

TRYPAREDOXIN PEROXIDASE

Introduction

Flagellated protozoan parasites of the family *Trypanosomatidae* are among the most prevalent human pathogens in tropical and subtropical areas. These organisms have complex life cycles and some of them are the causative agents of debilitating or life-threatening diseases, such as American Chagas' disease (*Trypanosoma cruzi*), African sleeping sickness (*T. brucei gambiense* and *T. b. rhodesiense*), oriental sore (*Leishmania tropica*), kala azar (*L. donovani*) and mucocutaneous leishmaniasis (*L. brasiliensis*). Others infect hosts as diverse as plants (*Phytomonas* species), insects (*Crithidia* and *Leptomonas* species) and livestock (*T. congolense*, *T. b. brucei*, *T. evansi*). Many of the human pathogens are also endemic in wildlife. Worldwide, more than 30 million people are estimated to suffer from trypanosomal and leishmanial infections (World Health Organisation, 1996). Vaccination strategies have so far failed and most of the chemotherapeutic drugs currently used for treatment are unsatisfactory in terms of both efficacy and toxicity (Risse, 1993). Nifurtimox, for instance, a drug widely used in the treatment of Chagas' disease, is an unspecific redox cyler affecting not only the peroxide sensitive parasites but also the host. Accordingly, the defense system against oxidants in

the trypanosomatids, which differs substantially from the analogous host metabolism, has been discussed as a potential target area for the development of more specific trypanocidal agents (Fairlamb, 1996; Jacoby et al., 1996).

El-Sayed et al. in Mol. Biochem. Parasitology, 73 (1995) 75 - 90 describe cDNAs from *Trypanosoma brucei rhodesiense* but do not make any suggestion how to cope with the problems mentioned.

As parasites, the trypanosomatids are inevitably exposed to various reactive oxygen species, such as superoxide radicals, hydrogen peroxide and myeloperoxidase products, generated during the host defense reaction. However, their ability to cope with such oxidative stress appears to be surprisingly weak. Although they possess an iron-containing superoxide dismutase to scavenge phagocyte-derived superoxide (LeTrant et al., 1983), they lack both catalase and glutathione peroxidase (Docampo, 1990), the major hydroperoxide metabolising enzymes of the host organisms (Chance et al., 1979; Flohé, 1989). They also contain conspicuously low concentrations of glutathione (GSH), the major antioxidant sulphhydryl compound in mammalian cells. Instead they form a unique GSH derivative known as trypanothione ($T(SH)_2$; N^1, N^8 -bis(glutathionyl)spermidine), which is believed to play a central role in their antioxidant defense system (Fairlamb et al., 1985; Fairlamb and Cerami, 1992). $T(SH)_2$ can be oxidized by H_2O_2 to the corresponding cyclic disulphide (TS_2) and is regenerated at the expense of NADPH by trypanothione reductase (Bailey et al., 1993; Jacoby et al., 1996). Whether the reaction of $T(SH)_2$ with H_2O_2 is enzymatically catalyzed has, however, been the subject of debate. A $T(SH)_2$ -dependent peroxidase activity was repeatedly reported for crude extracts of the trypanosomatids (Penketh and Klein, 1986; Henderson et al., 1987; Penketh et al., 1987). However, a pertinent enzymatic entity could never be purified (Henderson et al., 1987; Penketh et al., 1987) and doubts about its existence were raised (Penketh and Klein, 1986). A recent systematic investigation of the various developmental stages of *T. cruzi* even concluded that non-enzymatic oxidation of $T(SH)_2$ by H_2O_2 may fully account for the slow hydroperoxide metabolism in this species (Carnieri et al., 1993).

The present invention is based on the discovery that hydroperoxide metabolism in the trypanosomatids is enzymatic in nature, but distinct from any known metabolic pathway of the host organisms (Nogoceke et al., 1997). Apart from the previously known trypanothione reductase, the parasitic pathway comprises two novel proteins, called

tryparedoxin and tryparedoxin peroxidase, which together catalyse the reduction of hydroperoxides at the expense of NADPH as depicted in Fig. 1.

The uniqueness of this cascade of oxidoreductases offers the possibility to inhibit the parasitic metabolism without causing adverse effects in the host organism.

Thus, one embodiment of the invention concerns a protein, which is characterized by its capability of transferring reductive equivalence of trypanothione/trypanothione disulfide via a mediator such as tryparedoxin (trypanothione: peroxiredoxin oxidoreductase), mediating between trypanothione/trypanothione disulfide and the protein, to hydrogen peroxide and/or alkylhydroperoxide.

The protein according to the invention can be characterized in that it can be prepared by means of and/or isolated from a species of the family Trypanosomatidae.

Further, the protein according to the invention can be characterized in that the preparation and/or isolation can be carried out by genetic engineering especially by means of an oligonucleotide as probe wherein the sequence of said oligonucleotide codes for a peptide of SEQ ID NO 1:

MPWLAVPFAQSEAVQKLSK #FNVESIPTLIGVDADSGDVVTTR ARATLV.

Further, the protein according to the invention can be characterized by an oligomeric structure in its native state and by a molecular weight of 20 to 24 kDa in its monomeric state.

Further, the protein according to the invention can be a protein comprising or having the amino acid sequence of SEQ ID NO 1.

Further, the protein according to the invention can be characterized by at least one ValCysPro (VCP) motif and catalyzing the reduction of peroxides by means of tryparedoxin.

