data base X66817) (13). One of the clones (λ*EgTRX*) presenting a strong reaction, was chosen for further characterization.

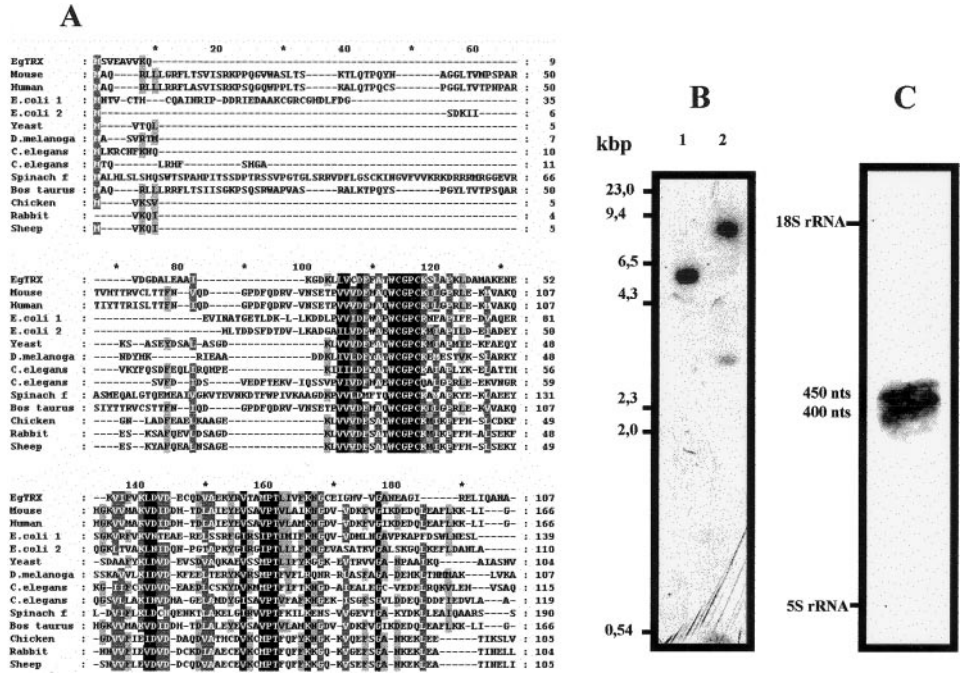

FIG. 1. (A) Sequence comparison among EgTRX and other TRXs, with their corresponding GenBank accession numbers given in brackets: mouse (U85089), human (U78678), *E.coli* (AE000344 and M54881), yeast (M59168), fruitfly (L27072), *C.elegans* (U23168 and B0024.9), spinach f (X14959), cow (D87741), chicken (A30006), rabbit (A28086) and sheep (Z25864). Sequence identities (100%) are shaded in black, those higher than 80% are shadowed in dark grey and those better than 60% are shadowed in light grey. Dashes represent gaps introduced in order to maximize sequence alignments. (B) Southern blot analysis using the λ*EgTRX* insert as a probe. DNA was isolated from *E. granulosus* protoscoleces and restricted with *Bgl*II (1) and *Eco*RI (2). The size of molecular weight marker fragments (λ-*Hind*III, in kbp) is indicated on the left of the panel. (C) Northern blot assay. The probe was the same as in (B). Ribosomal markers are shown on the left.

The insert of the isolated clone was 441 bp long and contained an open reading frame coding for a 107 amino acids long polypeptide. A 63 bp long poly A tail was present and preceded by a putative polyadenylation signal (AATAAA) located 14 bp upstream. A search with the deduced amino acid sequence of the λ*EgTRX* insert clone against the GeneBank/EMBL databases showed that this sequence does not match with any homeodomain-containing peptide, whereas it revealed a significant homology of EgTRX with thioredoxins, suggesting that the λ*EgTRX* clone contains a full length *E. granulosus* TRX cDNA.

Analysis of the Deduced Amino Acid Sequence

Alignment of EgTRX with prokaryotic and eukaryotic TRXs primary structures is shown in Fig. 1A. EgTRX presents a significant number of residues identical to all other described TRXs, in particular Val 26, Asp 28, Phe 29, Ala 31, the hexapeptide constituting the active site Trp 33-Cys 34-Gly 35-Pro 36-Cys 37 and Lys 38, Pro 42, Val 54, Lys 58, Asp 60, Asp 62, Ala 68, Pro 77, Thr 78, Lys 83, Gly 85 and Gly 93. Furthermore, the overall EgTRX sequence presents important similarities with TRXs, as evident in Fig. 1.

