HELMINTHOLOGIA, 42, 2: 57 – 61, 2005

γ-Glutamyl transpeptidase activity in adult *Setaria cervi* (filarial worms)

S. GUPTA, A. K. SRIVASTAVA, N. BANU[1]

Division of Biochemistry, Central Drug Research Institute, Lucknow 226 001, India, E mail: *drarv1955@yahoo.com*; [1]Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202 002, India

Summary

The bovine filarial worm *Setaria cervi* was found to have abundance of γ-glutamyl transpeptidase (γ-GT; EC 2.3.2.2) activity. The enzyme activity was almost evenly distributed in subcellular fractions. K_m and V_{max} of triton X-100 solubilized microsomal γ-GT towards L-γ-glutamyl-p-nitroanilide were calculated to be 0.13 ± 0.01 mM and 0.03 ± 0.002 μmol/min/mg protein, respectively. Similar kinetic parameters for cytosolic fraction were determined to be 0.21 ± 0.06 mM and 0.45 ± 0.17 μmol/min/mg protein, respectively. No remarkable effect by any of the studied amino acids at 1 mM concentration on the enzyme activity was found except L-cystine, which caused concentration dependent increase in enzyme activity while GSH and GSSG caused concentration dependent decrease in enzyme activity. The K_i values for GSH and GSSG were calculated to be around 3.5 ± 0.50 and 0.85 ± 0.05 mM, respectively, for microsomal enzyme, whereas 11.0 ± 1.41 and 0.9 ± 0.14 mM respectively, for cytosolic γ-GT. Filarial γ-GT was also inhibited by L-serine in the presence of borate, K_i being calculated to be 1.58 ± 0.38 mM and 1.13 ± 0.18 mM for microsomal and cytosolic fractions, respectively. K_i value for standard mammalian inhibitor acivicin was found 0.88 ± 0.18 mM and 0.28 ± 0.04 mM for microsomal and cytosolic fractions, respectively.

Key words: *Setaria cervi*; γ-glutamyl transpeptidase; γ-glutamyl cycle; glutathione; L-serine-borate, acivicin *Abbreviations:* γ-GT; γ-glutamyl transpeptidase; GSH; reduced glutathione; GSSG; oxidised glutathione

Introduction

γ-Glutamyl cycle is a series of six enzyme-catalysed reactions which accounts for the synthesis and degradation of one of the important bio- constituent glutathione (GSH; Meister, 1973). GSH is present in virtually all animal cells, serves several vital functions, including detoxifying electrophiles, maintaining the essential thiol status of proteins by preventing oxidation of –SH groups or by reducing disulphide bonds induced by oxidative stress, scavenging free radicals, providing a reservoir for cysteine and modulating critical cellular processes such as DNA synthesis, microtubular-related processes and immune function (Meister, 1991) The initial step in the breakdown of GSH is an amino acid/dipeptide dependent transpeptidation reaction which is catalysed by γ-GT (γ-glutamyl transpeptidase). γ-GT is predominantly a membrane bound glycoprotein in a studied nematode (Hussein & Walter, 1996) and many of the mammalian tissues (Tate & Meister, 1985), whereas it has also been reported to be a soluble protein in *Trypanosoma cruzi* (Repetto *et al.*, 1987). Under normal physiological conditions excess of GSH is released from the cell and the enzyme γ-GT then transfers the γ-glutamyl moiety of outgoing GSH to an incoming extracellular amino acid (Orlowski & Meister, 1970), the best acceptor being cystine, forming the γ-glutamyl amino acid and cysteinyl-glycine. Cysteinylglycine is broken down by dipeptidase to generate cysteine and glycine. Cysteine is readily taken up by most, if not all cells. Once inside the cell, the majority of cysteine is incorporated into GSH and some into protein, depending on the need of the cell (Anderson & Meister, 1987). Thus, the γ-glutamyl cycle allows the efficient utilization of GSH as cysteine storage (Lu, 1999). Although in the case of mammals cysteine can be synthesised from methionine *via* the transsulfuration pathway in the liver (Cooper, 1983) but in the absence of γ-GT the unabated loss of GSH from the body resulted in a depletion of cysteine that could not be compensated for by hepatic synthesis (Lieberman *et al.*, 1996). γ-GT thus must be very important in the case of nematodes where extremely low activity of γ-cystathionase may represent a bottleneck in transsulfuration pathway (Jaffe, 1980; Gomez-Bautista & Barrett, 1988). Since, it has been recognised that a continuous efflux of GSH occurs from a number of