Further, the protein according to the invention can be a protein

(a) having the amino acid sequence SEQ ID NO 2 (figure 9 positions 1 to 187):

```

      M   S   C   G   A   A   K   L   N   H   P   A   P   E   F   15
1  ATG TCC TGC GGT GCC GCC AAG TTG AAC CAC CCC GCG CCT GAG TTC
      D   D   M   A   L   M   P   N   G   T   F   K   K   V   S   30
46 GAC GAC ATG GCG CTC ATG CCC AAC GGC ACA TTC AAG AAG GTA AGC
      L   S   S   Y   K   G   K   Y   V   V   L   F   F   Y   P   45
91 CTG TCG TCC TAC AAG GGC AAG TAC GTC GTG CTC TTC TTC TAC CCC
      M   D   F   T   F   V   C   P   T   E   I   I   Q   F   S   60
136 ATG GAC TTC ACC TTC GTG TGC CCC ACC GAG ATC ATC CAG TTC TCC
      D   D   A   K   R   F   A   E   I   N   T   E   V   I   S   75
181 GAC GAC GCC AAG CGC TTC GCC GAG ATC AAC ACC GAG GTG ATC TCC
      C   S   C   D   S   E   Y   S   H   L   O   W   T   S   V   90
226 TGC TCC TGC GAC AGC GAG TAC AGC CAC CTG CAG TGG ACG TCC GTG
      D   R   K   K   G   G   L   G   P   M   A   I   P   M   L   105
271 GAC CGC AAG AAG GGC GGC CTC GGC CCC ATG GCC ATC CCC ATG CTG
      A   D   K   T   K   G   I   A   R   A   Y   G   V   L   D   120
316 GCC GAC AAG ACC AAG GGC ATC GCG CGC GCC TAC GGC GTG CTG GAC
      E   D   S   G   V   A   Y   R   G   V   F   I   I   D   P   135
361 GAG GAC AGC GGG GTC GCC TAC CGC GGC GTC TTC ATC ATC GAC CCC
      N   G   K   L   R   O   I   I   I   N   D   M   P   I   G   150
406 AAC GGC AAG CTG CGC CAG ATC ATC ATC AAC GAC ATG CCC ATC GGC
      R   N   V   E   E   V   I   R   L   V   E   A   L   O   F   165
451 CGC AAC GTC GAG GAG GTG ATC CGC CTG GTC GAG GCG CTG CAG TTC
      V   E   E   H   G   E   V   C   P   A   A   W   K   K   G   180
496 GTG GAG GAG CAC GGC GAG GTG TGC CCG GCC AAC TGG AAG AAG GGC
      D   A   K   K   K   E   G
541 GAC GCC AAG AAG AAG GAG GGC
      (G)   (C) (T)   (CC) (A)1

```

or

(b) having an amino acid sequence which is homologous to said according to (a), has the same number or a smaller or slightly smaller number of amino acids and is encoded by an oligonucleotide which is hybridizable with an oligonucleotide which encodes a protein having the amino acid sequence SEQ ID NO 2.

Further, the protein according to (b) can be a protein having an amino acid sequence which is homologous to said according to (a) by at least 70 % and especially at least 75 %.

Another embodiment of the invention concerns a plasmid for the expression of a protein according any of the preceding claims and comprising a nucleic acid sequence encoding said protein.

The plasmid according to the invention may comprise a DNA sequence encoding trypanothione peroxidase especially of *Crithidia fasciculata*.



Further, the plasmid according to the invention may comprise a DNA sequence encoding functionally active derivatives of tryparedoxin peroxidase designed for an isolation in a manner known per se.

Further, the plasmid according to the invention may comprise a DNA sequence encoding functionally active derivatives of tryparedoxin peroxidase wherein the tryparedoxin peroxidase is derivated by a His tag.

Still another embodiment of the invention concerns a process for the production of a protein according to the invention **characterized** in that it is produced by means of a DNA sequence encoding the amino acid sequence of SEQ ID NO 1 by genetic engineering in a manner known per se.

The process according to the invention can be **characterized** in that the production is carried out by means of a plasmid according to the invention.

Further, the process according to the invention can be **characterized** in that the host is selected from the group consisting of bacteria, fungi, yeast, plant cells, mammalian cells and cells cultures (heterologous expression).

Further, the process according to the invention can be **characterized** in that *Escherichia coli* is used as host.

Still another embodiment of the invention concerns a use of a protein according to the invention or obtained according to the process according to the invention for testing and recovering inhibitory substances which inhibit activities of said protein.

Still another embodiment of the invention concerns a test system for testing the catalytic activity of a protein according to the invention or obtained according to the process according to the invention, wherein the testing system contains or comprises trypanothione reductase, trypanothione, a tryparedoxin as mentioned before and, in addition, a hydroperoxide as indicator enzyme, mediator and substrate, respectively.

Finally, another embodiment of the invention concerns a pharmaceutical preparation having a trypanocidal activity and comprising an inhibitory substance inhibiting the catalytic activity of a protein according to the invention or of a protein which can be obtained according to the process according to the invention.

The pharmaceutical preparation according to the invention can be **characterized** in that it can be obtained by a use according to the invention and by using a test system according to the invention.

The invention is now described in greater detail by means of figures and examples.

Fig. 1 Flux of reducing equivalents from NADPH to hydroperoxide in *C. fasciculata*. TR = trypanothione reductase; $T(SH)_2$ = trypanothione; TS_2 = trypanothione disulphide; TXN = tryparedoxin; TXNPx = tryparedoxin peroxidase; ROOH = hydroperoxide.

Fig. 2 Components of the trypanothione-mediated hydroperoxidase metabolising system from *C. fasciculata* in silver-stained SDS-PAGE (8 - 25 %). Lane 2, extract of disrupted cells; lane 3, trypanothione reductase; lane 4, tryparedoxin peroxidase and lane 5, tryparedoxin. Lanes 1 and 6, molecular weight standards.

Fig. 3 Molecular mass determination by MALDI-TOF-MS of pure tryparedoxin peroxidase from *C. fasciculata*. The peaks at 20880 and 41829 correspond to the apparent molecular masses of the monomeric and dimeric forms of tryparedoxin peroxidase, respectively.

Fig. 4 Gel permeation chromatography of pure tryparedoxin peroxidase from *C. fasciculata*. Pure tryparedoxin peroxidase was chromatographed on a Superdex 200 HR 10/30 column in 0.2 M NH_4HCO_3 buffer, pH 7.8. The peak eluting at 11.8 ml corresponds to the homo-oligomeric form of tryparedoxin peroxidase with a molecular mass of more than 250000. The molecular mass of the second peak, eluting at 15.2 ml and corresponding to the dimeric form of tryparedoxin peroxidase, is approximately 42000. The monomeric form was not detected.

Fig. 5 NADPH -dependent hydroperoxide metabolism reconstituted from components isolated from *C. fasciculata*. Peroxidase activity depends on both isolated proteins, tryparedoxin (TXN) (A) and tryparedoxin peroxidase (TXNPx) (B), as well as $T(SH)_2$ (E) and trypanothione reductase (TR) (F). The comparatively high activity in (F) observed immediately after the addition of TR is due to the accumulation of its substrate, TS_2 . Note that the reaction is comparably fast with H_2O_2 (D) and t-bOOH (C). The tests were performed at 27 °C with 0.1 mM NADPH, 16.5. µg/ml tryparedoxin peroxidase, 12 µg/ml

tryparedoxin, 45 μ M T(SH)₂, 45 μ M hydroperoxide and 0.4 U/ml TR. NADPH consumption was measured photometrically at 340 nm.