Secondary structure calculations (not shown) predict that EgTRX active site would be located in a turn and at the beginning of an extended α-helix domain, in agreement with the already solved 3D structures of several thioredoxins (22).

Finally, phylogenetical analysis using the PAUP algorithm (23) (not shown) reveal that EgTRX is placed with animal TRXs, within the so-called f-type TRXs (11).

Detection of EgTRX Gene by Southern Blot

Results of genomic Southern blot analysis are shown in Fig. 1B. Equal amounts of genomic DNA were digested with either *Eco*RI or *Bgl*II and probed with the λ*EgTRX* insert. Under stringent conditions the probe detected a single band of 5600 bp in the *Eco*RI digest and two bands of 8300 and 3300 bp in the *Bgl*II digest.

Detection of EgTRX Gene Expression by Northern Blotting

Two transcripts of roughly 400 and 450 nucleotides, of similar intensities were found in high stringency Northern blotting (Fig. 2C). Their apparent lengths are in agreement with the size of the cDNA insert.

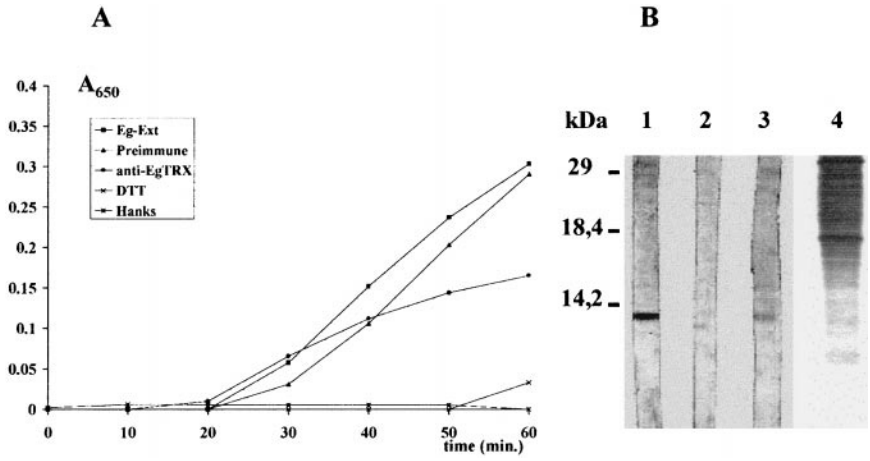

FIG. 2. (A) Thioredoxin activity in *E. granulosus* protoscolex extracts. Thioredoxin activity in protoscolex protein extracts were measured using the Holmgren assay (21). Eg-EXT: TRX activity in total protoscoleces extract. Preimmune: TRX activity in total protoscoleces extract with the addition of preimmune serum. Anti-EgTRX: TRX activity in total protoscolex extract with the addition of the monospecific anti-EgTRX antibody fraction. DTT: control assay (DTT without protein extracts). Hanks: control assay (Hanks' medium without protein extracts). (B) Protein Analysis and Western Blot. Western blotting of total protein extract from *E. granulosus* protoscoleces immunoprecipitated with the monospecific affinity-purified antibody assayed with: (1) the rabbit serum raised against a recombinant peptide containing the homeodomain of EgHbx1 protein (anti-EgHbx1), (2) the anti-EgHbx1 fraction depleted with the λ*EgTRX* clone and (3) the monospecific fraction prepared using the isolated λ*EgTRX* clone. Preimmune serum was used as a control. (4) SDS-PAGE stained with Coomassie blue to detect the immunoprecipitate of protoscolex total protein extract with the monospecific affinity purified antibody. Molecular weight markers are shown on the left of the panel.

Identification of the Parasite Protein

We were unable to identify the EgTRX protein in total protoscolex protein extracts with the monospecific serum fraction recognizing the λEgTRX clone. The identification of EgTRX was carried out by affinity precipitation from the total protein extract, using the monospecific antibody coupled to protein A-sepharose. SDS-PAGE revealed the presence of several bands in the immunoprecipitated fraction (Fig. 2B). Nevertheless, Western blotting of this fraction showed that the monospecific serum recognized only a 12 kDa protein (Fig. 2B).