cell types through specific out-transporters (Lu *et al.*, 1996) it is conceivable that the major function of γ-GT ectoactivity could be the salvage of extracellular GSH which would otherwise be lost from the cell. Another important function of γ-GT is in the cleavage of GSH conjugates which are formed during detoxification of xenobiotics (DeLeve & Kaplowitz, 1991).

In case of the nematode *Ascaris suum*, the enzyme γ-GT was demonstrated to be largely localised in the cuticle-hypodermis section of the parasite. Collectively the data relating to the uptake and metabolism of glutamine by the cuticular preparations suggest that the cuticle may not be impermeable to extracellular nutrients and this may be an indication that amino acid absorption could occur through the cuticle *via* the γ-glutamyl cycle (Dass & Donahue, 1986). The selective inhibition of cuticular absorption of these essential nutrients may provide a rational strategy for the design of chemotherapeutic agents directed towards the elimination of nematodes. In view of the above important functions of γ-GT, it is thought interesting to look its profile in the causative organisms of the dreadful disease filariasis. The present study describes the profile and kinetic properties of this enzyme in the bovine filarial nematode *Setaria cervi*.

Material and Methods

Chemicals

L-γ-glutamyl-p-nitroanilide, glycylglycine, triton X-100, GSH, GSSG, L-cystine, acivicin and L-serine were purchased from Sigma Chemical Co., USA. All the other amino acids and Hanks' balanced salt solution (HBSS) were procured from Hi Media Laboratories, Mumbai, India. All other chemicals used were of analytical grade.

Experimental models

The adults of bovine filarial worms *Setaria cervi* were collected from the peritoneal folds of freshly slaughtered naturally infected water buffaloes *Bubalus bubalis* (L.) at a local abbatoir and brought to laboratory in Ringer's saline. The thoroughly washed worms were incubated in Hanks' balanced salt solution fortified with glucose for 1 hr before these were used for enzyme preparation.

Preparation of γ-glutamyl transpeptidase from filarial worms

The adopted procedure is as described by Meister *et al.* (1981). A 10 % (w/v) homogenate of revived *S. cervi* worms was prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose with the help of Potter Elvejhem homogenizer fitted with a teflon pestle and was separated into various subcellular fractions by differential centrifugation. The mitochondrial and microsomal pellets were suspended in 10 mM Tris-HCl buffer of pH 7.5 to estimate particulate γ-GT. Crude and cytosolic fractions were used as such for activity determinations.

Optionally, for kinetic and inhibition studies, microsomal fraction was solubilised by triton X-100. Microsomal pellet was suspended and continuously shaken in 10 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and 1 % triton X-100 for 1 h and subsequently centrifuged at 100,000 x *g* for 1 h to obtain the solubilised γ-GT. Triton X-100 was removed by overnight dialysis against two changes of 200 volumes of Tris-HCl buffer (pH 7.5) containing 0.1 % triton X-100.

γ-GT activity determination

The activity of γ-GT was determined using the artificial substrate L-γ-glutamyl-p-nitroanilide. The assay mixture in a final volume of 1.0 ml contained 100 mM Tris-HCl (pH 8.0), 20 mM glycylglycine, 1 mM L-γ-glutamyl-p-nitroanilide and a suitable amount of the enzyme protein. The assay mixtures were incubated at 37°C for desired period and the reaction was terminated with 10 % acetic acid. p-Nitroaniline formed was estimated spectrophotometrically using a Shimadzu double beam UV-190 spectrophotometer at 410 nm (molar extinction coefficient = 8800 M^{-1} cm^{-1}).

Subcellular fractionation of rat (Wistar strain male) liver was done under similar conditions as stated above for *S. cervi* and under the specified assay conditions, the specific activity of γ-GT in solubilised microsomal fraction was calculated.