Fig. 6 Sequence alignment of the peptide fragments of tryparedoxin peroxidase with thiol-specific antioxidant protein from yeast (TSA/YEAST). Tryparedoxin peroxidase was digested with trypsin (Tryp) or endoproteinase Glu-C (Glu-C). The yeast TSA sequence was obtained from the SwissProt Data Bank (accession no. P34760). Asterisks denote conserved residues.

Fig. 7 Nucleotide and deduced amino acid sequences of the tryparedoxin peroxidase gene from *C. fasciculata*. The arrows delimit the PCR product used to screen the genomic library. The start and stop codons are in bold, as is the asparagine residue which was replaced by a threonine residue in direct peptide sequencing. Sequences confirmed by protein sequence analysis are underlined. The position of the Sac I site in the 1.1 kb fragment is heavily underlined. The differences in the coding region between the 1.5 kb and 1.1 kb fragments, and in the 5' flanking region between the 1.5 kb and 1.1 kb fragments are shown in brackets. The AG consensus splice leader sites and the polypyrimidine rich tract are double underlined.

Fig. 8 Tryparedoxin peroxidase specific activity determined in supernatants of sonicated *E. coli* BL21(DE3) pET/Tpod cells ($\blacktriangle$), *E. coli* BL21(DE3)pET/TpodH6 cells ($\bullet$) and *E. coli* BL 21(DE3) pET 24 a cells ($\blacksquare$). Gene expression induction, by IPTG addition is indicated by an arrow.

Fig. 9 Western blot analysis of expressed tryparedoxin peroxidase. SDS-PAGE was done under reducing conditions in 8-25 % gradient gels and the samples were electroblotted onto a PVDF membrane using a Pharmacia Phast System. Whole rabbit serum (1:250 dilution) containing antibodies raised against pure *C. fasciculata* tryparedoxin peroxidase was used as first antibody and anti-rabbit goat antibodies (Sigma) as second antibody. Lane 1, supernatant of *E. coli* BL21(DE3) pET/Tpod cells 5 h after induction; lane 2, supernatant of *E. coli* BL21(DE3)pET/TpodH6 cells 5 h after induction; lane 3 authentic tryparedoxin peroxidase from *C. fasciculata*

Fig. 10 SDS-PAGE of expressed tryparedoxin peroxidase. SDS-PAGE was done under reducing conditions in 8-25 % gradient gels on a Pharmacia Phast System and the gels were stained for protein with silver according to the manufacturers' recommendations. Lane 2, supernatant of *E. coli* BL21(DE3)pET/TpodH6 cells before induction; lane 4, supernatant of *E. coli* BL21(DE3)pET/TpodH6 cells 5 h after induction; lane 4, purified recombinant tryparedoxin peroxidase and lane 5, authentic tryparedoxin peroxidase from *C. fasciculata*. Lanes 1 and 6, molecular weight standards.

Fig. 11 Molecular mass determination by MALDI-TOF-MS of pure recombinant tryparedoxin peroxidase. The peaks at 21884 and 43766 correspond to the apparent molecular masses of the monomeric and dimeric forms of tryparedoxin peroxidase, respectively.

Example 1: Isolation of tryparedoxin peroxidase from *C. fasciculata*.

C. fasciculata was cultivated in a 100 l fermenter as described (Shim and Fairlamb, 1988). The cells were harvested in the late log phase, suspended in 50 mM sodium phosphate pH 5.8 (buffer B) containing 0.1 mM PMSF, then frozen and thawed twice to complete cell disruption. Cell debris was removed by centrifugation at 25,000 g for 30 min and the supernatant was applied on an S-Sepharose column pre-equilibrated with buffer B. Tryparedoxin peroxidase eluted at 150 mM NaCl in buffer B and was directly loaded on a hydroxyapatite (BioRad, USA) column pre-equilibrated with 10 mM sodium phosphate pH 6.8. Tryparedoxin peroxidase was eluted stepwise with 0.4 M potassium phosphate pH 6.8. The protein was extensively dialyzed against 20 mM Tris pH 7.6 (buffer C) and purified to homogeneity on a Resource Q column, eluting at 0.1 M NaCl in buffer C. The flow-through of the S-Sepharose column containing trypanothione reductase and tryparedoxin can be used to measure the enzymatic activity of tryparedoxin peroxidase (see example 2) or separated further to yield purified tryparedoxin and trypanothione reductase (Fig. 2) as described by Nogoceke et al. (1997).

MALDI-TOF-MS was performed with a Bruker Reflex-MALDI-TOF mass spectrometer in linear modus with an acceleration voltage of 30 kV after desalting the samples on Sephadex G-25 columns. SDS-PAGE was done under reducing conditions in 8-25% gradient gels on a

Pharmacia Phast System and the gels were stained for protein with silver according to the manufacturer's recommendations. Gel filtration was performed on a Superdex 200 HR 10/30 column in 0.2 M NH_4HCO_3 buffer pH 7.8.

Table 1 Yields and purification factors during the isolation of tryparedoxin peroxidase.

	Volume (ml)	Activity (U)	Protein (mg)	U/mg	Yield (%)	Purification factor
Cell extract	180	1268	3322	0.38	(100)	(1.0)
S-Sepharose	600	685	557	1.23	54	3.2
Hydroxyapatite	400	503	87	5.78	40	15.1
Resource Q	54	373	64	5.83	29	15.3

The overall yields of the final purification scheme are shown in Table 1. Based on the purification factors yielding homogeneous products the minimum concentrations of tryparedoxin and tryparedoxin peroxidase in the starting material were estimated to amount to 5% and 6% of the total soluble protein, respectively. The homogeneity and approximate molecular masses of the purified proteins are shown in Figure 1. The apparent subunit masses deduced by SDS-PAGE (about 21000) were compatible with those obtained by MALDI, 20880 ± 120 (Fig. 3). Freshly prepared tryparedoxin peroxidase was partially dimerised and contained higher molecular mass oligomeric species according to MALDI-MS. Rechromatography of purified tryparedoxin peroxidase on Superdex 200 revealed the enzymatically active protein to be homo-oligomeric with an approximate molecular mass beyond 250,000 (Fig. 4). The monomeric species was not detected and a trace of the dimeric form obtained by gel permeation was enzymatically inactive. Analyses of the spectral properties of the two proteins confirmed the absence of any chromophoric cofactors absorbing in the visible region.

