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Research brief

Setaria cervi: Kinetic studies of filarial glutathione synthetase by high performance liquid chromatography

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Abstract

The bovine filarial worm *Setaria cervi* was found to have abundance of glutathione synthetase (GS; EC 6.3.2.3) activity, the enzyme being involved in catalysing the final step of glutathione (GSH) biosynthesis. A RP-HPLC method involving precolumn derivatization with *o*-phthalaldehyde has been followed for the estimation of GS activity in crude filarial preparations. Subcellular fractionation of the enzyme was undertaken and it was confirmed to be a soluble protein residing mainly in cytosolic fraction. Attempts to determine the K_m value for L-γ-glutamyl-L-cysteine gave a distinctly nonlinear double-reciprocal plot in which data obtained at relatively high dipeptide concentrations (>1 mM) extrapolate to a K_m value of about 400 μM whereas data obtained at lower concentrations (<0.1 mM) extrapolate to a value of about 33 μM. K_m was determined to be around 950 and 410 μM for ATP and glycine, respectively. The effect of various amino acids was studied on enzyme activity at 1 mM concentration. L-Cystine caused a significant enzyme inhibition of 11%. Preincubation with *N*-ethylmaleimide also resulted in significant inhibition of GS activity.

Index Descriptors and Abbreviations: GS, glutathione synthetase (EC 6.3.2.3); GSH, glutathione; GCL, glutamate cysteine ligase (EC 6.3.2.2); RP-HPLC, reversed phase-high performance liquid chromatography; HBSS, hanks' balanced salt solution; ROS, reactive oxygen species; DTT, dithiothreitol; *S. cervi*, *Setaria cervi*; *O. volvulus*, *Onchocerca volvulus*; Filariasis; Nematode

1. Introduction

The role of GSH as an important antioxidant in filarial nematodes is well established (Selkirk et al., 1998). It protects filariae from the oxidative stress of ROS produced by the host immune cells, thus aiding their long term survival in the host body. The ultimate step for GSH biosynthesis is attributed to GS and is well characterized in mammals (Gali and Board, 1995; Gali and Board, 1997; Huang et al., 1995; Luo et al., 2000; Oppenheimer et al., 1979). However, GS has not been characterized in nematodes and filariids. The present study is a preliminary step in this direction in support of future work.

In most of previous research, emphasis has been given to studies of filarial GCL. This enzyme is well characterized in *Onchocerca volvulus* and *Setaria cervi* (Lüersen et al., 2000; Tiwari et al., 2003). Less emphasis has been placed on GS, except for the effect of some synthetic diglycosylated diaminoalkanes and coumarin derivatives in our own studies (Gupta et al., 2004). In the present work GS has been confirmed as a cytosolic enzyme in *S. cervi* filarial worms and its kinetic properties have been studied.

Classical methods for GS assay involve quantitative determination of inorganic phosphate (Pi) produced during the reaction or the coupled enzyme procedure which allows the formation of ADP to be followed during the reaction (Beutler and Gelbart, 1986; Seelig and Meister, 1985). These methods can be used on fully/partially purified enzyme, but are unsuitable for crude

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preparations. In the present study a RP-HPLC method has been employed for the accurate detection of GS and investigation of its kinetics in crude preparations.

2. Materials and methods

2.1. Materials

L-γ-Glutamylcysteine, glycine, ATP, KCl, DTT, $MgCl_2$, acivicin, *o*-phthalaldehyde, GSH, and Tris were purchased from Sigma Chemical, USA. All the amino acids and HBSS were procured from Hi Media Laboratories, India. Methanol (HPLC grade) and sodium acetate were acquired from Merck India, India. All other chemicals were of analytical grade.

2.2. Instrumentation

HPLC was performed using Waters Millennium[32] chromatography management system which includes Waters 474 scanning fluorescence detector, Waters 515 HPLC pumps and chromatographic data acquisition and processing computer with Millennium[32] chromatography manager software. Reversed phase analytical Spherisorb S5 ODS2 (4.0×125 mm) column, S5 ODS2 (4.6×10 mm) guard cartridges, and accessories were also purchased from Waters.