Determination of kinetic parameters K_m, V_{max} and K_i and their relevance

K_m, the Michaelis constant, represents a valuable constant that relates the velocity of an enzyme-catalysed reaction to the substrate concentration. K_m is equivalent to the substrate concentration that yields half-maximal velocity. Numerical value of K_m is of interest for several reasons. Firstly, the K_m establishes an approximate value for the intracellular level of substrate. Secondly, it determines the affinity of substrate for enzyme. Thirdly, K_m is constant for a given enzyme. V_{max} is the maximal velocity of an enzyme catalysed reaction. V_{max} is not a constant but depends upon k_p (catalytic rate constant) and the concentration of enzyme in the assay.

Lineweaver-Burk reciprocal plot (1/v verses 1/[S]) was used for the determination of K_m and V_{max}. At varying L-γ-glutamyl-p-nitroanilide concentrations (0.125 mM-1.5 mM), the velocities were determined and plotted. Intercept on x axis gives $-1/K_m$ and intercept on y axis gives $1/V_{max}$.

The inhibitor constant (K_i) is the dissociation constant of the enzyme-inhibitor complex or in other words, the reciprocal of the affinity of the enzyme for the inhibitor. K_i is not equivalent to the inhibitor that yields 50 % inhibition but it is equivalent to the concentration of inhibitor that doubles the slope of the 1/v verses 1/[S] plot. The lower the value of K_i, the greater is the degree of inhibition at any substrate and inhibitor concentration.

The determination of K_i involves measurements of initial velocities with a number of different concentrations of inhibitor, keeping the substrate concentration constant. The Dixon plot of 1/v versus [I] was used for the determination of K_i. The effect of amino acids, GSH, GSSG, serine-borate and acivicin on γ-GT was studied by directly adding

them in the assay system 10 min prior to the addition of substrate.

Protein estimation

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. Proteins in the homogenates were precipitated with an equal volume of 10 % trichloroacetic acid (TCA). The precipitate was washed twice with 5 % TCA. TCA was discarded by centrifugation and the precipitate was dissolved in sodium hydroxide (0.1 N). Protein was then measured in different aliquots of this solution. The tubes contained 0.25 ml aliquot (sample/ standard and triple distilled water) and 2.5 ml of a mixture of protein reagent A (8 % sodium carbonate) and B (copper sulphate and sodium potassium tartarate) in the ratio of 1:1. After incubation for 10 min at 37°C, 0.25 ml of Folin Ciocalteu's reagent (1 N) was also added and the tubes were incubated for 30 min at 37°C. A blank tube was also set up which contained all the reagents except the protein. The absorbance of the colour developed was read at 660 nm.

Results

Under the specified assay conditions the specific activities of *S. cervi* γ-GT in the particulate microsomal and triton X-100 solubilised microsomal fraction were determined to be around 0.052 ± 0.002 µmol/min/mg protein and 0.025 ± 0.001 µmol/min/mg protein, respectively. The average specific activity of cytosolic enzyme was determined to be around 0.050 ± 0.01 µmol/min/mg protein (Table 1). It is evident from the table 1 that the activity of γ-GT was almost uniformly distributed in all subcellular fractions. Under the defined assay conditions the specific activity of γ-GT in solubilised microsomal fraction of rat liver was calculated to be 3.431 ± 0.06 µmol/min/mg protein.

