Tryparedoxin, Expression plasmid, process of production, use, test system and pharmaceutical preparation

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 cycler 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).

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), most of them 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 sulfhydryl compound in mammalian cells. Instead they form a unique GSH derivative known as trypanothione ($T(SH)_2$; N^1,N^8-bis(glutathionyl)spermidine) (Fig. 1), 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) (Fig. 1) and is regenerated at the expense of NADPH by trypanothione reductase (TR; Bailey et al., 1993; Jacoby et al., 1996) (Fig. 1). 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 (TR), the parasitic pathway comprises two novel proteins, called tryparedoxin (TXN) and tryparedoxin peroxidase (TXNPx), which together catalyse the reduction of

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hydroperoxides at the expense of NADPH as depicted in Fig. 1. Isoforms of tryparedoxin exist in one species.

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 proteins, which we called tryparedoxins (trypanothione: peroxiredoxin oxidoreductases) and which are **characterized** by their capability of transferring reductive equivalents of trypanothione to a peroxiredoxin-type protein such as tryparedoxin peroxidase. As regards peroxi-redoxins and peroxi-redoxin-type proteins, reference is made to Chae et al. in J. Biol. Chem., 269(1994) 27670-24678 and in PNAS USA, 91 (1994) 7017-7021 and to EP 96 120 016.9 or PCT/EP 97/04 990. Tryparedoxins exhibit a catalytic site similar to that of thioredoxin, an ubiquituous redox mediator with pleiotropic functions. Typical thioredoxins have never been found in any trypanosomatid. It therefore appears conceivable that in the trypanosomatids the tryparedoxins substitute for thioredoxin in such diverse metabolic functions as reduction of ribonucleotides, differentiation, regulation of transcription or other regulatory processes depending on the cellular thiol/disulphide equilibrium. The possibility of multiple biological functions of tryparedoxins is further suggested by the coexistence of more than one tryparedoxin in the same species, as described below.

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

Further, the proteins according to the invention can be **characterized** in that their preparation and /or isolation can be carried out by genetic engineering, especially by means of an oligonucleotide as probe having the oligonucleotide sequence and encoding the amino acid sequence of SEQ ID NO 1 (Fig. 2) or any useful part thereof.

Further, the proteins according to the invention can be **characterized** by a molecular weight of 15-19 kDa.

Further, a protein according to the invention can be **characterized** by a WCPPC motif and catalyzing the reduction of protein disulphide bonds by means of trypanothione.

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

(a) having the amino acid sequence SEQ ID NO 2 (Fig. 3, positions 1 to 150)

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 or larger number of amino acids than SEQ ID

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NO 2 and is encoded by an oligonucleotide which is hybridizable with an oligonucleotide which encodes a protein comprising or having the amino acid sequence SEQ ID NO 1or SEQ ID NO 2.

Further, the protein according to (b) can be a protein having an amino acid sequence which is homologous to SEQ ID NO 1or SEQ ID NO 2 by at least 70% and especially at least 75%.

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

The plasmids according to the invention may comprise DNA sequences encoding tryparedoxin especially of *Crithidia fasciculata*.

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

Further, a plasmid according to the invention may comprise a DNA sequence encoding functionally active derivatives of tryparedoxin wherein the tryparedoxin is derivatised 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 2 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, insect cells, mammalian cells and cell 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 the use of a protein 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

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according to the invention, wherein the testing system contains or comprises trypanothione, trypanothione reductase, a tryparedoxin peroxidase, a tryparedoxin 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 Nucleotide and deduced amino acid sequences of the PCR product used to screen a genomic library for inserts containing the tryparedoxin gene. Sequences confirmed by protein sequence analysis are underlined and the sequences corresponding to the primers used to obtain the PCR product are double underlined.

Fig. 3 Nucleotide and amino acid sequences of tryparedoxin II isolated from a *C. fasciculata* genomic library. The start and stop codons are in bold. The position of the Pvu II site from which one third of the gene at the 5' end of the open reading frame was determined is double underlined. The WCPPC thioredoxin-like motif is underlined.

Fig. 4 Components of the trypanothione-mediated hydroperoxide 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 I. Lanes 1 and 6, molecular weight standards.

