J Parasit Dis
DOI 10.1007/s12639-012-0138-8

ORIGINAL ARTICLE

J P D

Purification and characterization of glutathione reductase (E.C. 1.8.1.7) from bovine filarial worms *Setaria cervi*

Kavita Arora · Rumana Ahmad · Arvind K. Srivastava

Received: 28 April 2012 / Accepted: 15 June 2012

Abstract Antioxidant enzymes are the parasite's premier resource to defend themselves against reactive oxygen species generated by macrophages, neutrophils and eosinophils of the host. These enzymes may be particularly important for parasites involved in chronic infections, such as parasitic helminths. Glutathione (GSH) and glutathione reductase (GR) are parts of the GSH redox cycle, which protects cells against damage by oxidants. Both GSH and GR are present in significant amounts in *Setaria cervi* female worms. GR has a central role in glutathione metabolism and as such is a potential target for chemotherapy. The aim of the work was to purify and characterize GR from *S. cervi* and to compare the properties of the helminth enzyme with its mammalian counterpart. GR was purified from filarial parasites *S. cervi* and preliminary steady state kinetics was performed. The purified protein was observed to be a dimer of 55 kDa subunit as evident from SDS-PAGE analysis. Kinetic studies revealed significant differences in the properties of *S. cervi* GR from its mammalian counterpart which may be exploited in chemotherapy of filariasis. Filarial GR is thus proposed as a potential drug target.

Present Address:
K. Arora
K&S Partners, Intellectual Property Attorneys, 109,
Sector 44, Gurgaon 122 003, National Capital Region, India
e-mail: kavita@knspartners.com

K. Arora · R. Ahmad · A. K. Srivastava (✉)
Division of Biochemistry, Central Drug Research Institute,
P.O. Box No. 173, Chattar Manzil Palace, Lucknow 226 001,
UP, India
e-mail: drarv1955@yahoo.com

R. Ahmad
e-mail: rumana_ahmad@yahoo.co.in

Keywords Enzyme inhibition · Glutathione reductase · Helminth parasites · Purification · *Setaria cervi*

Introduction

Lymphatic filariasis, a disease caused by filarial worms, is a major public health and socioeconomic problem in most of the developing countries of south-east Asia. Worldwide, about 120 million people are infected with *Wuchereria bancrofti* and *Brugia malayi*. The mainstay of filarial control is chemotherapy, and this is likely to remain so the foreseeable future. The treatment of filariasis consists of chemotherapy directed against the adult worms (macrofilaricidal) and against the microfilariae (microfilaricidal) combined with symptomatic treatment to relieve the damage caused by the body's immunological reaction to dead and dying worms. Control of filariasis remains disappointing due to lack of appropriate one-shot chemotherapeutic agents capable of eliminating adult parasites (Ottesen and Ramchandran 1995). Therefore, an antifilarial drug is required which can kill the adult parasites slowly, has microfilaricidal activity and at the same times sterilize the female worms.

The tripeptide glutathione (GSH) has been identified as an important part of the antioxidant system of filarial worms to protect them from the oxidative stress created by the host. The significance of GSH and related metabolism to filariae has been indicated by the finding that sustained severe reduction in endogenous GSH pools within adult filariae accelerated a drop in microfilarial output and eventually led to the death of the parasites. It has been shown that interference with the filarial GSH metabolism has curative effect on filarial infections in mice (Bhargava et al. 1983). The finding that filariae in vitro are also able to salvage exogenous GSH, unlike most, if not all, mammalian cell

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138 LE TYPESET
MS Code : CP DISK

populations, points to a promising target for selective inhibition. In view of published findings (Bhargava et al. 1983; Meister and Anderson 1983) suggesting that maintenance of a characteristic and relatively high intracellular ratio of reduced to oxidized glutathione (GSH/GSSG) is vital to adult filariae, the enzymes involved in its de novo synthesis (glutamate cysteine ligase), and its replenishment (glutathione reductase, GR) can thus be harnessed as important chemotherapeutic targets.

The flavoenzyme GR (glutathione: $NADP^+$ oxidoreductase, GR, E.C. 1.8.1.7), is the central enzyme of antioxidative defence and catalyzes the regeneration of the reducing tripeptide Glutathione (GSH) from its disulfide GSSG

$$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH$$

GR creates an intracellular GSH/GSSG ratio of 20–1,000 depending on the respective metabolic conditions (Schirmer and Schulz 1987). A high GSH/GSSG ratio is essential for protection against oxidative stress (Carlberg and Mannervik 1985). Due to its central role in cellular redox metabolism, inhibition of the GR from the parasites represents an important approach to antiparasitic drug development. If the enzyme from host and parasite differ in their susceptibility to inhibitors, it will be of practical relevance. The wide distribution of reduced glutathione (Jocelyn 1972) reflects the general importance of GR.

GR is present in numerous micro-organisms, protozoa, plants and higher animals (Krauth-Siegel et al. 1982; Tutic et al. 1990; Collinson and Dawes 1995; Stevens et al. 1997). The protein is a homodimer that possesses two identical subunits, each containing 1 FAD and 1 redox-active disulfide/dithiol as components of the catalytic apparatus (Schirmer et al. 1989; Deonarain et al. 1990; Argyrou et al. 2004). The human protein has been isolated from erythrocytes (Worthington and Rosemeyer 1974) and its three dimensional structure determined (Schulz et al. 1975; Karplus and Schulz 1987). The structures and the reaction mechanisms of human enzyme and *E. coli* GR have been intensively examined (Schirmer et al. 1989; Mittl and Schulz 1994; Perham et al. 1996). The crystallographic analysis of GR clarifies the construction of the protein from four different domains: an N-terminal FAD binding domain, the subsequent nicotinamide adenine dinucleotide phosphate (NADPH) binding domain, the central domain as well as interface domain responsible for dimerization (Pai et al. 1988; Karplus and Schulz 1989; Perham et al. 1996; Savvides et al. 2002).

GR has been purified from many different sources such as rat liver (Carlberg and Mannervik 1975; Carlberg et al. 1981), calf liver (Carlberg and Mannervik 1981), gerbil liver (Le Trang et al. 1983), human erythrocytes (Worthington and Rosemeyer 1974; Krohne-Ehrich et al. 1977), bovine erythrocytes (Erat et al. 2003), porcine erythrocytes (Boggaram et al. 1979), sheep brain (Açan and Tezcan 1989), sheep liver (Erat and Ciftci 2003), rodent malaria parasite *Plasmodium berghei* (Kapoor et al. 2008) and some of its characteristic properties have been determined. With few exceptions, GR is a homodimeric protein with subunits of about 55 kDa (Worthington and Rosemeyer 1975; Halliwell and Foyer 1978; Libreros-Minotta et al. 1992).