Example 2: Determination of tryparedoxin peroxidase activity.

In essence, the activity of tryparedoxin peroxidase activity is measured by coupling the catalytic reduction of hydroperoxide by tryparedoxin to NADPH consumption by means of trypanothione and trypanothione reductase. For example, an assay sample may contain 0.1 mM NADPH in 50 mM Hepes pH 7.6, 1 mM EDTA, 50 M H_2O_2 or *t*-butyl hydroperoxide (*t*-BOOH), 45 M T(SH)_2 , 0.6 μM tryparedoxin, and 0.34 U trypanothione reductase. Unless otherwise stated, the reaction is started with the addition of the hydroperoxide. Dihydro-

trypanothione is obtained by chemical reduction of TS_2 (Bachem, Switzerland) as described (Fairlamb et al., 1986). In the specificity studies, $T(SH)_2$ and trypanothione reductase were replaced by GSH and glutathione reductase. t-BOOH may be replaced by other hydroperoxides such as H_2O_2 , linoleic acid hydroperoxide or phosphatidylcholine hydroperoxide. Phosphatidylcholine hydroperoxide is prepared from phosphatidylcholine by oxidation with soybean lipoxygenase (both Sigma, Germany) as described (Maiorino et al., 1990). Accordingly, linoleic acid hydroperoxide is prepared by enzymatic oxidation, extracted with ether at pH 4 and identified as 13-linoleic acid hydroperoxide by HPLC (Thomas and Jackson, 1991).

Figure 5 demonstrates that trypanothione reductase, $T(SH)_2$, tryparedoxin and tryparedoxin peroxidase are indispensable for the efficient reduction of H_2O_2 or alkyl hydroperoxides by NADPH. The $T(SH)_2$ -mediated "NADPH peroxidase activity" of *C. fasciculata* is thus achieved by the concerted action of three distinct proteins; the well characterized trypanothione reductase (Bailey et al., 1993), tryparedoxin and tryparedoxin peroxidase. A "trypanothione peroxidase" as a single enzymatic entity does not exist but is an enzymatic system composed of the two distinct proteins, tryparedoxin and tryparedoxin peroxidase.

Example 3: Characterisation of tryparedoxin peroxidase by partial proteins sequencing.

Since the N-terminus of tryparedoxin peroxidase was blocked, the protein was digested with bovine trypsin or endoproteinase Glu-C from *Staphylococcus aureus* (both sequencing grade, Promega) according to Stone and Williams (1993). The peptides were separated by HPLC (Applied Biosystems 172A) on an Aquapore OD-300 RP-18 column. Automated Edman degradation was performed with an Applied Biosystems, Inc. sequencer with an on-line C-18 reverse phase HPLC. Database searches were performed with the BLAST and FASTA programs. Peptides were aligned with the Bestfit program, Genetics Computer Group (GCG), Madison, Wisconsin, USA.

Eight fragments could be sequenced and could be aligned to a thiol-specific antioxidant protein (TSA) of yeast first described by Kim et al. (1988) (Fig. 6). TSA, in turn, belongs to the peroxiredoxin family of proteins, which comprises thioredoxin peroxidases, alkyl hydroperoxide reductase of bacteria, and a large number of proteins of undefined function (Chae et al., 1994) including a deduced protein sequence of *Trypanosoma brucei rhodesiense* (El-Sayed et al., 1995).

Example 4: Use of sequenced fragments of tryparedoxin peroxidase to elucidate the encoding DNA.

Cells culture and DNA extraction: *C. fasciculata* (HS6) was grown as described by Shim and Fairlamb (1988). The cells were harvested by centrifugation for 15 min at 7000 rpm, washed twice with saline solution (0.9% NaCl) and resuspended in 5 ml buffer (50 mM TrisCl, 100 mM EDTA, 15 mM NaCl, 0.5% SDS, 100 µg ml⁻¹ Proteinase K, pH 8.0). Resuspended cells were preincubated at 50°C for 40 min. The genomic DNA was extracted twice with equivalent volumes of phenol (incubation: 60 °C for 45 min; centrifugation: 20 min, 4500 rpm) followed by phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol extraction (24:1). Genomic DNA was precipitated with sodium acetate and ethanol.

Primers, hybridization probes and sequence analysis: Based on the peptide sequences of tryparedoxin peroxidase (Nogoceke et al., 1997) degenerate oligodeoxyribonucleotides 5'-TCGAATTCGAYATGGCSCTIATGC-3' and 5'-CTGGATCCCRATIGGCATRTC-3' were synthesized. Polymerase chain reaction (PCR) amplification was performed using the GeneAmp PCR Core kit (Perkin Elmer) using 0.8 µg of *C. fasciculata* genomic DNA as template, 10 µl of 10x reaction buffer, 8 µl 25mM MgCl₂, 2 µl of each 10 µM dNTP, 100 pmol of each primer and 0.5U Taq polymerase. An annealing temperature of 50°C was used. The PCR product was analysed by agarose gels and purified using the QIAquick PCR purification kit (QIAGEN Inc.). Sequencing was performed on a 373A DNA Sequencer (Applied Biosystems) using the PRISM Ready Reaction DyeDeoxy Terminator Sequencing Kit (1550V, 19 mA, 30 W, 42°C). When used as a hybridization probe the PCR product was labelled with digoxigenin using the DIG DNA Labeling Kit (Boehringer Mannheim) according to the instructions provided by the supplier.

Library construction and screening procedure: The genomic DNA was partially digested for 5 - 30 min with a ratio unit Sau3A / µg DNA of 0.005. The efficiency of the digestion was monitored by electrophoresis on agarose gels. Proteins were removed from the DNA using StrataClean Resin (Stratagene). The Sau3A sites were partially refilled with dATP and dGTP and Klenow fragment. The genomic DNA was ligated into Lambda GEM-11 Xho I half site arms (Promega) at a molar ratio of DNA to genomic DNA (average size 15 kb) of 1:0.7. The ligated DNA was packaged using the Packagene Lambda DNA Packaging System (Promega) according to the suppliers' instructions. The phages were used to infect the *E. coli* host strain LE392 (Promega) according to the standard protocol. 5.1×10^3



pfu of the genomic library gave positive PCR signals for tryparedoxin peroxidase and were plated on agar. The plaques were transferred to 9 cm diameter Biodyne-A nylon membranes and screened with the DIG-labelled PCR probes following the instructions provided by the supplier but using a hybridization temperature of 58 C. DIG labelled nucleic acids were detected colorimetrically with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim). Positive clones were rescreened, amplified and suspended in SM buffer. The phages were precipitated by PEG 8000 and purified in CsCl gradients. The isolated DNA was used for restriction analyses (Sau I, Sac I) or as template for PCR reactions. The digestion products were eluted from agarose gels and ligated into pBluescript II KS (+/-) phagemids (Stratagene). The ligated DNA was used to transform *E. coli* LE392. Transformed cells were selected by ampicillin resistance, plasmids were purified using QIAprep Spin Plasmid Kit (Qiagen Inc.) and analyzed by restriction enzyme digestion and sequencing.