The antiserum obtained against the recombinant EgTRX, also recognized a single band of 12 kDa in protoscolex extracts by Western blotting (Fig. 2B, 4). The recombinant EgTRX presents a higher molecular weight because it has a N-terminal amino acid tag: (ISESEDPGTMGLG) that was not removed by factor Xa (Fig. 2B, 3).

Analysis of Thioredoxin Activity

The reduction of insulin obtained with the protoscolex extracts sample was faster and higher than that produced by incubation with 0.33 mM DTT. Furthermore, insulin reduction was significantly inhibited by addition of the monospecific anti-λEgTRX antibody, whereas it was not affected by the incubation with preimmune serum (Fig. 2A). The recombinant EgTRX also showed thioredoxin activity as shown in Fig. 3A. In both cases, a significant lag precedes the appearance of the catalytic activity.

DISCUSSION

In this study, we report the cloning and characterization of a cDNA that contains the complete coding sequence for an *E. granulosus* thioredoxin (*EgTRX*). This is supported by the following facts: the length of the cDNA clone agrees with the molecular weight of the specific mRNAs identified by Northern blotting; the deduced amino acid sequence presents an extensive conservation with thioredoxins; the corresponding affinity-purified monospecific antibody fraction reveals a single band of an apparent molecular weight of 12 kDa in Western blotting and is able to inhibit TRX activity of protoscolex protein extracts; the recombinant fusion peptide rEgTRX presents thioredoxin activity; finally, the antiserum raised against rEgTRX recognizes a single band of 12 kDa in protoscolex extracts in Western blotting.

The primary structure of EgTRX presents a complete identity with all described TRXs at the active site level (Trp-Cys-Gly-Pro-Cys). Conservative substitutions are present throughout the EgTRX sequence, shared with TRXs from most organisms. A phylogenetic analysis using the PAUP software clearly shows that among the four groups described for TRXs, EgTRX belongs to the f TRXs group (11), and that it is grouped with invertebrate and vertebrate TRXs (not shown).

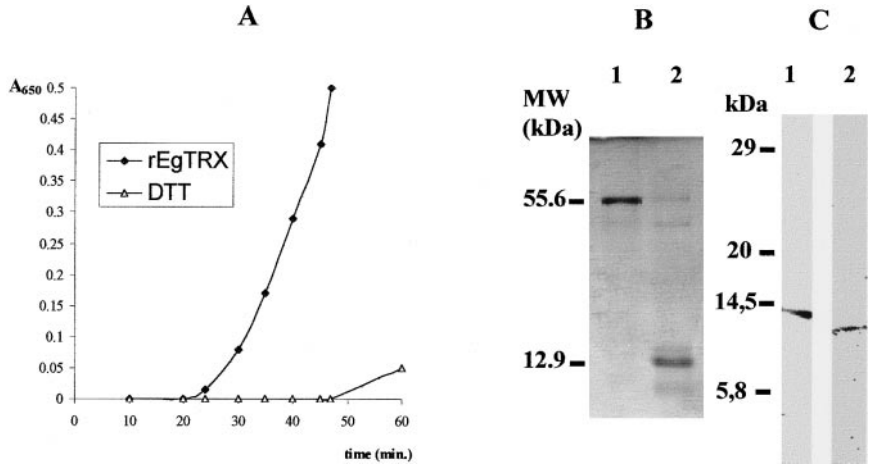

FIG. 3. Analysis of rEgTRX activity and Western Blot. (A) rEgTRX: recombinant EgTRX assayed for its ability to reduce insulin in presence of DTT. DTT: control assay (DTT without recombinant protein). (B) SDS-PAGE stained with Coomassie blue showing rEgTRX purification. (1) rEgTRX-MBP fusion peptide (55.6 kDa) prior to factor Xa treatment. (2) purified rEgTRX (12.9 kDa). (C) Immunoblotting analysis of recombinant EgTRX (80 ng) (1) and total protoscolex protein extract (30 μg) (2), with the antiserum raised against rEgTRX. Molecular weight markers are shown on the left of the panel.