GSH and GR have been analyzed and detected in many filarial species viz. *Acanthocheilonema viteae*, *Litomosoides carinii* and *Setaria cervi* (Singh et al. 1997; Gupta et al. 2002). The *O. volvulus* GR (*Ov*GR) gene has been cloned and sequenced (Müller et al. 1997). GRs from two cattle filariae (*S. digitata* and *O. gutturosa*) have been isolated and their properties have been compared to those of human erythrocyte GR. Although their physical and kinetic properties are very similar, studies on the inhibition of the enzymes by the trivalent melaminophenyl arsenical melarsen oxide revealed that human GR is less susceptible to inhibition by the arsenical than the filarial enzymes (Müller et al. 1995). Moreover, the mechanism of inhibition was found to be different in host and filarial enzymes, competitive inhibition was found in the case of human enzyme where as filarial GRs were inhibited in two stages: an immediate partial inactivation followed by a time-dependent stage with saturable pseudo-first-order kinetics.

The present work describes the isolation and purification of native GR from adult females *S. cervi* and subsequent biochemical and kinetic studies on the native enzyme that reveal significant differences between the parasitic and mammalian enzyme. These differences between host and parasite enzyme might reflect differences in the primary and secondary structure of the proteins that may be exploited for the design of new specific macrofilaricidal drugs (Müller et al. 1995).

Materials and methods

Chemicals

Oxidized and reduced glutathione (GSSG and GSH), NADPH, Tris, sodium L-glutamate, phenyl methyl sulfonyl fluoride (PMSF), trisodium citrate, EDTA, ammonium sulfate, sodium chloride (NaCl), Sepharose 6B, cyanogen bromide (CnBr), ethanolamine, sodium borate, potassium chloride (KCl), sodium azide, GR, dithiothreitol (DTT), sodium sulfate, and BSA were purchased from Sigma Chemical Co., USA. All reagents used in electrophoresis and activity staining were purchased from Sigma Chemical Co., USA. 2′,5′ ADP was purchased from Sigma Chemical Co., USA. Folin Ciocaltaeu's phenol reagent and 2,6-dichlorophenol indophenol (DCIP) were purchased from Sisco Research Laboratories, Mumbai, India. Carboxymethyl-Sepharose (CM-Sepharose) and diethylaminoethane-

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138 LE TYPESET
MS Code : CP DISK

Sepharose (DEAE-Sepharose) were purchased from Amersham Pharmacia and Hanks balanced salt solution (HBSS) was from Himedia Laboratories Pvt. Ltd. Mumbai, India. All other chemicals used were of analytical grade.

Biological materials

Collection of filarial worms S. cervi

Adult bovine *S. cervi* females of average body weight 35 ± 5 mg and length 6.0 ± 1.0 cm were collected from the peritoneal cavity of freshly slaughtered naturally infected water buffaloes *Bubalus bubalis* (Linn.) at a local abattoir. They were thoroughly washed with saline and kept in HBSS with sodium bicarbonate and glucose (5.55 mM) for 60 min at 37 °C in Dubnoff metabolic shaker for complete revival before being used in enzymatic studies.

Preparation of GR from filarial worms S. cervi

A 10 % homogenate of actively motile bovine filarial worms was prepared in Tris–HCl buffer, 50 mM, pH 7.5 containing 1.0 mM glutamate, 5.0 mM $MgCl_2$ and 0.1 mM PMSF using a Potter Elvehjem glass homogenizer fitted with a Teflon pestle. Glutamate was added to stabilize the enzyme. The homogenate was centrifuged at 1,000×*g* for 15 min, 10,000×*g* for 30 min, and subsequently at 100,000×*g* for 60 min to obtain mitochondrial, post mitochondrial and cytosolic fractions, respectively.

GR activity determination

GR activity in various dialyzed fractions obtained as a result of subcellular fractionation was determined spectrophotometrically at 340 nm (Carlberg and Mannervik 1985). All enzyme kinetics experiments were carried out at room temperature (37 °C) using a Shimadzu double beam UV spectrophotometer. Enzyme activity was spectrophotometrically monitored from the rate of conversion of NADPH to NADP ($e_{340} = 6.2\ mM^{-1}\ cm^{-1}$). For each assay the absorbance of NADPH in the reaction mixture was measured over a period of 5 min. Enzyme aliquots were preincubated in the presence of NADPH for 5 min in order to trace the non-enzymatic oxidation, after which GSSG was added to start the reaction. To correct for non-specific oxidation of NADPH, the blank cuvette contained all the assay components except GSSG.

A unit of enzyme activity was expressed as the amount that catalyzes the consumption of 1 µmol of substrate per minute, using a molar extinction coefficient of 6.2 $mM^{-1}\ cm^{-1}$ for NADH/NADPH.

Protein estimation

Protein was estimated in the crude homogenate/sub-cellular fractions using BSA as standard (Lowry et al. 1951).

Biochemical and functional characterization of GR from *S. cervi*

Ammonium sulfate precipitation

The cytosolic fraction was subjected to ammonium sulfate fractionation. For the goal, the fraction was brought to a final concentration of 0–80 % saturation with solid ammonium sulfate. All the samples were analyzed for GR activity, which was found enriched in the 65–80 % ammonium sulfate saturated fraction. This fraction was stored at 4 °C and used as the source of GR.

Ultrafiltration

For further purification of GR, the 65–80 % ammonium sulfate saturated fraction was concentrated by ultrafiltration. For this, precipitate obtained after ammonium sulfate cut was dissolved in minimum amount of homogenization buffer and was then centrifuged through a microcon centrifugal filter device fitted with a 30 kDa molecular mass cut-off filter at 12,000×*g* for 15 min at 4 °C. The retentate obtained was divided into two batches of (2.5 ml each) and each of them was subjected to ion-exchange chromatography.

Ion-exchange chromatography

Solvents A (0.05 M citrate buffer, pH 5.5) and B (0–3.0 M NaCl in 0.05 M citrate buffer, pH 5.5) were degassed for 5 min. For cation-exchange chromatography, CM-Sepharose preswollen in 20 % ethanol was prepared by decanting 20 % ethanol solution and replacing it with starting buffer (0.05 M citrate buffer pH 5.5). The CM-Sepharose slurry was degassed and packed in a glass column upto a vertical height of 12 cm. About two bed-volumes of citrate buffer were passed through the column for equilibration. Three milliliters of 65–80 % ammonium sulfate precipitate from *S. cervi* was loaded on exchanger bed. The flow rate was adjusted between 10 and 15 ml/h (0.3 ml/min) and 1.0 ml fractions were collected in different tubes. The column was subsequently washed with citrate buffer and 1.0 ml fractions were collected in different tubes. Finally, the bound proteins were eluted by applying a linear gradient of NaCl from 0 to 3.0 M in starting buffer.

For anion-exchange chromatography, the above procedure was repeated using DEAE-Sepharose as anion exchanger

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138 LE TYPESET
MS Code : CP DISK

and replacing solvent A with 0.02 M Tris buffer, pH 8.0 and solvent B with 0–1.0 M NaCl in 0.02 M Tris buffer, pH 8.0. The bound proteins were eluted by applying a linear gradient of NaCl from 0 to 1.0 M in starting buffer.