Isolation and sequencing of the tryparedoxin peroxidase gene from *C.*

fasciculata: Sequenced peptide fragments obtained from isolated tryparedoxin peroxidase of *C. fasciculata* (Nogoceke et al., 1997) could be aligned along the established deduced amino acid sequence of the thiol-specific antioxidant protein of yeast (Chae et al., 1993). This enabled appropriate degenerate PCR primers to be designed for the generation of a PCR product from the *C. fasciculata* genomic DNA. This PCR product was subsequently used to screen a genomic library for inserts containing the full length DNA encoding the tryparedoxin peroxidase. A clone containing a 15 kb insert with the presumed tryparedoxin peroxidase gene was isolated and sequenced. This, however, led to the detection of equal quantities of different nucleotides at several positions towards the 3' end of the gene, implying the presence of similar but not identical genes in the insert. This observation was not unexpected since the genome of the *Trypanosomatidae* is known to contain repetitive structural genes separated by intergenic sequences (Tschudi et al., 1985; Wong et al., 1993; Field and Field, 1997). The DNA was consequently digested with the restriction enzyme Sac I to separate the phage arms from the insert. A southern blot was performed and three fragments (1.1 kb, 1.5 kb and 11 kb) were hybridized with the labelled PCR product. Each of the three fragments was subcloned into pBluescript II KS (+/-) phagemids and sequenced. Sequencing of the 11 kb fragment revealed an open reading frame containing coding sequences for the previously sequenced peptides of tryparedoxin peroxidase (Fig. 3). Nevertheless, as with the 15 kb insert, the bases at positions 542, 548, 551, 556, 557, 560, 563, 564 and 565 could not be unequivocally identified. The nucleotide sequence of the 1.5 kb fragment contained a largely identical open reading frame to the one of the 11 kb

fragment, except for the presence of an additional cytosine at position 30. As a consequence of the resulting frameshift, the deduced amino acid sequence no longer complied with the established peptide sequences. Hence, the 1.5 kb fragment contained a pseudogene. The 1.1 kb fragment also contained an open reading frame but encoded only part of the tryparedoxin peroxidase since a Sac I restriction site was present at position 472 - 477. This reading frame was therefore not sequenced to completion.

The full length encoding DNA and the deduced amino acid sequence is shown in Fig. 7.

Example 5: Heterologous expression of tryparedoxin peroxidase in *Escherichia coli*.

The tryparedoxin peroxidase gene contained in the cloned 1.5 kb fragment was amplified by PCR with a forward primer A (5'-CCACCACTTGGCGCACATATGTCCTGCGGTGCC GCC-3') that contains an Nde I site and overlaps the 5' end of the coding sequence, and a reverse deletion primer a (5'-CGCGGGGTGGTTCAACTTGGCGGCACCGCAGGAC-3') to delete the extra cytosine base at position 30. Amplification was also performed with a forward deletion primer b (5'-CAAGTTGAACCACCCGCGCCTGAGTTCGACGAC-3') and a reverse primer B (5'-GCCACGCCTGCTTCTCTCCTCGAGGCCCTCCTTCTTCTTGG-3') which overlaps the 3' end of the coding sequence and contains an Xho I site. Consequently the last coded amino acid is changed to a leucine, a glutamate residue is added, the stop codon is deleted and the protein will contain 6 histidine residues at its carboxyl-terminal end. Consequently amplification was performed as above but using the Expand High Fidelity polymerase mixture and buffer (Boehringer Mannheim) at an annealing temperature of 58°C with the extension temperature being increased in 10 sec increments per cycle during cycles 10 - 20. PCR products of the expected size were obtained and used as template for a second PCR amplification with the forward primer A and the reverse primer B. The amplified coding region was digested with Nde I and Xho I and ligated to a pET24a(+) vector (Novagen) treated with the same enzymes and dephosphorylated. The resulting plasmid (pET/TpodH6) was used to transform *E. coli* BL21(DE3). Transformed cells were selected by kanamycin resistance, the plasmids purified and sequenced. The same procedure, but using a reverse primer C (5'-GGCCACGCCTGTCTGACT TACTAGTGGCCCTCCTTCTTCTTGG-3') instead of reverse primer B was used to express tryparedoxin peroxidase with no changes at the carboxyl-terminal end. In this case the reverse primer contained an extra stop codon and a

Sal I site at the 5'-end of the extra stop codon, with the digestions for the cloning step being performed with Nde I and Sal I. The resulting plasmid was called pET/Tpod.

E. coli BL21(DE3) pET/Tpod and *E. coli* BL21(DE3) pET/TpodH6 were grown to A_{600} of 0.9 - 1.0 at 36°C and 180 rpm in LB medium with 30 µg kanamycin/ml, then expression of the tryparedoxin peroxidase gene was induced with 1 mM isopropyl- β -D-thiogalactopyranoside. *E. coli* BL21(DE3) containing the pET24a plasmid was grown in the same way. Samples taken at different times were centrifuged, resuspended in 50 mM Tris-HCl pH 8.0, 1 mM EDTA buffer, sonicated and centrifuged. Enzyme activity was determined as in Nogoceke et al. (1997); protein concentration was determined using Coomassie Brilliant Blue-G reagent (BioRad) with bovine serum albumin as standard. After induction of the transformed bacteria, tryparedoxin peroxidase activity was detected in supernatants of sonicated cells. Activity increased to a maximum after 5 hours induction and no activity was found in the control (Fig. 8). Induction resulted in the accumulation of a new protein with an apparent molecular mass of 21000, which was recognised by the anti-tryparedoxin peroxidase antibodies raised against pure *C. fasciculata* tryparedoxin peroxidase (Fig. 9).

Example 6: Purification and characterization of recombinant tryparedoxin peroxidase.