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Fig. 5 Molecular mass determination by MALDI-TOF of pure tryparedoxin I from *C. fasciculata* (trace A), tryparedoxin I exposed to iodoacetamide (trace B), $T(SH)_2$-reduced tryparedoxin I derivatized with iodoacetamide (trace C) and with iodoacetamide plus N-ethylmaleimide (trace D). The mass increments shown in traces C and D correspond to the addition of one carboxyamidomethyl residue (observed 54 Da, theoretical 57 Da) and one carboxyamidomethyl plus one N-ethylsuccinimide residue (observed 185 Da, theoretical 182 Da), respectively.

Fig. 6 NADPH-dependent hydroperoxide metabolism reconstituted from components isolated from *C. fasciculata*. Peroxidase activity depends on both isolated proteins, tryparedoxin I (TXN I) (**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 g/ml tryparedoxin peroxidase, 12 g/ml tryparedoxin I, 45 M $T(SH)_2$, 45 M hydroperoxide and 0.4 U/ml TR. NADPH consumption was measured photometrically at 340 nm.

Fig. 7 Sequence alignment of the peptide fragments of tryparedoxin I with thioredoxin-like protein of *Caenorhabditis elegans* (TLP/CE).Tryparedoxin I was digested with trypsin (Tryp) or endoproteinase Glu-C (Glu-C). Asterisks denote conserved residues.

Fig. 8 Tryparedoxin II containing a His tag specific activity determined in the supernatants of sonicated *E.coli* BL21(DE3)pET24a cells (black square) and *E. coli* BL21(DE3)pET/TXN II H6 cells (black circle). Gene expression induction by isopropyl- -D-thiogalactopyranoside addition is indicated by an arrow.

Fig. 9 Western blot analysis of expressed tryparedoxin II containing a His tag. SDS-PAGE was done under reducing conditions in 8-25% gradient gels on a Pharmacia Phast System and the samples were electroblotted onto a PVDF membrane using a Pharmacia Phast System. Whole rabbit serum (1:500 dilution) containing antibodies raised against pure *C. fasciculata* tryparedoxin I was used as primary antibody and anti-rabbit goat antibodies (Sigma) as

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secondary antibody. Lane 1, supernatant of *E. coli* BL21(DE3) pET/TXN II H6 cells 6h after induction; lane 2, purified recombinant tryparedoxin II; lane 3, authentic tryparedoxin I from *C. fasciculata.*

Fig. 10 Tryparedoxin II specific activity determined in the supernatants of sonicated *E.coli* BL21(DE3)pET24a cells (black square) and *E.coli* BL21(DE3)pET/TXN II cells (black rhomb). Gene expression induction by isopropyl- -D-thiogalactopyranoside addition is indicated by an arrow.

Fig. 11 Western blot analysis of expressed tryparedoxin II. SDS-PAGE was done under reducing conditions in 8-25% gradient gels on a Pharmacia Phast System and the samples were electroblotted onto a PVDF membrane using a Pharmacia Phast System. Whole rabbit serum (1:500 dilution) containing antibodies raised against pure *C. fasciculata* tryparedoxin I was used as primary antibody and anti-rabbit goat antibodies (Sigma) as secondary antibody. Lane 1, authentic tryparedoxin I from *C. fasciculata*; lane 2, supernatant of *E. coli* BL21(DE3) pET/TXN II cells 6h after induction.

Fig. 12 SDS-PAGE of expressed tryparedoxin II containing a His tag. 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) pET24a cells after 6h induction; lane 2, supernatant of *E. coli* BL21(DE3) pET/TXN II H6 cells 6h after induction; lane 3, purified recombinant tryparedoxin II; lane 4, authentic tryparedoxin I from *C. fasciculata.* Lanes 1 and 6, molecular weight standards.

Example 1: Isolation of tryparedoxin 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 25,000xg 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). The flow-through of the S-Sepharose column, containing trypanothione reductase and tryparedoxin, was adjusted to pH 7.2 with 1 M NaOH. The extract was adjusted to 3% (w/v) streptomycin sulphate, brought to 50% ammonium sulphate saturation, and centrifuged for 10 min at 11,000xg. The supernatant was adjusted to 80% ammonium sulphate saturation and recentrifuged. The pellet was dissolved in, then dialyzed extensively, against 20 mM bis-Tris propane pH 7.2 containing 1mM EDTA and 1mM DTT (buffer D). The enzyme extract was loaded on a DEAE-Sepharose column and eluted with a linear gradient of 0.4 M KCl in buffer D. The sample eluting at 80-120 mM KCl was concentrated by ultrafiltration (Omegacell, Filtron, Germany), washed with 20 mM potassium phosphate pH 7.2 containing 1 mM EDTA and 1 mM DTT (buffer E) and loaded on a 2'5'ADP-Sepharose 4B column. Trypanothione reductase was eluted with 5 mM NADP in buffer E and purified to homogeneity on a Sephacryl S-200 column. The unbound fraction was concentrated by ultrafiltration and fractionated on an Ultrogel AcA54 (LKB, Sweden) gel filtration column in 50 mM Hepes pH 7.6 containing 150 mM NaCl, 1 mM EDTA and 1mM DTT to yield homogeneous tryparedoxin. The authentic tryparedoxin, thus isolated, is termed tryparedoxin I (TXN I).
The overall yields of the final purification scheme are shown in Table 1.