GSH–Sepharose affinity chromatography

Coupling of ligand to Sepharose

The GSH affinity column was prepared according to published procedure (Simons, and Vander Jagt 1977). The coupled gel was washed with 100 ml of water and the remaining active groups were blocked by allowing the gel to stand in 1.0 M ethanolamine for 4 h.

Chromatographic procedures

The affinity column was used at 4 °C. The protein sample was applied to the GSH–Sepharose affinity column (15 ml volume), previously washed with 0.5 M KCl and equilibrated with buffer A (10 mM Tris–HCl, 1.0 mM EDTA and 3.0 mM DTT, pH 8.0) at a flow rate of 30 ml/h. After sample application, the column was sequentially washed with buffer B (10 mM Tris–HCl, 1.0 mM EDTA, 3.0 mM DTT and 0.7 M NaCl, pH 8.0) and buffer C (10 mM Tris–HCl, 1.0 mM EDTA, 3.0 mM DTT and 10 mM GSH, pH 8.0). Fractions of 2.0 ml each were collected and tested for conjugation activity towards NADPH and absorbance at 280 nm.

ADP–Sepharose affinity chromatography

Coupling of ligand to Sepharose

Sepharose 4B was activated by the CnBr method (Axén et al. 1967), using a 1 M buffered solution of potassium carbonate (pH 11) and 10 g CnBr/100 ml gel. The coupled gel was exhaustively washed with 0.1 M $NaHCO_3$–1.0 M NaCl and stored in 50 % glycerol and 0.1 % sodium azide (4 °C) until use.

Chromatographic procedures

The ADP–Sepharose affinity column was used at 4 °C. The protein sample in 50 mM potassium phosphate buffer containing 1.0 mM EDTA, pH 6.0 (buffer A) was loaded onto the column and washed with 25 ml 0.1 M potassium phosphate, pH 6.0 and 25 ml 0.1 M potassium phosphate, pH 7.85 (buffer B). Bound proteins were then eluted with two column volumes of 50 mM potassium phosphate buffer containing 1.0 mM EDTA, pH 6.0, 0.5 mM GSH and 1.0 mM NADPH (buffer C). Fractions of 1.0 ml each were collected and tested for activity. The peak of activity determined by conjugation activity towards NADPH and absorbance at 280 nm was collected and active fractions were pooled and concentrated by ultrafiltration.

Electrophoresis

SDS-PAGE

SDS-PAGE was performed on a 12 % gel according to the method of Laemmli (1970). All reagents for SDS-PAGE were prepared and stored under specified conditions. The following molecular mass standards were used: trypsinogen (24 kDa), glyceraldehyde-3-phosphate (36 kDa), ovalbumin (45 kDa), glutamic dehydrogenase (55 kDa), BSA (66 kDa), and β-galactosidase (116 kDa).

Activity staining

Native PAGE was performed on a 7.5 % gel according to the method of Laemmli (1970). The activity staining method for GR (Okpodu and Waite 1997) is based on the fact that GSH can reduce DCIP. Following electrophoresis, native gels were removed from glass plates with water, and assayed for GR activity by incubating in 25 mM Tris–Cl (pH 7.6), 3.4 mM GSSG, 0.4 mM NADPH, 1.2 mM MTT and 0.04 mM DCIP for 25 min at room temperature after which purple insoluble formazan appeared on the gel surface at points where GR activity was present.

Kinetic and inhibition studies

All kinetic and inhibition studies were carried out using dialyzed preparations of enzyme to avoid interference due to ammonium sulfate and Tris–HCl. The K_m value of native GR for substrate GSSG was determined using varying GSSG concentrations and a fixed NADPH concentration of 1.0 mM. The K_m value for substrate NADPH was determined using varying substrate concentrations and a fixed GSSG concentration of 1.0 mM. Data were plotted as double reciprocal Lineweaver–Burk plots to determine the apparent K_m values.

Inhibition of GR activity by the products of the GR reaction i.e. NADP and GSH was studied by adding them directly in separate assay systems 10 min prior to addition of the substrate. For determination of dose-dependent effects of NADP and GSH and their respective inhibitor constants (K_i), the purified enzyme was incubated with varying concentrations of each inhibitor for 10 min at room temperature in presence of 100 mM potassium phosphate buffer, pH 7.0.

For determining the type of inhibition, enzyme was incubated with varying concentrations of inhibitors at different substrate concentrations for 10 min at room

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138 LE TYPESET
MS Code : CP DISK

temperature in the presence of 100 mM potassium phosphate buffer, pH 7.0. Percent inhibition of enzyme activity was calculated as follows:

$$\text{Percent inhibition} = \frac{\text{Absorbance/min in the absence of inhibitor} - \text{absorbance/min of experimental tube}}{\text{Absorbance/min in the absence of inhibitor}} \times 100$$

Statistical analysis

Results were expressed in terms of mean ± standard deviation (SD) based on all experiments performed in either triplicates or quadruplicates.

Results

GR localization and characterization

Setaria cervi GR activity was found to be maximal in the cytosolic fraction. The specific activity profile of *S. cervi* cytosolic fraction showed enrichment of GR activity in 65–85 % ammonium sulfate fraction. GR, which was purified around fivefold in the ammonium sulfate fraction, was further enriched to around ninefold by ultrafiltration.

GR activity was found nearly 1.3-fold enriched on CM-Sepharose column (Fig. 1), and nearly 1.8-fold enriched on DEAE-Sepharose column (Fig. 2).

When partially purified GR was applied to GSH–Sepharose 6B affinity matrix, the enzyme activity eluted with buffer B alone, indicating that GR did not bind to the GSH–Sepharose column. On the other hand, when partially purified GR was applied to 2′5′-ADP–Sepharose 6B affinity matrix, the enzyme activity eluted with 1.0 mM NADPH in phosphate buffer, and was found enriched around 37.7-fold (Fig. 3). This purification procedure delivered a highly purified enzyme, exhibiting a single band on SDS-PAGE (Fig. 4a). The enzyme was found to retain its biological activity and activity staining was also detected when PAGE was done under non-denaturing conditions on a 7.5 % gel (Fig. 4b).

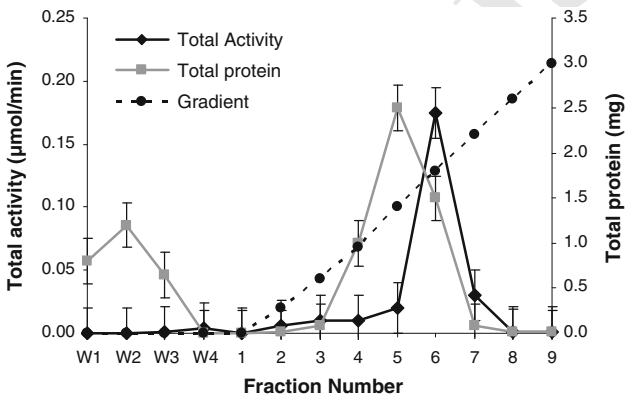

Fig. 1 Elution profiles for total protein and total activity of *S. cervi* GR from CM-Sepharose column. *W1–W4* 1.0 ml fractions after washing the column with citrate buffer, *1–9* 1.0 ml fractions (proteins) eluted by applying a linear gradient of NaCl from 0 to 3.0 M in starting buffer. Activity was expressed as µmol NADP released/min ± SD based on experiments done in quadruplicates

Stability

The purified enzyme was found to be fairly stable at 0–4 °C, and there was no appreciable effect of freezing and thawing on enzyme activity for approximately a month.