E. coli BL21(DE3) pET/TpodH6 was grown at 36°C and 180 rpm in LB medium with 30 µg kanamycin/ml to A_{600} of 0.9 - 1.0, then expression of the tryparedoxin peroxidase gene was induced with 1 mM isopropyl- β -D-thiogalactopyranoside. *E. coli* BL21(DE3) containing the pET24a plasmid was grown in the same way. Samples taken at different times were centrifuged, resuspended in 50 mM Tris-HCl pH 8.0, 1 mM EDTA buffer, sonicated and centrifuged. After 5 h the culture was centrifuged and either stored at -20°C or the cells were resuspended in 0.05 culture volume of binding buffer (5mM imidazole, 500 mM NaCl and 20 mM Tris-HCl pH 7.9). The cell suspension was sonicated on ice and centrifuged for 40 min at 4°C, 35000g. The supernatant was applied to a His Bind resin (Novagen) column charged with Ni^{2+} and equilibrated with binding buffer, at a flow rate of about 10 column volumes per hour. The column was washed with 10 volumes of binding buffer, 6 volumes of 500 mM NaCl, 20 mM Tris-HCl pH 7.9 buffer containing 60 mM imidazole and 6 volumes of the same buffer with 100 mM imidazole. Tryparedoxin peroxidase eluted in the buffer containing 500 mM imidazole. Active fractions were pooled and immediately dialysed against 50 mM Tris-HCl pH 7.6 buffer containing 100 mM NaCl and 1 mM EDTA. Tryparedoxin peroxidase eluted at 500 mM imidazole and was shown by SDS-PAGE and subsequent silver staining to be pure (Fig. 10). N-terminal sequencing of this protein showed the initial methionine to be

missing and allowed us to confirm the first 30 amino acids. The expressed tryparedoxin peroxidase showed nearly the same molecular mass of about 21000 as the authentic tryparedoxin peroxidase in SDS-PAGE (Fig. 12). MALDI-MS analysis demonstrated the molecular mass of the recombinant tryparedoxin peroxidase to be 21884 ± 22 (Fig. 11). The difference in the molecular mass between the recombinant and authentic tryparedoxin peroxidase corresponded to the additional amino acids (leucine, glutamate and 6 histidine residues) added at the C-terminal end of the recombinant enzyme.

Whereas tryparedoxin peroxidase purified from *C. fasciculata* contained several isoforms ranging from pI 4.9 to 5.8, the recombinant protein showed two bands of pI 6.2 and 6.3. The higher alkalinity may be attributed to the additional histidines residues.

The purified recombinant enzyme had a specific activity of 2.51 U/mg compared to 5.83 U/mg for the authentic enzyme. This difference may be attributed to the additional amino acids at the caboxyl-terminal end of the recombinant tryparedoxin peroxidase. The kinetic pattern was essentially identical to that observed with the authentic tryparedoxin peroxidase. The numerical values of the kinetic parameters of the pure recombinant tryparedoxin peroxidase are shown in Table 2.

Table 2. Dalziel coefficients (k_1 and k_2) and apparent second order rate constants (k_1 and k_2) for the reaction of recombinant tryparedoxin peroxidase with *t*-butyl hydroperoxide (t-bOOH) and tryparedoxin (TXN). Data are the means of two independent measurements. All k_1 values are calculated per subunit concentration.

acceptor substrate	TXN [μ M]	k_1 [10^{-6} M sec]	k_1 [10^5 M ⁻¹ sec ⁻¹]	k_2 [10^{-7} M sec]	k_2 [10^6 M ⁻¹ sec ⁻¹]
t-bOOH	0.607	12.7 $\pm$ 0.1	0.8 $\pm$ 0.0	1.2 $\pm$ 0.0	0.8 $\pm$ 0.0
	0.303	10.2 $\pm$ 0.0	0.1 $\pm$ 0.0	1.0 $\pm$ 0.0	1.0 $\pm$ 0.1
	0.200	13.5 $\pm$ 0.5	0.8 $\pm$ 0.0	1.0 $\pm$ 0.1	1.0 $\pm$ 0.0

Example 7: Inhibition studies.

The test system described in example 2 is easily adapted to screen compounds for specific inhibition of tryparedoxin peroxidase. As an example the inhibition of tryparedoxin peroxidase by S-alkylating agents such as N-ethylmaleimide (NEM) is described (Table 3). Tryparedoxin peroxidase was preincubated in 50 mM Hepes, 1 mM EDTA, pH 7.6 with or

without presumed reducing substrate ($T(SH)_2$), then reacted with NEM and activity was checked at 22°C essentially as described in example 2, but using 1 mM $T(SH)_2$ with 1.0 μ M tryparedoxin peroxidase and 0.6 μ M tryparedoxin. Changes in molecular mass were determined by MALDI-TOF-MS. The large standard deviation of tryparedoxin peroxidase masses is explained by polymerisation equilibria resulting in a broadening of mass peaks.

Table 3 Derivatisation scheme of tryparedoxin peroxidase (TXNPx).

Protein	Pretreatment	Derivatisation	mass increment	residual activity [%]
TXNPx (5.7 μ M)	725 μ M $T(SH)_2$; 30 sec at 4°C	--	--	(100)
	725 μ M $T(SH)_2$; 8.3 μ M TXN; 30 sec at 4°C	--	--	132 $\pm$ 5*
	none	1.9 mM NEM; 30 min at 4°C	n. d.	93 $\pm$ 5
	725 μ M $T(SH)_2$; 30 sec at 4°C	1.9 mM NEM; 150 sec at 4°C	7 $\pm$ 120 (0)	81 $\pm$ 5
	725 μ M $T(SH)_2$; 8.3 μ M TXN; 30 sec at 4°C	1.9 mM NEM; 150 sec at 4°C	232 $\pm$ 120 (250)	3 $\pm$ 5

* apparent increase of activity due to reduction of tryparedoxin peroxidase.