K_m and V_{max} of solubilised microsomal and cytosolic γ-GT with respect to the substrate L-γ-glutamyl-p-nitroanilide were determined as described. Microsomal K_m was found to be around 0.13 ± 0.01 mM and V_{max} to be around 0.03 ± 0.002 µmol/min/mg protein. On the other hand cytosolic K_m and V_{max} were calculated to be around 0.21 ± 0.06 mM and 0.45 ± 0.17 µmol/min/mg protein, respectively. The effect of various amino acids, GSH and GSSG on triton X-100 solubilised microsomal and cytosolic γ-GT was studied. All studied amino acids *viz* L-Met, L-Gln, L-Asn, L-Ala, L-Cys, L-Lys, L-Arg, L-His, L-Leu, DL-Ile, DL-Val, DL-B-Phe, DL-Ser, DL-Thr, L-Pro, L-Hyp, L-Glu, L-Asp, L-Tyr and DL-Trp at 1 mM concentration showed no remarkable effect on enzyme activity except L-cysti-ne. The effect of L-cystine was studied over a wide concentration range (0.25 – 2 mM). A concentration dependent increase in enzyme activity can be observed from table 2 for solubilised microsomal and cytosolic frac-tions respectively. Both GSH and GSSG caused concen-tration dependent decrease in enzyme activity when stu-died for their effect on solubilised microsomal and cyto-solic γ-GT in the concentration range of 0.25 – 5 mM. K_i of GSH and GSSG for solubilised microsomal γ-GT was found to be 3.5 ± 0.50 and 0.85 ± 0.05 mM, respectively. For cytosolic γ-GT, K_i was calculated to be 11.0 ± 1.41 and 0.9 ± 0.14 mM, respectively. The effect of different concentrations of L-serine (0.25 – 5 mM) in the presence of borate (10 mM)

Table 1. Distribution of γ-glutamyl transpeptidase activity in various subcellular fractions of *Setaria cervi* homogenate

Fraction	Specific Activity [a]
Crude	0.049 ± 0.003
Cytosolic	0.050 ± 0.010
Mitochondrial (particulate)[b]	0.038 ± 0.003
Microsomal (particulate)[b]	0.052 ± 0.002
Microsomal (triton X-100 solubilised)	0.025 ± 0.001

a – Specific Activity is expressed as µmol p-nitroaniline released/ min/mg protein; b – The mitochondrial and microsomal pellets were suspended in 10 mM Tris-HCl buffer (pH 7.5) whereas crude and cytosolic fractions were used as such for activity determinations

Table 2. Effect of various concentrations of L-cystine on γ-glutamyl transpeptidase[a] from *Setaria cervi*

[Cystine, mM]	% Increase in enzyme activity[b] (solubilised microsomal γ-GT)	% Increase in enzyme activity[b] (cytosolic γ-GT)
0.25	6.20 ± 2.6	23.0 ± 7.3
0.5	12.8 ± 3.0	43.4 ± 13.7
0.75	21.0 ± 3.6	54.3 ± 7.1
1	28.4 ± 3.3	78.0 ± 9.5
1.5	36.3 ± 8.0	98.7 ± 6.4
2	45.3 ± 3.23	119.4 ± 11.4

a – Enzyme activity was estimated in a reaction mixture (1 ml) containing 0.1 M Tris-HCl buffer (pH 8.0), 20 mM glycylglycine and 1 mM L-γ-glutamyl-p-nitroanilide; b – Results are the mean of three independent experiments; c – In this study, the range of specific activity for solubilised microsomal and cytosolic γ-GT in control was 0.025 – 0.039 and 0.12-0.13 µmol/min/mg protein, respectively

Table 3. Comparison of kinetic parameters of solubilised microsomal and cytosolic γ-glutamyl transpeptidase from *S. cervi*

	K_m (mM)	V_{max} (µmoles/min/mg)	K_i (mM)			
			GSH	GSSG	Serine Borate	Acivicin
Microsomal*	0.13 ± 0.01	0.03 ± 0.002	3.5 ± 0.5	0.85 ± 0.05	1.58 ± 0.38	0.88 ± 0.18
Cytosolic	0.21 ± 0.06	0.45 ± 0.17	11.0 ± 1.41	0.9 ± 0.14	1.13 ± 0.18	0.28 ± 0.04

* Solubilised by triton X-100

on solubilised microsomal γ-GT was studied and Ki was calculated to be around 1.58 ± 0.38 mM. Similar inhibition studies were also done using cytosolic γ-GT and Ki was determined to be 1.13 ± 0.18 mM. Similarly the K_i calculated for acivicin was around 0.88 ± 0.18 and 0.28 ± 0.04 mM for solubilised microso-mal and cytosolic fractions respectively. All the kinetic parameters are compared in Table 3.