Effect of pH

The pH profile of *S. cervi* GR was determined with NADPH as cofactor. In order to determine the optimum pH, phosphate and Tris–HCl buffers were used within the pH range of 5.7–8.0 and 8.5–10.0, respectively; the results

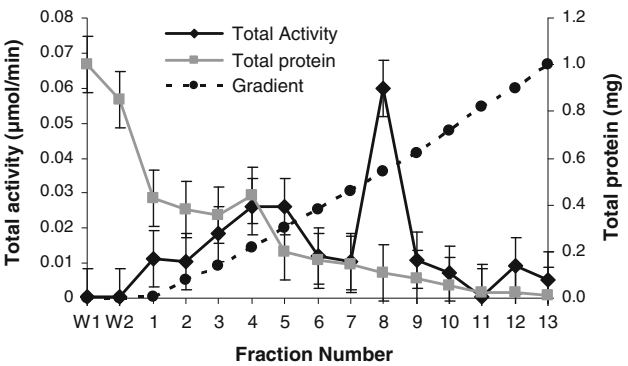

Fig. 2 Elution profile for total protein and total activity of *S. cervi* GR from DEAE-Sepharose column. *W1–W2* 1.0 ml fractions after washing the column with Tris buffer, *1–13* 1.0 ml fractions (proteins) eluted by applying a linear gradient of NaCl from 0 to 1.0 M in starting buffer. Activity was expressed as µmol NADP released/min ± SD based on experiments done in quadruplicates

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138 LE TYPESET
MS Code : CP DISK

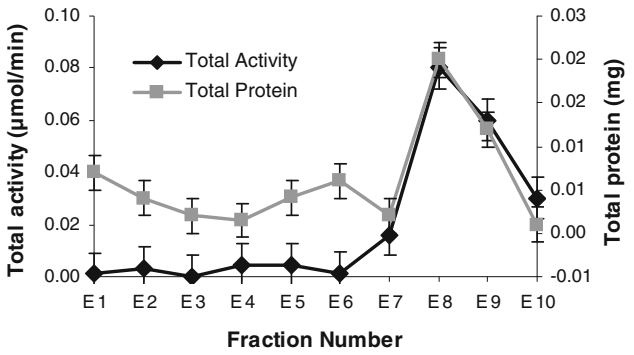

Fig. 3 Elution profiles for total protein and total activity of *S. cervi* GR from ADP–Sepharose column. *E1–E10* 1.0 ml fractions collected by applying 50 mM potassium phosphate buffer containing 1 mM EDTA, pH 6.0, 0.5 mM GSH and 1.0 mM NADPH. Activity was expressed as µmol NADP released/min ± SD based on experiments done in quadruplicates

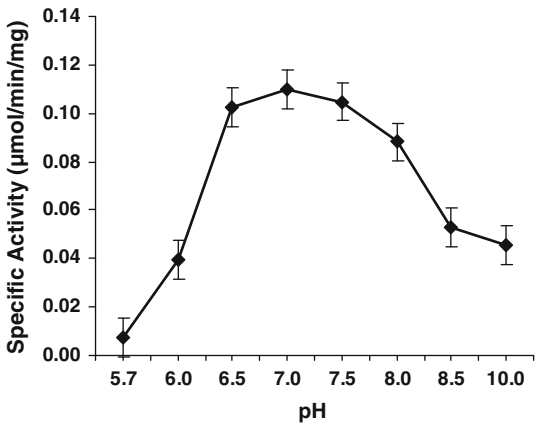

Fig. 5 Effect of pH of the assay buffer on *S. cervi* GR activity. Specific activity was expressed as µmol NADP released min^{-1} mg^{-1} protein ± SD based on experiments done in quadruplicates

showed a broad pH optimum centered around pH 7.0 (Fig. 5).

Effect of buffer strength

Setaria cervi GR activity increased linearly with increasing concentration of phosphate buffer with maxima at 100 mM (Fig. 6a).

Effect of Salts

The monovalent salts like NaCl, KCl, and NH_4Cl caused an inhibition in enzyme activity when added in the assay system in 0.1–1.0 M concentration range (Fig. 6b).

Kinetic studies

The reaction catalyzed by cytosolic GR from *S. cervi* was found to be fairly linear with respect to time (30 s to 5 min) and amount of enzyme protein (4.23–42.3 µg). GR from *S. cervi* displayed Michaelis–Menten behavior with regards to the standard substrate CDNB and co-substrate GSH. Reciprocal plots of 1/v versus 1/[S] gave the kinetic parameters relating to one substrate in presence of saturating concentration of the second substrate. The K_m values of native *S. cervi* GR with respect to NADPH and GSSG were found to be 0.019 and 0.047 mM respectively. The K_m of the purified enzyme with respect to NADPH (Fig. 7a) and GSSG (Fig. 7b) showed little difference from that obtained with the crude enzyme (0.021 and 0.05 mM respectively).

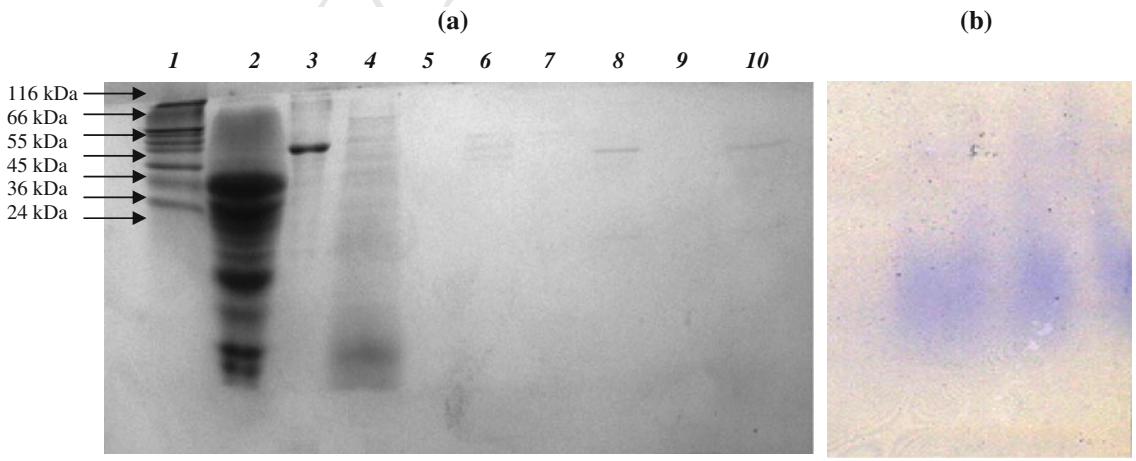

Fig. 4 **a** SDS-PAGE analysis of purified GR from *S. cervi* on 12 % gel. *Lane 1* marker, sigma, *Lane 2* ovalbumin (45 kDa), *Lane 3* BSA (66 kDa), *Lane 4* cytosolic fraction, *Lane 6* sample obtained from ultrafiltration, *Lanes 8*, *10* purified GR from *S. cervi* (obtained by elution from 2′5′-ADP–Sepharose 4B affinity column). **b** Activity staining of purified GR from *S. cervi* on 7.5 % gel