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

	Volume (ml)	Activity (U)	Protein (mg)	U/mg	Yield (%)	Purification factor
Cell extract	180	386	3322	1e+10	(100)	(1.0)
$(NH_4)_2SO_4$ precipitation	86	134	1728		35	0.7
DEAE-Sepharose	270	136	176		36	6.4
Ultrogel AcA54	13	51	22		13	20.2

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 4. The apparent subunit masses deduced by SDS-PAGE (about 16000) were compatible with those obtained by MALDI, 16393 ± 10 (Fig. 5).

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 activity.

In essence, the activity of tryparedoxin activity is measured by coupling the catalytic reduction of hydroperoxide mediated by tryparedoxin peroxidase 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 $T(SH)_2$, 16.5 µg/ml tryparedoxin peroxidase and 0.34 U trypanothione reductase and an unknown amount of tryparedoxin. 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). t-BOOH may be replaced by other hydroperoxides, such as H_2O_2, linoleic acid hydroperoxide or phosphatidylcholine hydroperoxide.

Figure 6 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.

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

Since the N-terminus of tryparedoxin I was blocked, the protein was digested with either 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.

Seven fragments could be sequenced and could be aligned to a thioredoxin-like protein of *C. elegans* (Fig. 7).

Example 4: Use of sequenced fragments of tryparedoxin I 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 TrisHCl, 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 I (Nogoceke et al., 1997) degenerate oligodeoxyribonucleotides were synthesized. Polymerase chain reaction (PCR) amplification was performed using the GeneAmp PCR Core kit (Perkin Elmer) using 0.2 µg of *C. fasciculata* genomic DNA as template, 5 µl of 10x reaction buffer, 3µl 25mM $MgCl_2$, 1µl of each 10µM dNTP, 100 pmol of each primer and 0.25U Taq polymerase. An annealing temperature of 52°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 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 54 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 (Sac I, EcoR I, BamH I, Xho I, Nco I) or as template for PCR reactions. The digestion products were eluted from agarose gels and ligated into pBluescript II KS (+/-) phagemids (Stratagene) or pET24d(+) vector (Novagen). The ligated DNA was used to transform *E. coli* LE392. Transformed cells were selected by ampicillin (pBluescript II KS (+/-) phagemid) or kanamycin (pET24d(+) vector) resistance, plasmids were purified using QIAprep Spin Plasmid Kit (Qiagen Inc.) and analyzed by restriction enzyme digestion and sequencing.

Isolation and sequencing of tryparedoxin genes from *C. fasciculata*: Sequenced peptide fragments obtained from isolated tryparedoxin I of *C. fasciculata* (Nogoceke et al., 1997) could be aligned along the established deduced amino acid sequence of a thioredoxin-like protein of *Caenorhabditis elegans*. 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, which coded for approximately the 50 % of tryparedoxin I (Fig.2), was subsequently used to screen a genomic library for inserts containing the full length DNA encoding tryparedoxin I. A clone containing a 22 kb insert with the presumed tryparedoxin I gene was isolated. The DNA was digested with the restriction enzyme Sac I to separate the phage arms from the insert and with the restriction enzyme Nco I. A southern blot was performed and a fragment of 6 kb hybridized with the labelled PCR product. The fragment was subcloned into the pET24d(+) vector (Novagen). Subsequent digestion of the cloned fragment with the restriction enzyme Pvu II led to the isolation and cloning of a 1 kb fragment. When this fragment was sequenced it contained only one third of the gene coding for tryparedoxin at the 5' end of the open reading frame (Fig.3). The DNA sequence thus

obtained was in full agreement with the peptide sequence derived from the isolated tryparedoxin I.