2.3. Preparation of sample

Adult *S. cervi* were collected from the peritoneal folds of naturally infected water buffaloes (*Bubalus bubalis* Linn) at a local abbatoir and brought to laboratory in Ringer's saline. Filarial worms were revived in sterilized HBSS containing gentamycin (1 μg/ml) to be used for further studies. A 10% (w/v) homogenate of filarial worms was prepared in 50 mM imidazole–HCl buffer containing 10 mM $MgCl_2$ (Oppenheimer et al., 1979). The homogenate was then subjected to subcellular fractionation by differential centrifugation. The homogenate was centrifuged at 1000*g* for 15 min to obtain the crude fraction in the supernatant. The crude fraction was subsequently centrifuged at 10,000*g* for 30 min to get the postmitochondrial and mitochondrial fractions in the form of supernatant and pellet, respectively. The postmitochondrial fraction was then centrifuged at 100,000*g* for 60 min to obtain cytosolic and microsomal fractions in the form of supernatant and pellet, respectively.

Homogenization and subcellular fractionation of rat (Wistar strain-male) liver was also done under similar conditions and under the specified assay conditions, the specific activity of GS in cytosolic fraction was calculated. The animals were housed in plastic cages with proper care according to the guidelines of our institutional animal house ethics committee. Under standard laboratory conditions of temperature (22 ± 1 °C) and humidity (50–60%), the animals were maintained on commercially available pellet diet supplemented with soaked grains. Water was provided ad libitum. Breeding colonies of animals were maintained under a SPF (specific pathogen free) environment in standard housing condition.

2.4. Enzyme assay

The assay system used was modified form as described by Nardi et al. (1990). It contained 0.1 M Tris–HCl buffer (pH 8.2), 6 mM ATP, 50 mM KCl, 6 mM DTT, 20 mM $MgCl_2$, 0.3 mM L-γ-glutamylcysteine, 3 mM glycine, and 5 mM acivicin in the total reaction volume of 1 ml. Acivicin was added to prevent γ-glutamyl transpeptidase to break the reaction product, GSH. The reaction was initiated by the addition of an enzyme aliquot. After incubation at 37 °C, the reaction was stopped by the addition of 10% sulfosalicylic acid. After centrifugation at 10,000*g* for 10 min, the supernatant was derivatized with *o*-phthalaldehyde.

2.5. Derivatization with o-phthalaldehyde

Derivatization is as described by Neuschwander-Tetri and Roll (1989) with slight alterations. Fifty microlitres of 10,000*g* supernatant was mixed with 50 μl of *o*-phthalaldehyde reagent (40 mM *o*-phthalaldehyde and 0.4 M sodium tetraborate, pH 9.0) and the reaction was stopped after 1 min with 100 μl of 0.1 M potassium phosphate–H_3PO_4 buffer (pH 7.0). From this solution, 50 μl aliquot was introduced to the HPLC column using a Rheodyne injector for product (GSH) analysis.

2.6. Preparation of standard and control

Different known concentrations of GSH were added to the reaction mixtures in place of enzyme aliquot and were treated exactly as described above for *o*-phthalaldehyde derivatization to obtain a standard curve for GSH (10–150 μM). Controls contained the same amount of enzyme aliquot but no L-γ-glutamylcysteine. Control values were subtracted from the experimental values to obtain the exact amount of GSH formed.

2.7. High performance liquid chromatography

Separation was performed at a flow rate of 1 ml/min with solvent A (0.15 M sodium acetate, pH adjusted to 7.0 with acetic acid/methanol (1/24, v/v)) and solvent B (100% methanol). Gradient (expressed as percentages of solvent B) used was: 8 min, 0%; 12 min, 10%; 13 min, 25%; 27 min, 90%; 35 min, 90%; 40 min, 0%; 50 min,