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138
MS Code :
☐ LE ☑ CP ☐ TYPESET ☑ DISK

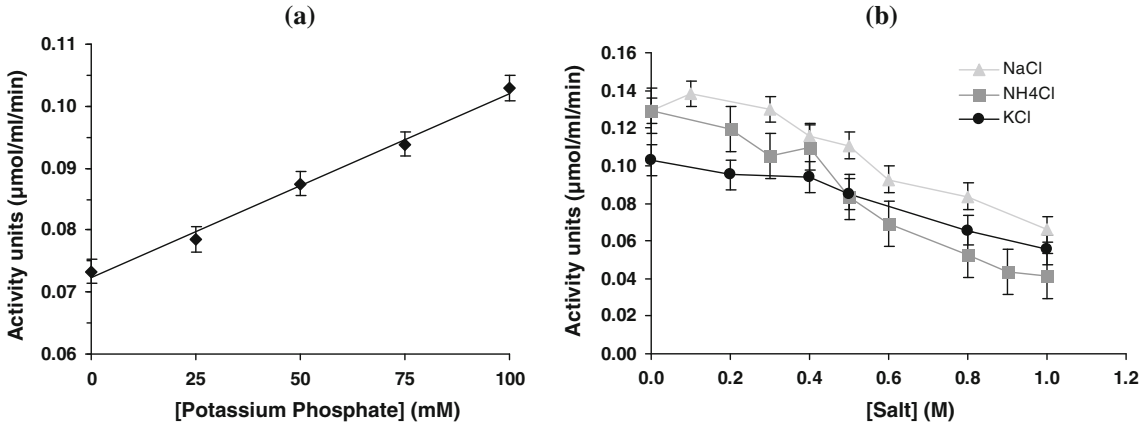

Fig. 6 Effect of potassium phosphate (**a**) and various salts (**b**) on purified *S. cervi* GR activity

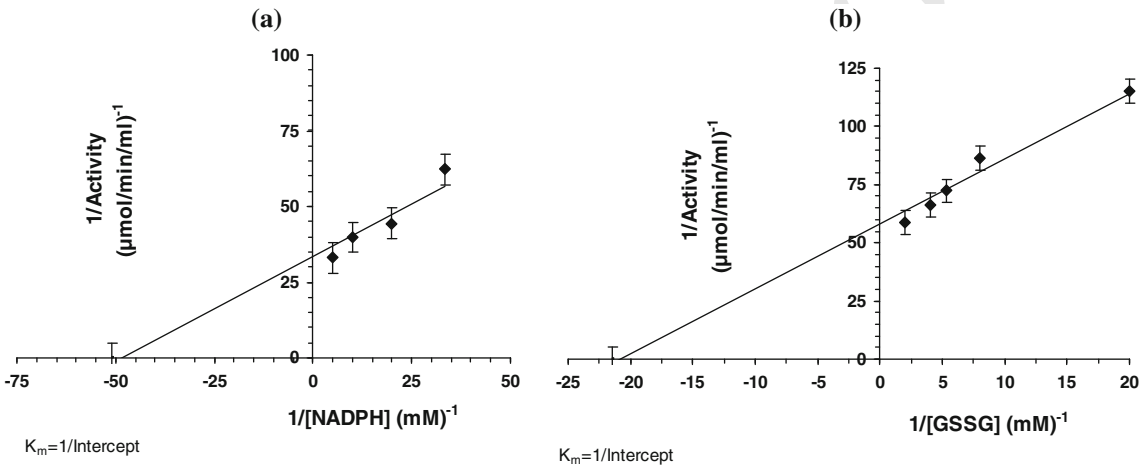

Fig. 7 Lineweaver–Burk double reciprocal plots for K_m of purified GR from *S. cervi* with respect to NADPH (**a**) and GSSG (**b**). Activity was measured in terms of µmol NADP released/min/ml ± SD based on experiments done in triplicates

Inhibition studies

The K_i values obtained for product inhibition by NADP (0.03 mM) and GSH (5.9 mM), however, were slightly lower than those for the crude enzyme (0.048 and 6.8 mM respectively). From the reciprocal plots, the inhibition by NADP was clearly competitive with respect to NADPH and non-competitive with respect to GSSG (Fig. 8). The other product of the GR reaction i.e. GSH inhibited the enzyme in a non co-operative manner.

Discussion

The present study describes the purification and characterization of GR from adult filarial worms *S. cervi*. The enzyme was purified by a combination of salt fractionation, ion exchange and affinity chromatography and further characterized by gel electrophoresis. Activity Staining revealed the presence of a single isoenzyme of GR in *S. cervi*. The enzyme was purified 37.7-fold and with a yield of 42.0 %. Enzyme activity was affected by buffer concentration and the presence of monovalent salts as reported for the enzyme from yeast (Moroff and Brandt 1975) and the cestode *Moniezia expansa* (McCallum and Barrett 1995). The pH optimum was found to be 7.0 and the K_m values with respect to GSSG and NADPH were found to be 0.047 and 0.019 mM respectively. The parasitic enzyme has been found to be inhibited by a variety of compounds including arsenic derivatives, 2,4,6 trinitrobenzene sulfonate 1,3-bis(2-chlorethyl)-1-nitrosourea, 1-chloro-2,4-dinitrobenzene, methylene blue and oxidized glutathione (results not shown).

The substrate specificity and inhibition profile appears to be similar to GR from mammalian sources (Carlberg and Mannervik 1985). The results indicate that for both the enzymes, the affinity towards NADPH is higher than GSSG. Results are in agreement with those reported for GR

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138
MS Code :
LE TYPESET
CP DISK