Example 5: Isolation of a new tryparedoxin gene (TXN II)

New primers were designed using the information obtained in example 4 and the complete gene was sequenced directly from the 6 kb fragment. The previously obtained PCR product is about 60% identical in its amino acid sequence to the correponding region of the isolated gene. The full length encoding DNA and the deduced amino acid sequence are shown in Fig. 3. The gene encodes for a protein with a molecular mass of 17000, while the molecular mass of natural tryparedoxin I isolated from *C. fasciculata* is 16393±10 (Nogoceke et al.,1997).

Example 6: Heterologous expression of tryparedoxin II in *Escherichia coli.*

The tryparedoxin gene contained in the cloned 6 kb fragment was amplified by PCR with a forward primer A (5'-TCGTGATTCCGTTCCGCATATGTCAGGGC-3') that contains an Nde I site and overlaps the 5' end of the coding sequence, and a reverse primer B (5'-GCAACTCAATCGCTCCCCTCGAGCTTCTTGGCCTCC-3') which overlaps the 3' end of the coding sequence and contains an Xho I site. Consequently a leucine and a glutamate residue are added, the stop codon is deleted and the protein will contain 6 histidine residues at its carboxyl-terminal end. Amplification was performed as above but using the Expand High Fidelity polymerase mixture and buffer (Boehringer Mannheim) at an annealing temperature of 50°C with the extension temperature being increased in 10 sec increments per cycle during cycles 10 - 20. 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/TXN II H6) 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'- CAGCAACTCAATGGATCC TCATTACTTCTTGGCC-3') instead of reverse primer B, was used to express tryparedoxin II with no changes at the carboxyl-terminal end. In this case the reverse primer contained an extra stop codon and a BamH I site at the 5'-end of the extra stop codon, with the digestions for the cloning step being performed with Nde I and BamH I. The resulting plasmid was called pET/TXN II and was used to transform *E. coli* BL21(DE3). Transformed cells were selected by kanamycin resistance, the plasmids purified and sequenced.

E. coli BL21(DE3) pET/TXN IIH6 were grown to A_{600} of 0.9 - 1.0 at 25°C and 180 rpm in LB medium with 30µg kanamycin/ml, then expression of the tryparedoxin II gene was induced with 1 mM isopropyl- -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, a marked increase in tryparedoxin activity was detected in supernatants of sonicated cells. Activity increased to a maximum 6 hours after 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 18000, which was recognised by the anti-tryparedoxin antibodies raised against pure *C. fasciculata* tryparedoxin I (Fig. 9).

E. coli BL21(DE3) pET/TXN II were grown to A_{600} of 0.9 - 1.0 at 25°C and 180 rpm in LB medium with 30µg kanamycin/ml, then expression of the tryparedoxin II gene was induced with 1 mM isopropyl- -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, a marked increase in tryparedoxin activity was detected in supernatants of sonicated cells. Activity increased to a maximum 6 hours after induction and no activity was found in the control (Fig. 10). Induction resulted in the accumulation of a new protein with an apparent molecular mass of 18000, which was recognised by the anti-tryparedoxin antibodies raised against pure *C. fasciculata* tryparedoxin I (Fig. 11)

Example 7: Purification and characterization of recombinant tryparedoxin II.

E. coli BL21(DE3) pET/TXN II H6 was grown at 25°C and 180 rpm in LB medium with 30µg kanamycin/ml to A_{600} of 0.9 - 1.0, then expression of the tryparedoxin II gene was induced with 1 mM isopropyl- -D-thiogalactopyranoside. After 6 h the culture was centrifuged and either stored at -20°C or the cells were resuspended in 0.05 culture volumes 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, 13000 rpm. 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 and 6 volumes of 500 mM NaCl, 20 mM Tris-HCl pH 7.9 buffer containing 100 mM imidazole. Tryparedoxin 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 1 mM DTT and 1 mM EDTA. Tryparedoxin II eluted at 500 mM imidazole and was shown to be pure by SDS-PAGE and subsequent silver staining (Fig. 12). N-terminal sequencing of this protein showed the initial methionine to be missing and allowed us to confirm the first 20 amino acids. The expressed tryparedoxin II showed a molecular mass of about 18000 in SDS-PAGE (Fig. 12), being slightly bigger than the authentic tryparedoxin I. The difference in the molecular mass between the recombinant and authentic tryparedoxin peroxidase corresponded mainly to the additional amino acids (leucine, glutamate and 6 histidine residues) added at the C-terminal end of the recombinant enzyme.

The purified recombinant enzyme had a specific activity of 7.7 U/mg compared to 2.3 U/mg for the authentic enzyme.