REFERENCES

- Bailey, S., Smith, K., Fairlamb, A.H. and Hunter, W.H. (1993) Substrate interactions between trypanothione reductase and N¹-glutathionylspermidine disulphide at 0.28-nm resolution. *Eur. J. Biochem.* 213, 67-75.
- Carnieri, E.G.S., Moreno, S.N.J. and Docampo, R. (1993) Trypanothione-dependent peroxide metabolism in *Trypanosoma cruzi* different stages. *Mol. Biochem. Parasitol.* 61, 79-86.
- Chae, H.Z., Kim I, Kim K and Rhee, S.G. (1993) Cloning, sequencing, and mutation of thiol-specific antioxidant gene of *Saccharomyces cerevisiae*. *J. Biol. Chem* 268, 16815-16821.
- Chae, H.Z., Robinson, K., Poole, L.B., Church, G., Storz, G. and Rhee, S.G. (1994) Cloning and sequencing of thiol-specific antioxidant from mammalian brain: Alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc. Natl. Acad. Sci. USA* 91, 7017-7021.
- Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* 59, 527-605.
- Dalziel, K. (1957) Initial steady state velocities in the evaluation of enzyme-coenzyme-substrate reaction mechanisms. *Acta Chem. Scand.* 11, 1706-1723.
- Docampo, R. (1990) Sensitivity of parasites to free radical damage by antiparasitic drugs. *Chem. Biol. Interactions* 73, 1-27.
- El-Sayed, N.M.A., Alarcon, C.M., Beck, J.C., Sheffield, V.C. and Donelson, J.E. (1995) cDNA expressed sequence tags of *Trypanosoma brucei rhodesiense* provide new insights into the biology of the parasite. *Mol. Biochem. Parasitol.* 73, 75-90.
- Fairlamb, A.H. (1996) Pathways to drug discovery. *The Biochemist* 18 (Feb/Mar), 11-16.
- Fairlamb, A.H., Blackburn, P., Ulrich, P., Chait, B.T. and Cerami, A. (1985) Trypanothione: A novel bis (glutathionyl) spermidine cofactor for glutathione reductase in trypanosomatids. *Science* 227, 1485-1487.
- Fairlamb, A.H. and Cerami, A. (1992) Metabolism and functions of trypanothione in the kinetoplastida. *Annu. Rev. Microbiol.* 46, 695-729.
- Fairlamb, A.H., Henderson, G.B. and Cerami, A. (1986) The biosynthesis of trypanothione and N¹-glutathionylspermidine in *Crithidia fasciculata*. *Mol. Biochem. Parasitol.* 21, 247-257.

- Field, H. and Field, M.C. (1997) Tandem duplication of rab genes followed by sequence divergence and acquisition of distinct functions in *Trypanosoma brucei*. *J. Biol. Chem.* 272, 10498-10505.
- Flohé, L. (1989) The selenoprotein glutathione peroxidase. In: Glutathione - chemical, biochemical, and medical aspects. D. Dolphin, R. Poulson, and O. Avramovic, eds. (New York, USA: J. Wiley and Sons, Inc.) pp. 643-731.
- Flohé, L., Loschen, G., Günzler, W.A. and Eichele, E. (1972) Glutathione peroxidase. V. The kinetic mechanism. *Hoppe-Seyler's Z. Physiol. Chem.* 353, 987-999.
- Forstrom, J.W., Stults, F.H., and Tappel, A.L. (1979) Rat liver cytosolic glutathione peroxidase: reactivity with linoleic acid hydroperoxide and cumene hydroperoxide. *Arch. Biochem. Biophys.* 193, 51-55.
- Henderson, G.B., Fairlamb, A.H. and Cerami, A. (1987) Trypanothione dependent peroxide metabolism in *Crithidia fasciculata* and *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 24, 39-45.
- Jacoby, E.M., Schlichting, I., Lantwin, C.B., Kabsch, W., and Krauth-Siegel, R.L. (1996) Crystal structure of the *Trypanosoma cruzi* trypanothione reductase-mepacrine complex. *Proteins* 24, 73-80.
- Kim, K., Kim, I.H., Lee, K.-Y., Rhee, S.G. and Stadtman, E.R. (1988) The isolation and purification of a thiol-specific protector protein which inhibits enzyme inactivation by a thiol Fe (III)/O₂ mixed-function oxidation system. *J. Biol. Chem.* 263, 4704-4711.
- LeTrant, N., Meshnick, S.R., Kitchener, K., Eaton, J.W. and Cerami, A. (1983) Iron-containing superoxide dismutase from *Crithidia fasciculata*. *J. Biol. Chem.* 258, 125-130.
- Maiorino, M., Gregolin, C., and Ursini, F. (1990) Phospholipid hydroperoxide glutathione peroxidase. *Methods Enzymol.* 186, 448-457.
- Nogoceke, E. Gommel, D. U., Kieß, M., Kalisz, H. M. and Flohé, L. (1997) A unique cascade of oxidoreductases catalyses trypanothione-dependent peroxide metabolism in *Crithidia fasciculata*. *Biol. Chem.*, 378, in press.
- Penketh, P.G., Kennedy, W.P.K., Patton, C.L. and Sartorelli, A.C. (1987) Trypanosomatid hydrogen peroxidase metabolism. *FEBS Lett.* 221, 427-431.
- Penketh, P.G. and Klein, R.A. (1986) Hydrogen peroxide metabolism in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 20, 111-121.
- Risse, H.J. (1993) Afrikanische Trypanosomen - Meister im Austricksen des Immunsystems. In: Extremophile. K. Hausmann, and B.P. Kremer, eds. (Weinheim, Germany: VCH) pp. 361-380.

- Shim, H. and Fairlamb, A.H. (1988) Levels of polyamines, glutathione, and glutathione-spermidine conjugates during growth of the insect trypanosomatid *C. fasciculata*. J. Gen. Microbiol. 134, 807-817.
- Stone, K.L. and Williams, K.R. (1993) Enzymatic digestion of proteins and HPLC peptide isolation. In: A practical guide to protein and peptide purification for microsequencing, 2nd ed. P. Matsudaira, ed. (London, England: Academic Press Inc.) pp. 45-69.
- Thomas, C.E. and Jackson, R.L. (1991) Lipid hydroperoxide involvement in copper-dependent and independent oxidation of low-density lipoproteins. J. Pharmacology Exp. Ther. 256, 1182-1188.
- Tschudi, C., Young A.S., Ruben, L., Patton, C.L. and Richards F.F. (1985) Proc. Nat. Acad.Sci.USA 82, 3998-4002
- Ursini, F., Maiorino, M., Brigelius-Flohé, R., Aumann, K.D., Roveri, A., Schomburg, D. and Flohé, L. (1995) The diversity of glutathione peroxidase. Methods Enzymol. 252, 38-53.
- Wong, S., Morales, T.H., Neigel, J.E. and Campbell D.A. (1993) Mol. Cell Biol. 13, 207-216
- World Health Organization (1996) Fighting disease, fostering development. World Health Report 1996 (Geneva, Switzerland: WHO).