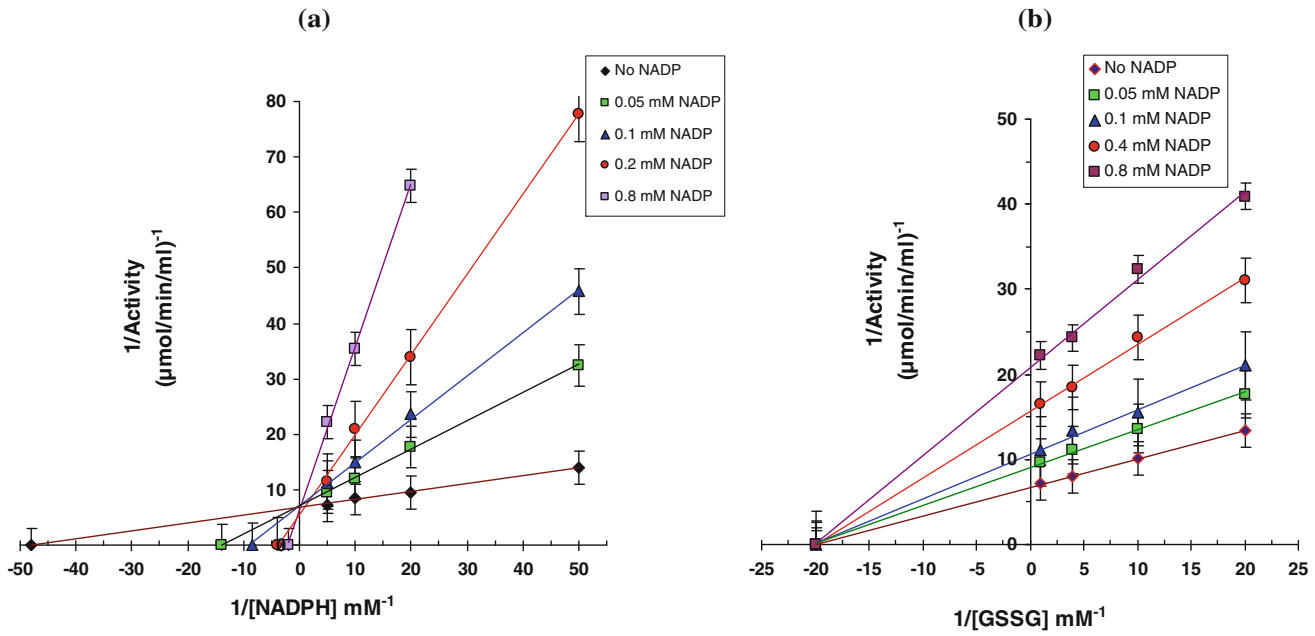

Fig. 8 Pattern of inhibition of NADP on purified GR from *S. cervi* with respect to NADPH (**a**) and GSSG (**b**)

Table 1 Comparison of properties of GR from human erythrocytes, *P. falciparum*, *P. berghei*, *C. elegans*, *O. volvulus*, *S. digitata* and *S. cervi*

	Human RBCs[a]	Human RBCs[b]	*P. falciparum*[b]	*P. berghei*[c]	*C. elegans*	*O. volvulus*[a]	*S. digitata*[a]	*S. cervi*
Subunit molecular mass (kDa)	50	52.4	55.5	25	50	54	51	55
Native molecular mass (kDa)	98	105	111	ND	100	110	97	110
Specific activity (U/mg)	246	240	204	0.617 ± 0.08	68 ± 3[d]	78 ± 3	27.3	0.0362 ± 0.0058
K_m GSSG (µM)	58 ± 2.1	65	69 ± 2	20	34.1 ± 4[d]	130.7 ± 13.0	24.0 ± 0.24	46.5 ± 5
K_m NADPH (µM)	9.6 ± 0.2	8.5	4.8 ± 0.4	ND	12.9 ± 1.2[d]	10.9 ± 2.4	ND	19 ± 5

ND not determined

[a] Data obtained from Müller et al. (1995)

[b] Data obtained from Krauth-Seigel et al. (1996)

[c] Data obtained from Kapoor et al. (2008)

[d] Data shown represent mean ± SE of four independent experiments

from other sources (Krohne-Ehrich et al. 1977; Carlberg and Mannervik 1981; Lamotte et al. 2000). The K_m values obtained with respect to the substrates GSSG and NADPH i.e. 0.047 and 0.019 mM, respectively for *S. cervi*, were different from known values for calf liver (0.10 and 0.021 mM), rat liver (0.026 and 0.008 mM) and human erythrocytes (0.065 and 0.0085 mM). Ulusu et al. (2005) reported K_m value of 0.02 mM for GSSG and 0.025 mM for NADPH while V_{max} was 0.026 and 0.027 mM for GSSG and NADPH respectively in sheep liver GR.

Product inhibition of GR by NADP is in agreement with previous work on this enzyme (Scott et al. 1963; Staal and Veeger 1969; Worthington and Rosemeyer 1976). The *S. cervi* enzyme had a lower K_i (0.03 mM) for NADP from the competition with NADPH, as compared to that obtained for GR of human erythrocytes (0.07 mM).

The enzyme was a homodimer of native molecular mass 110 kDa, subunit mass 55 kDa. These results correspond well with data for purified GR from human erythrocytes (Schirmer et al. 1989; Tutic et al. 1990; Becker et al. 1991; Müller et al. 1995), mouse liver (Lopez-Barea and Lee 1979), calf brain (Gutterer et al. 1999), fish (Speers-Roesch and Ballantyne 2005), *O. volvulus* (Müller et al. 1997), as well as *S. digitata* (Müller et al. 1995), where the protein has been found to have an apparent molecular mass of 97 kDa as shown in Table 1. The protein was found to be active and stable at 0 °C. The specific activity of the protein was found to be 0.0362 ± 0.0058 µmol min^{-1} mg^{-1} protein in the cytosolic fraction. It appears to be lower than that of *Ov*GR (78 µmol min^{-1} mg^{-1}) and *Hs*GR (240 µmol min^{-1} mg^{-1}), indicating a lower efficiency of catalysis for the nematode enzyme (Müller et al. 1997). The *Sc*GR activity

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138
MS Code :
☐ LE ☑ CP ☐ TYPESET ☑ DISK

was also found to be three times lower than the purified native protein of *S. digitata* and *Ascaris suum* GR which have specific activities of 24 and 32 μmol min^{-1} mg^{-1} protein, respectively. Regarding its substrates GSSG and NADPH, the purified GR displayed Michaelis–Menten behavior. Compared with *Hs*GR, the affinity of *Sc*GR to the substrate GSSG is slightly higher (46.5 μM compared to 58 μM) and much higher than *Ov*GR (130 μM). With respect to substrate binding, the K_m value of the *Sc*GR domain for NADPH is within the range of values seen with K_m values of other GRs: 19.0 μM for *Sc*GR, 9.6 μM for *Hs*GR and 10.9 μM for *Ov*GR.

Setaria cervi adult females as well as microfilariae (mf) have been found to possess an active antioxidant system. GR, glucose-6-phosphate dehydrogenase and glutathione-S-transferase have been reported to be present in low amounts, while gamma-glutamyl transpeptidase showed significant activity. These enzymes activities were reported to be higher in mf stage as compared to adult parasite.

The thioredoxin and glutathione antioxidant systems play a central role in thiol-disulfide redox homeostasis in many organisms by providing electrons to essential enzymes, and defence against oxidative stress. These systems have recently been characterized in platyhelminth parasites, and the emerging biochemical scenario is the existence of linked processes with the enzyme thioredoxin–GR supplying reducing equivalents to both pathways (Maggioli et al. 2004). Analysis of published data and expressed-sequence tag databases indicates the presence of linked thioredoxin–glutathione systems in the cytosolic and mitochondrial compartments of platyhelminths (Salinas et al. 2004).