Example 8: Inhibition studies.

The test system described in example 2 is easily adapted to screen compounds for specific inhibition of tryparedoxin I. As an example the inhibition of tryparedoxin peroxidase by S-modifying agents such as N-ethylmaleimide (NEM), iodoacetamide (IAM) and phenylarsine oxide (PAO) is described (Table 2). Tryparedoxin was preincubated in 50 mM Hepes, 1 mM EDTA, pH 7.6 with or without presumed reducing substrate ($T(SH)_2$), then reacted with inhibitors and activity was checked at 22°C essentially as described in example 2. Changes in molecular mass were determined by MALDI-TOF-MS (Fig. 5).

Table 2 Derivatisation scheme of tryparedoxin.

Protein	Pretreatment	Derivatisation	mass increment[b]	residual activity [%]
tryparedoxin (9.5 µM)	1.16 mM $T(SH)_2$; 15 min at 22°C	--	--	~100
	none[a]	3 mM IAM; 30 min at 22°C	4±3 (0)	83±5
	1.16 mM $T(SH)_2$; 15 min at 22°C	3 mM IAM; 30 min at 22°C	57±3 (57)[c]	0±5
		3 mM NEM; 30 min at 22°C	251±3 (250)[d]	24±5
		3 mM PAO; 30 min at 22°C	154±3 (152)[c]	n. d.[e]

Tryparedoxin was preincubated in 50 mM Hepes, 1 mM EDTA, pH 7.6 with presumed reducing substrates, then reacted with iodoacetamide (IAM), NEM or phenylarsine oxide (PAO). Changes in molecular mass were determined my MALDI-TOF-MS. Residual activity was measured at 22°C using 1 mM $T(SH)_2$ with 1.0 µM tryparedoxin peroxidase and 0.6 µM tryparedoxin.

[a] stored under non-reducing conditions

[b] values in brackets represent predicted mass increments

[c] one molecule of derivatising agent

[d] two molecules of derivatising agent

[e] inhibition reversible: activity regained within the timescale of the test

The disclosure comprises also that of EP 96 120 015.1 a copy of which is attached.

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Claims

1. A protein, **characterized** by its capability of transferring reductive equivalents of trypanothione ($T(SH)_2$) to a peroxiredoxin.

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 of the following sequence SEQ ID NO 1 or a part thereof or by a partial sequence of the following sequence SEQ ID NO 1 as probe especially a partial sequence of SEQ ID NO 1wherein the probe is characterized by a simple or double line above the sequence.

G K V L F F Y F S A S W C P P 15

1 GGS AAR CTR GTS TTC TTC TAC TTC TCC GCG AGC TGG TGC CCG CCG

C R G F T P O L I E F Y D K F 30

46 TGC GCG GGC TTC ACG CCG CAG CTG ATC GAG TTC TAC GAC AAG TTC

H E S K N F E V V F C S W D E 45

91 CAC GAG TCG AAG AAC TTC GAG GTT GTG TTC TGC ACG TGG GAC GAG

E E D G F R G Y F A K M P W L 60

136 GAG GAG GAC GGC TTT GCG GGC TAC TTC GCG AAG ATG CCG TGG CTT

A V P F A O S E A V O K L S K 75

181 GCG GTG CCG TTT GCG CAG AGC GAG GCG GTG CAG AAG CTG TCG AAS

H F N V E 80

226 CAS TTC AAC GTC GAG

4. A protein according to any of the preceding claims, **characterized** by a molecular weight of 15 to 19 kDa.

5. A protein comprising or having the amino acid sequence of SEQ ID NO 1 according to claim3.

6. A protein comprising or having the amino acid sequence of SEQ ID NO 2 (Fig. 3).

7. A protein according to any of the preceding claims, **characterized** by a WCPPC motif (Fig. 3) and catalyzing the reduction of protein disulphides, especially of a peroxiredoxin, especially of tryparedoxin peroxidase (TXNPx), by means of trypanothione ($T(SH)_2$).