Together with protein disulphide-isomerases and glutaredoxins, TRXs are members of a growing class of well characterized proteins that share a common fold (the "thioredoxin fold") and an exposed dithiol-disulphide active site that in the oxidized form is located at the end of a β-strand, followed by an α-helix (22). Using several different algorithms for secondary structure prediction, EgTRX active site and its surrounding domains seem to share this structure (not shown).

The reported results suggest that there might be one or two *EgTRX* genes in the parasite's genome. If *EgTRX* is present as a single copy gene, the presence of two bands in the *Bgl*II digest could be due to the presence of the corresponding cleavage site in an intron. Likewise, two bands of comparable length in high stringency Northern blotting we observed analyzing protoscolex mRNA, could correspond then to transcripts differing in their processing.

The immunoprecipitation and subsequent Western blotting with monospecific anti-λEgTRX fraction, revealed a protein with an apparent MW of 12 kDa, which matches those reported for TRXs. It is interesting to note that after immunoprecipitation and subsequent SDS-PAGE analysis, an important number of bands appeared that were not further recognized by anti-λEgTRX in Western blotting. This suggests that TRX is probably associated to several cell proteins, in agreement with already reported tight complexes between TRX and different proteins, observed in several organisms (24). Furthermore, the antiserum raised against rEgTRX recognizes a single band of 12 kDa in protoscolex extracts in Western blotting.

Finally, concerning the analysis of thioredoxin activity in protoscolex extracts, it is worth mentioning that the lag preceding the turbidity increase produced by the protoscolex protein fraction, is comparable to those described in other TRX assays (21). It could correspond either to an isomerization step preceding the catalytic process and/or to a previous reduction of the TRX present in the extract by DTT or other protoscolex molecules (possibly TRX reductase among others, see (25). Interestingly, a similar lag is also present when using the rEgTRX.

After parasite invasion, host immune-dependent defences are elicited. Effector mechanisms include the release of toxic molecules from granules of inflammatory cells and the production of oxygen radicals (26). Parasite damage can occur in many ways: granule proteins can attack lipid membranes resulting in loss of membrane integrity and/or function (27). Also, released oxygen or nitric oxide-derived free radicals may directly inactivate or cause un/misfolding of intracellular proteins, degrade nucleic acids, and initiate membrane lipid peroxidation, causing the discharge of lipid hydroperoxides and cytotoxic carbonyls into the parasite cells (28).

Parasites can respond to this type of immunological-mediated stress by deploying a series of defences aimed at controlling an immune offensive at the effector level. Many molecules (enzymes as catalase, peroxidases and superoxide dismutases, and different compounds and cofactors) are able to scavenge or quench oxidants, thus constituting a short-term primary line of defence (29). A secondary detoxification level can be achieved by back-up defences, such as selenium-dependent glutathione peroxidase and non-selenium-dependent α-type glutathione transferase enzyme activities. Even if this secondary defence fails, cytotoxic carbonyls (the breakdown products of peroxides), may be neutralized by

parasite I-type NADPH/NADH carbonyl reductases or through glutathione conjugation catalysed via phase II glutathione transferase isoenzymes (28).

Besides the antioxidant enzymes and molecules, parasites might have some repair enzymes that regenerate the oxidatively damaged proteins. The function of TRX as an oxireductase enzyme and its ubiquitous distribution, suggest that this protein is one of the candidates for such *in vivo* repair mechanisms (30). Noteworthy, it has been shown (31) that TRX exerts a regenerative effect on proteins inactivated by oxidative stress.

It has been suggested that cestodes have only weak primary (anti-ROS) defences, thus they should rely mainly upon secondary defences (3). Nevertheless, the presence of a superoxide dismutase activity has been recently described in *E. granulosus* cyst wall (32), and a thioredoxin peroxidase gene has recently been isolated from *E. granulosus* protoscoleces (33). Concerning secondary defences, the presence of a glutathione S-transferase activity has been characterized (34), and more recently a 24 kDa protein whose N-terminal amino acid sequence resembles the μ class of enzymes was isolated from protoscoleces (35).

In all organisms where thioredoxins were studied, they participate in a cascade of redox exchanges, contributing to the maintaining of cell homeostasis. Thus, EgTRX could contribute to ensure a reductive environment in *E. granulosus*. Therefore, it could be a key molecule in parasite survival, development and growth, both as a defence barrier and as an adaptive molecule.

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