Parasitic helminths have a coexistence with mammalian hosts whereby they survive for several years in known hostile conditions of their hosts. Many explanations exist describing how these parasitic helminths are able to survive. In the last years, a lot of studies have focused on both enzymatic and non-enzymatic antioxidant systems now shown to exist in these parasites and which may serve as defence tactics against the host-generated oxygen radicals (Chiumiento and Brushchi 2009).

Many parasites appear to be more sensitive to oxidative stress than their mammalian hosts (Krauth-Siegel et al. 1991). Clinical observations and experimental evidence suggest that oxidative stress plays a dominant role in the defence against parasitic infections (Schirmer et al. 1987; Fairlamb and Cerami 1985; Penketh and Klein 1986), and the participation of the redox systems of the parasite is critical to its survival in a hostile environment (Rendóna et al. 2004). In helminths, this is of particular interest with respect to protection against the respiratory burst of activated cells of the host's immune system and susceptibility to drugs whose mode of action involves the generation of free radicals (Brophy and Pritchard 1992). Due to its central role in cellular redox metabolism, inhibition of the GR of filarial worms represents an important target in the design and synthesis of antifilarial agents.

In conclusion the GR of *S. cervi* resembles the mammalian enzyme in its general physical properties and its substrate and inhibitor profile. However, the parasite enzyme appears to be more sensitive to inhibition by sodium ions as compared to the mammalian enzyme. It is well known that organic arsenicals have macro-filaricidal effects but are discarded because of their toxic effect to mammals. Other compounds targeting filarial GR are thus required. The central role of GR in maintenance of the thiol redox state and in anti-oxidative defense has to be evaluated in more detail in order to establish the essential function of this enzyme in the survival of the filarial parasite.

Acknowledgments Financial assistance from Council for Scientific and Industrial Research (CSIR), New Delhi (India) in the form of Senior Research Fellowships to KA and RA are gratefully acknowledged.

References

Açan NL, Tezcan EF (1989) Sheep brain glutathione reductase: purification and general properties. FEBS Lett 250:72–74

Argyrou A, Vetting MW, Blanchard JS (2004) Characterization of a new member of the flavoprotein disulfide reductase family of enzymes from *Mycobacterium tuberculosis*. J Biol Chem 279: 52694–52702

Axén R, Porath J, Ernback S (1967) Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halide. Nature 214:1302–1304

Becker K, Krebs B, Schirmer RH (1991) Protein-chemical standardization of the erythrocyte glutathione reductase activation test. Application to hyperthyroidism. Int J Vitam Nutr Res 61: 180–187

Bhargava KK, Le Trang N, Cerami A, Eaton JW (1983) Effect of arsenical drugs on glutathione metabolism of *Litomosoides carinii*. Mol Biochem Parasitol 9:29–35

Boggaram V, Brobjer T, Larson K, Mannervik B (1979) Purification of glutathione reductase from porcine erythrocytes by the use of affinity chromatography on 2′,5′-ADP–Sepharose 4B and crystallization of the enzyme. Anal Biochem 98:335–340

Brophy PM, Pritchard DI (1992) Immunity to helminths: ready to tip the biochemical Balance? Parasitol Today 8:419–422

Carlberg I, Mannervik B (1975) Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 250:5475–5480

Carlberg I, Mannervik B (1981) Purification and characterization of glutathione reductase from calf liver. An improved procedure for affinity chromatography on 2′,5′ ADP Sepharose 4B. Anal Biochem 116:531–536

Carlberg I, Mannervik B (1985) Glutathione reductase. Methods Enzymol 113:484–490

Carlberg I, Altmejd B, Mannervik B (1981) Purification and immunological studies of glutathione reductase from rat liver: evidence for an antigenic determinant at the nucleotide-binding domain of the enzyme. Biochim Biophys Acta 677:146–152

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138
MS Code :
☐ LE ☐ TYPESET
☑ CP ☑ DISK

Chiumiento L, Brushchi F (2009) Enzymatic antioxidant systems in helminth parasites. Parasitol Res 105(3):593–603

Collinson LP, Dawes IW (1995) Isolation, characterization and overexpression of the yeast gene, GLR1, encoding glutathione reductase. Gene 156:123–127

Deonarain MP, Scrutton NS, Berry A, Perham RN (1990) Directed mutagenesis of the redox-active disulphide bridge in glutathione reductase from *Escherichia coli*. Proc Biol Sci 241:179–186

Erat M, Ciftci M (2003) In vitro effects of some antibiotics on glutathione reductase from sheep liver. J Enzym Inhib Med Chem 18:545–550

Erat M, Sakiroglu H, Ciftci M (2003) Purification and characterization of glutathione reductase from bovine erythrocytes. Prep Biochem Biotechnol 33:283–300

Fairlamb AH, Cerami A (1985) Identification of a novel, thiol containing cofactor essential for glutathione reductase enzyme activity in Trypanosomatids. Mol Biochem Parasitol 14:187–198

Gupta S, Tripathi R, Srivastava AK, Iqbal J (2002) Depletion of intracellular glutathione of *Setaria cervi* by buthionine sulfoximine, carmustine and their analogues. In: Callaos N, He Y, Perez-Perez JA (eds) Proceedings of International Institute on Informatics and Systemics (IIIS) and International Federation of Systems Research (IFSR), vol XVII. Orlando, Florida, pp. 491–496

Gutterer JM, Dringen R, Hirrlinger J, Hamprecht B (1999) Purification of glutathione reductase from bovine brain, generation of an antiserum, and immunocytochemical localization of the enzyme in neural cells. J Neurochem 73:1422–1430

Halliwell B, Foyer CH (1978) Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta 139:9–17

Jocelyn PC (1972) Biochemistry of the SH group. Academic Press, London, pp 240–278

Kapoor G, Bagai U, Banyal HS (2008) Purification and characterization of *Plasmodium berghei* glutathione reductase. J Parasit Dis 32:22–29

Karplus PA, Schulz GE (1987) Refined structure of glutathione reductase at 1.54 Å resolution. J Mol Biol 195:701–729

Karplus PA, Schulz GE (1989) Substrate binding and catalysis by glutathione reductase as derived from refined enzyme: substrate crystal structures at 2 Å resolution. J Mol Biol 210:163–180

Krauth-Siegel RL, Blatterspiel R, Saleh M, Schiltz E, Schirmer RH, Untucht-Grau R (1982) Glutathione reductase from human erythrocytes: the sequences of the NADPH domain and of the interface domain. Eur J Biochem 121:259–267

Krauth-Siegel RL, Lohrer H, Bucheler US, Schirmer RH (1991) In: Coombs GH, North MJ (eds) Biochem protozoology. Taylor and Francis, London, pp 493–503

Krauth-Siegel RL, Muller JG, Lottspeich F, Schirmer RH (1996) Glutathione reductase and glutamate dehydrogenase of *Plasmodium falciparum*, the causative agent of tropical malaria. Eur J Biochem 235:345–350