Discussion

γ-GT is of central importance in the degradation of GSH. It catalyses the initial step in the breakdown of GSH. γ-GT catalyses transfer of the γ-glutamyl moiety of GSH, S-substituted glutathione derivatives and other γ-glutamyl compounds to a number of acceptors *viz* amino acids and dipeptides as well as GSH in case of autotranspeptidation (Meister *et al.*, 1981).

K_m for the substrate L-γ-glutamyl-p-nitroanilide determined from our studies on *S. cervi* γ-GT was found to be lower as compared to the K_m for the rat and human kidney enzyme. The apparent K_m value for the L-γ-glutamyl-p-nitroanilide derived from rat kidney and human kidney enzyme was 0.9 mM and 0.8 mM respectively (Tate and Meister, 1974; Miller *et al.*, 1976) whereas it is 0.13 and 0.21 mM in our studies on *S. cervi* microsomal and cytosolic γ-GT.

Our studies also reveal that L-cystine is a good acceptor of γ-glutamyl group as compared to all the studied amino acids as well as the dipeptide glycylglycine. Increase in enzyme activity after addition of L-cystine confirms that the affinity of L-cystine for γ-GT is greater than glycylglycine. It seems of interest that our studies are consistent with previous studies. Thompson & Meister (1976) found that the amino acid specificity of γ-GT was maximal in the case of L-cystine. Moreover, specificity of the dipeptide glycylglycine was found lower as compared to L-cystine (Thompson & Meister, 1975).

Decrease in enzyme activity with GSH and GSSG is because both are natural γ-glutamyl group donors and thus are preventing the artificial substrate L-γ-glutamyl-p-nitroanilide from donating its γ-glutamyl group to the enzyme. Similar studies were done previously on human kidney γ-GT and it was found that both GSH and GSSG competitively inhibited the activity of γ-GT when γ-glutamyl-p-nitroanilide was used as the substrate (Miller *et al.*, 1976). According to our studies microsomal γ-GT has higher affinity for GSH as compared to cytosolic one as evident from the K_i values of GSH for microsomal and cytosolic γ-GT *viz* 3.5 and 11.0 mM, respectively.

Serine-borate complex is a known competitive transition state inhibitor of γ-GT. L-Serine and its analogues bind to the γ-glutamyl site of the enzyme by linkages involving the α-amino and α-carboxyl groups. Such binding is enhanced by complex formation between the serine hydroxyl group, borate and a hydroxyl group at the active centre of the enzyme that is probably a seryl/threonyl residue (Tate & Meister, 1978). K_i was determined to be 300 and 20 µM respectively in previous studies on *Ascaris suum* (Hussein & Walter, 1996) and mammalian enzyme (Tate & Meister, 1978). The K_i estimated in our studies (1580 and 1130 µM for microsomal and cytosolic fractions, respectively) is close to that found for the helminth as compared to the mammalian system.

Acivicin on the other hand is the known irreversible inhibitor of γ-GT. It binds to a site close to, but different from that at which γ-glutamylation occurs. Inhibition of the enzyme by acivicin involves conversion of acivicin on the enzyme to a form which is attached, apparently as an ester, to an enzyme hydroxyl group. The very slow rate of release of this species (to form threo-β-hydroxyglutamic acid) appears to account for the inhibition (Stole *et al.*, 1994). K_i was determined to be 0.42 mM in the nematode *A. suum* which is comparable to that of 0.88 and 0.28 mM found in our case. Previous findings indicate that acivicin inhibits the rat, pig and human enzyme by esterification of Thr-523, Ser-405 and Ser-406 respectively (Smith *et al.*, 1995).

Although mammalian (Hughey & Curthoys, 1976) and microbial (Suzuki *et al*, 1986) γ-GT have been extensively studied and their biochemical as well as physiological roles investigated, there is only scanty information about the enzyme in parasitic nematodes. The present study, therefore, is an initiative in this direction for further work.

Acknowledgements

Financial assistance in the form of Senior Research Fellowship to one of our authors (SG) from CSIR, New Delhi (India) and Volkswagen Stiftung, Hannover (Germany) in the form of ad-hoc research grant to AKS for carrying out this research are gratefully acknowledged.

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RECEIVED JANUARY 10, 2005

ACCEPTED MAY 5, 2005