8. A protein

(a) having the amino acid sequence SEQ ID NO 2 (figure 3 positions 1 to 150):

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```
    M   S   G   L   K   K   F   F   P   Y   S   T   N   V  14
1   ATG TCA GGG CTG AAG AAG TTC TTC CCT TAC AGC ACA AAC GTG
    L   K   G   D   A   A   D   I   A   L   P   S   L   A  28
43  CTG AAG GGT GCT GCT GCG GAT ATC GCG CTC CCC TCG CTG GCG
    G   K   T   V   F   F   Y   F   S   A   S   W   C   P  42
85  GGC AAG ACC GTA TTC TTC TAC TTC TCC GCG AGC TGG TGC CCG
    P   C   R   A   F   T   P   Q   L   I   E   F   Y   K  56
127 CCG TGC CGG GCC TTC ACG CCG CAG CTG ATC GAT TTT TAC AAG
    A   H   A   E   K   K   N   F   E   V   M   L   I   F  70
169 GCC CAC GCG GAG AAG AAG AAC TTC GAG GTG ATG CTC ATC TCC
    W   D   E   S   A   E   D   F   K   D   Y   Y   A   K  84
211 TGG GAT GAG TCA GCA GAG GAC TTT AAG GAC TAC TAC GCG AAG
    M   P   W   L   A   L   P   F   E   D   R   K   G   M  98
253 ATG CCG TGG CTG GCA TTG CCG TTT GAA GAC CGC AAA GGG ATG
    E   F   L   T   T   G   F   D   V   K   S   I   P   T 112
295 GAG TTC TTG ACG ACC GGC TTC GAT GTG AAG TCG ATC CCA ACC
    L   V   G   V   E   A   D   S   G   N   I   I   T   T 126
337 TTG GTG GGC GTC GAG GCG GAC TCG GGA AAC ATC ATC ACA ACG
    Q   A   R   T   M   V   V   K   D   P   E   A   K   D 140
378 CAG GCG CGT ACG ATG GTG GTG AAG GAC CCG GAA GCA AAG GAT
    F   P   W   P   N   V   E   A   K   K   *             150
421 TTT CCG TGG CCC AAC GTG GAG GCC AAG AAG TAA
```

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 or larger number of amino acids and is encoded by an oligonucleotide which is hybridizable (at 1 M NaCl concentration and a temperature of at least 25 °C) with an oligonucleotide which encodes a protein having the amino acid sequence SEQ ID NO 1 or SEQ ID NO 2.

9. A protein according to claim 8 (b) having an amino acid sequence which is homologous to said according to claim 8 (a) by at least 70% and especially at least 75% and/or differing in its number of amino acids from SEQ ID NO 2 by up to 30 and especially up to 25 and preferably up to 20 amino acids.

10. A plasmid for the expression of a protein according to any of the preceding claims and comprising a nucleic acid encoding said protein.

11. A plasmid according to claim 10, containing a DNA sequence encoding a protein according to claim 8 (tryparedoxin = TXN) especially of *Crithidia fasciculata.*

12. A plasmid for the exprcssion of a functionally active derivative of a protein according to claim 8 (tryparedoxin = TXN) designed for an isolation in a manner known per se, wherein the plasmid contains a DNA sequence which encodes said derivative.

13. A plasmid according to claim 12, containing a DNA sequence encoding a functionally active derivative of a protein according to claim 8 (tryparedoxin = TXN) wherein the tryparedoxin is derivatised by a His tag.

14. A process for the production of a protein according to any claims 1 to 9, **characterized** in that it is produced by genetic engineering in a manner known per se by means of an oligonucleotide of sequence SEQ ID NO 1 or any part thereof or by means of a partial sequence of sequence SEQ ID NO 1 as probe according to claim 3.

15. A process for the production of a protein according to any claims 1 to 9, **characterized** in that the production is carried out by means of a plasmid according to any of claims 10 to 13.

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16. A process according to 15, **characterized** in that the host is selected from the group consisting of bacteria, fungi, yeast, plant cells, insect cells, mammalian cells or cell cultures (heterologous expression).

17. A process according to 15, **characterized** in that *Escherichia coli* is used as host.

18. Use of a protein according to any of claims 1 to 9 or obtained according to the process according to any of the claims 14 to 17 for testing and recovering inhibitory substances which inhibit activities of said protein.

19. A test system for testing the catalytic activity of a protein according to any of the claims 1 to 9 or obtained according to the process according to any of claims 14 to 17, wherein the testing system contains or comprises trypanothione ($T(SH)_2$), trypanothione reductase (TR), a tryparedoxin peroxidase (TXNPx), the protein the activity of which is to be tested and, in addition, a hydroperoxide as indicator enzyme, mediator and substrate, respectively.

20. 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 9 or of a protein which can be obtained according to any of claims 14 to 17.

21. A pharmaceutical preparation according to claim 20, **characterized** in that it can be obtained by a use according to claim 17 or 18 and/or by using a test system according to claim 19.