Krohne-Ehrich G, Schirmer RH, Untucht-Grau R (1977) Glutathione reductase from human erythrocytes. Isolation of the enzyme and sequence analysis of the redox-active peptide. Eur J Biochem 80:65–71

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–685

Lamotte F, Liaud NV, Duviau MP, Kobrehel K (2000) Glutathione reductase in wheat grain: isolation and characterization. J Agric Food Chem 48:4978–4983

Le Trang N, Bhargava KK, Cerami A (1983) Purification of glutathione reductase from gerbil liver in two steps. Anal Biochem 133:94–99

Libreros-Minotta CA, Pardo JP, Mendoza-Hernández G, Rendón JL (1992) Purification and characterization of glutathione reductase from *Rhodospirillum rubrum*. Arch Biochem Biophys 298:247–253

Lopez-Barea J, Lee CY (1979) Mouse-liver glutathione reductase. Purification, kinetics, and regulation. Eur J Biochem 98:487–499

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–276

Maggioli G, Piacenza L, Carambula B, Carmona C (2004) Purification, characterization, and immunolocalization of a thioredoxin reductase from adult *Fasciola hepatica*. J Parasitol 90(2): 205–211

McCallum MJ, Barrett J (1995) The purification and properties of glutathione reductase from the cestode *Moniezia expansa*. Int J Biochem Cell Biol 27:393–401

Meister A, Anderson ME (1983) Glutathione. Annu Rev Biochem 52:711–760

Mittl PR, Schulz GE (1994) Structure of glutathione reductase from *Escherichia coli* at 1.86 Å resolution: comparison with the enzyme from human erythrocytes. Protein Sci 3:799–809

Moroff G, Brandt KG (1975) Yeast Glutathione reductase: studies of the kinetics and stability of the enzyme as a function of pH and salt concentration. Biochim Biophys Acta 410:21–31

Müller S, Walter RD, Fairlamb AH (1995) Differential susceptibility of filarial and human erythrocyte glutathione reductase to inhibition by the trivalent organic arsenical melarsen oxide. Mol Biochem Parasitol 7:211–219

Müller S, Gilberger TW, Fairlamb AH, Walter RD (1997) Molecular characterization and expression of *Onchocerca volvulus* glutathione reductase. Biochem J 325:645–651

Okpodu CM, Waite KL (1997) Method for detecting glutathione reductase activity on native activity gels which eliminates the background diaphorase activity. Anal Biochem 244:410–413

Ottesen EA, Ramchandran CP (1995) Lymphatic filariasis infection and disease: control strategies. Parasitol Today 11:129–131

Pai EF, Karplus PA, Schulz GE (1988) Crystallographic analysis of the binding of NADPH, NADPH fragments, and NADPH analogues to glutathione reductase. Biochemistry 27:4465–4474

Penketh PG, Klein RA (1986) Hydrogen peroxide mechanism in *Trypanosoma brucei*. Mol Biochem Parasitol 20:111–121

Perham RN, Leistler B, Solomon RG, Guptasarma P (1996) Protein engineering of domains in flavoprotein disulphide oxidoreductases: contributions to folding and assembly. Biochem Soc Trans 24:61–66

Rendóna JL, del Arenal IP, Guevara-Flores A, Uribe A, Plancarte A, Mendoza-Hernández G (2004) Purification, characterization and kinetic properties of the multifunctional thioredoxin–glutathione reductase from *Taenia crassiceps* metacestode (cysticerci). Mol Biochem Parasitol 133:61–69

Salinas G, Selkirk ME, Chalar C, Maizels RM, Fernández C (2004) Linked thioredoxin–glutathione systems in platyhelminths. Trends Parasitol 20:340–346

Savvides SN, Scheiwein M, Bohme CC, Arteel GE, Karplus PA, Becker K et al (2002) Crystal structure of the antioxidant enzyme glutathione reductase inactivated by peroxynitrite. J Biol Chem 277:2779–2784

Schirmer RH, Schulz GE (1987) Chemical, biochemical and medical aspects. In: Dolphin D, Poulson R, Avramouic O (eds) Coenzymes and cofactors, pyridine nucleotide coenzymes. Wiley, New York, pp 333–339

Schirmer RH, Schollhammer T, Eisenbrand G, Krauth-Siegel RL (1987) Oxidative stress as a defense mechanism against parasitic infections. Free Radic Res Commun 3:3–12

Schirmer RH, Krauth-Siegel RL, Schulz GE (1989) Glutathione. In: Dolphin D, Poulson R, Avramovic O (eds) Coenzymes and cofactors, vol III. Wiley, New York, pp 553–596

Schulz GE, Zappe HA, Worthington DJ, Rosemeyer MA (1975) Crystals of human erythrocyte glutathione reductase. FEBS Lett 54:86–88

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138
MS Code :
☐ LE ☑ CP ☐ TYPESET ☑ DISK

Scott EM, Duncan IW, Ekstrand V (1963) Purification and properties of glutathione reductase of human erythrocytes. J Biol Chem 238:3928–3933

Simons PC, Vander Jagt DL (1977) Purification of GST(s) from human liver by glutathione affinity chromatography. Anal Biochem 82:334–341

Singh SN, Srivastava AK, Chatterjee RK (1997) Presence of glutathione, glutathione reductase and glutathione peroxidase in filarial parasites. Helminthologia 34:70–73

Speers-Roesch B, Ballantyne JS (2005) Activities of antioxidant enzymes and cytochrome c oxidase in liver of Arctic and temperate teleosts. Comp Biochem Physiol A 140:487–494

Staal GEG, Veeger C (1969) The reaction mechanism of glutathione reductase from human erythrocytes. Biochim Biophys Acta 185: 49–62

Stevens RG, Creissen GP, Mullineaux PM (1997) Cloning and characterization of a cytosolic glutathione reductase cDNA from pea (*Pisum sativum* L.) and its expression in response to stress. Plant Mol Biol 35:641–654

Tutic M, Lu XA, Schirmer RH, Werner D (1990) Cloning and sequencing of mammalian glutathione reductase cDNA. Eur J Biochem 188:523–528

Ulusu G, Ciftci M, Erat M, Sakiroglu H, Bakan E (2005) Purification and characterization of glutathione reductase from sheep liver. Turk J Vet Anim Sci 29:1109–1117

Worthington DJ, Rosemeyer MA (1974) Human glutathione reductase: purification of the crystalline enzyme from erythrocytes. Eur J Biochem 48:167–177

Worthington DJ, Rosemeyer MA (1975) Glutathione reductase from human erythrocytes. Eur J Biochem 60:459–466

Worthington DJ, Rosemeyer MA (1976) Glutathione reductase from human erythrocytes: catalytic properties and aggregation. Eur J Biochem 67:231–238

Journal : Large_Springer-India12639 Dispatch : 10-7-2012 Pages : 11
Article No. : 138 ☐ LE ☐ TYPESET
MS Code : ☑ CP ☑ DISK