Claims

1. A protein, characterized by its capability of transferring reductive equivalence of trypanothione/trypanothione disulfide via a mediator such as tryparedoxin (trypanothione: peroxiredoxin oxidoreductase), mediating between trypanothione/trypanothione disulfide and the protein, to hydrogen peroxide and/or alkylhydroperoxide.
2. A protein according to claim 1, characterized in that it can be prepared by means of and/or isolated from a species of the family Trypanosomatidae.
3. A protein according to claims 1 or 2, characterized in that the preparation and/or isolation can be carried out by genetic engineering especially by means of an oligonucleotide as probe wherein the sequence of said oligonucleotide codes for a peptide of SEQ ID NO 1.

MPWLAVPFAQSEAVQKLSK^HFNYESIPTLIGVDADSGDVVTTR ARATLV.

4. A protein according to any of the preceding claims, characterized by an oligomeric structure in its native state and by a molecular weight of 20 to 24 kDa in its monomeric state.



5. A protein comprising or having the amino acid sequence of SEQ ID NO 1 according to claim 3.

6. A protein according to any of the preceding claims, **characterized** by at least one ValCysPro (VCP) motif and catalyzing the reduction of peroxides by means of trypanredoxin.

7. A protein

(a) having the amino acid sequence SEQ ID NO 2 (figure 9 positions 1 to 187):

```

      M   S   C   G   A   A   K   L   N   H   P   A   P   E   F   15
1  ATG TCC TGC GGT GCC GCC AAG TTG AAC CAC CCC GCG CCT GAG TTC
      D   D   M   A   L   M   P   N   G   T   F   K   K   V   S   30
46 GAC GAC ATG GCG CTC ATG CCC AAC GGC ACA TTC AAG AAG GTA AGC
      L   S   S   Y   K   G   K   Y   V   V   L   F   F   Y   P   45
91 CTG TCG TCC TAC AAG GGC AAG TAC GTC GTG CTC TTC TTC TAC CCC
      M   D   F   T   F   V   C   P   T   E   I   I   Q   F   S   60
136 ATG GAC TTC ACC TTC GTG TGC CCC ACC GAG ATC ATC CAG TTC TCC
      D   D   A   K   R   F   A   E   I   N   T   E   V   I   S   75
181 GAC GAC GCC AAG CGC TTC GCC GAG ATC AAC ACC GAG GTG ATC TCC
      C   S   C   D   S   E   Y   S   H   L   O   W   T   S   V   90
226 TGC TCC TGC GAC AGC GAG TAC AGC CAC CTG CAG TGG ACG TCC GTG
      D   R   K   K   G   G   L   G   P   M   A   I   P   M   L   105
271 GAC CGC AAG AAG GGC GGC CTC GGC CCC ATG GCC ATC CCC ATG CTG
      A   D   K   T   K   G   I   A   R   A   Y   G   V   L   D   120
316 GCC GAC AAG ACC AAG GGC ATC GCG CGC GCC TAC GGC GTG CTG GAC
      E   D   S   G   V   A   Y   R   G   V   F   I   I   D   P   135
361 GAG GAC AGC GGG GTC GCC TAC CGC GGC GTC TTC ATC ATC GAC CCC
      N   G   K   L   R   O   I   I   I   N   D   M   P   I   G   150
406 AAC GGC AAG CTG CGC CAG ATC ATC ATC AAC GAC ATG CCC ATC GGC
      R   N   V   E   E   V   I   R   L   V   E   A   L   O   F   165
451 CGC AAC GTC GAG GAG GTG ATC CGC CTG GTC GAG GCG CTG CAG TTC
      V   E   E   H   G   E   V   C   P   A   A   W   K   K   G   180
496 GTG GAG GAG CAC GGC GAG GTG TGC CCG GCC AAC TGG AAG AAG GGC
      D   A   K   K   K   E   G
541 GAC GCC AAG AAG AAG GAG GGC
      (G)      (C) (T)      (CC) (A)

```

or

(b) having an amino acid sequence which is homologous to said according to (a), has the same number or a smaller or a slightly smaller number of amino acids and is encoded by an oligonucleotide which is hybridizable with an oligonucleotide which encodes a protein having the amino acid sequence SEQ ID NO 2.

This Sheet Has Been Replaced





8. A protein according to claim 7 (b) having an amino acid sequence which is homologous to said according to claim 7 (a) by at least 70 % and especially at least 75 %.
9. A plasmid for the expression of a protein according any of the preceding claims and comprising a nucleic acid sequence encoding said protein.
10. A plasmid according to claim 9, comprising a DNA sequence encoding tryparedoxin peroxidase especially of *Crithidia fasciculata*.
11. A plasmid according to claim 9, comprising a DNA sequence encoding functionally active derivatives of tryparedoxin peroxidase designed for an isolation in a manner known per se.
12. A plasmid according to claim 11, comprising a DNA sequence encoding functionally active derivatives of tryparedoxin peroxidase wherein the tryparedoxin peroxidase is derivated by a His tag.
13. A process for the production of a protein according to any of claims 1 to 8, **characterized** in that it is produced by means of a DNA sequence encoding the amino acid sequence of SEQ ID NO 1 according to claim 3 by genetic engineering in a manner known per se.
14. A process for the production of a protein according to any of claims 1 to 8, **characterized** in that the production is carried out by means of a plasmid according to any of claims 9 to 12.
15. A process according to claim 14, **characterized** in that the host is selected from the group consisting of bacteria, fungi, yeast, plant cells, mammalian cells and cells cultures (heterologous expression).
16. A process according to claim 15, **characterized** in that *Escherichia coli* is used as host.
17. Use of a protein according to any of claims 1 to 8 or obtained according to the process according to any of the claims 13 to 16 for testing and recovering inhibitory substances which inhibit activities of said protein.





18. A test system for testing the catalytic activity of a protein according to any of claims 1 to 8 or obtained according to the process according to any of claims 13 to 16, wherein the testing system contains or comprises trypanothione reductase, trypanothione, a tryparedoxin according to claim 1 and, in addition, a hydroperoxide as indicator enzyme, mediator and substrate, respectively.

19. A pharmaceutical preparation having a trypanocidal activity and comprising an inhibitory substance inhibiting the catalytic activity of a protein according to any of claims 1 to 8 or of a protein which can be obtained according to the process according to any of claims 13 to 16.

20. A pharmaceutical preparation according to claim 18, characterized in that it can be obtained by a use according to claim 17 and by using a test system according to claim 18.

This Sheet Has Been Replaced

