Urea cycle of *Fasciola gigantica*: Purification and characterization of arginase

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Abstract

The ornithine–urea cycle has been investigated in *Fasciola gigantica*. Agrinase had very high activity compared to the other enzymes. Carbamoyl phosphate synthetase and ornithine carbamoyltransferase had very low activity. A moderate enzymatic activity was recorded for argininosuccinate synthetase and argininosuccinate lyase. The low levels of *F. gigantica* urea cycle enzymes except to the arginase suggest the urea cycle is operative but its role is of a minor important. The high level of arginase activity may benefit for the hydrolysis of the exogenous arginine to ornithine and urea. Two arginases Arg I and Arg II were separated by DEAE-Sepharose column. Further purification was restricted to Arg II with highest activity. The molecular weight of Arg II, as determined by gel filtration and SDS-PAGE, was 92,000. The enzyme was capable to hydrolyze L-arginine and to less extent L-canavanine at arginase : canavanase ratio ($>$10). The enzyme exhibited a maximal activity at pH 9.5 and K_m of 6 mM. The optimum temperature of *F. gigantica* Arg II was 40 °C and the enzyme was stable up to 30 °C and retained 80% of its activity after incubation at 40 °C for 15 min and lost all of its activity at 50 °C. The order of effectiveness of amino acids as inhibitors of enzyme was found to be lysine $>$ isoleucine $>$ ornithine $>$ valine $>$ leucine $>$ proline with 67%, 43%, 31%, 25%, 23% and 15% inhibition, respectively. The enzyme was activated with Mn^{2+}, where the other metals Fe^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+} and Mg^{2+} had inhibitory effects.
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1. Introduction

The trematode parasites *Fasciola hepatica* (temperate) and *Fasciola gigantica* (tropical) are the causative agents of liver fluke disease (fasciolosis) in cattle and sheep and even humans, leading to chronic infections (Milbourne and Howell, 1993). They are of substantial commercial importance in agricultural countries where they parasitizes livestock (Shubkin et al., 1992). Due to their economic importance they have been the subject of many scientific investigations.

Nitrogen excretion had been studied extensively in vertebrates while far less is known for invertebrates. Insects and many other invertebrates independently evolved the ability to excrete ammonium ions as uric acid. Such organisms are collectively designated uricotelic. Spiders and other arachnids also use the purine biosynthetic pathway, but stop at guanine as the disposal product (Jenkinson et al., 1996). Urea and proline were excreted from *F. hepatica* (Kurelec, 1975). Urea could be derived from any of the following four sources: dietary arginine which is broken down by arginase, dietary urea which is excreted unchanged, purines which are degraded via uric acid, and finally from ammonia and bicarbonate by ornithine–urea cycle (Krebs and Henseleit, 1932). Despite the fact that excreted urea may be derived in any of these four ways, the majority of research has been directed towards determining whether the urea cycle, a major pathway in mammals, is functional in invertebrates. The urea cycle is an essential

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metabolic pathway for the removal of highly toxic ammonium ions from the body (Knox and Greengard, 1965). Arginase is one of the five key enzymes of the urea cycle, the others being ornithine carbamoyltransferase, argininosuccinate synthetase, argininosuccinate lyase and carbamoyl phosphate synthetase.

Lack of carbamoyl phosphate synthetase and ornithine carbamoyltransferase in the liver fluke (Kurelec, 1972) eliminates "true" ureotelism (Cohen, 1970), and together with the absence of argininosuccinate synthetase (Janssens and Bryant, 1969) makes it dependent on arginine from the diet, which means that the arginine found in a free amino acid pool (Kurelec and Rijavec, 1966) is of exogenous origin. It would first be used for the synthesis of proteins, while the remainder will be available for the action of arginase present in the liver fluke (Rijavec and Kurelec, 1965; Kurelec, 1974). This raised the question of the physiological function of arginase from Fasciola. Kurelec (1975) reported that the great activity of the arginine catabolic path explains the nature of the production and excretion of urea and proline noticed in F. hepatica. This pathway includes hydrolyses of arginine to ornithine and urea by very active arginase, followed by conversion of ornithine into proline via very active ornithine–alanine transaminase and Δ^1-pyrroline-5-carboxylate reductase. The only benefit for the parasite from these reactions could be the regeneration of NAD during the conversion of Δ^1-pyrroline-5-carboxylate into proline. In insect, arginase had been reported in several insect species (Cochran, 1985). The enzymology for a complete ornithine-cycle appears to be missing in insects (Cochran, 1985; Urich, 1990). The occurrence of the urea cycle in camel tick Hyalomma dromedarii developing embryos based on the identification of intermediates and enzymes involved in this cycle was established except carbamoyl phosphate synthetase (Fahmy et al., 1994a). Wilkie et al. (1999) demonstrated that larval sea lampreys excreted physiological relevant amounts of urea and the presence of key ornithine–urea cycle enzymes such as carbamoyl phosphate synthetase and ornithine carbamoyl transferase were also demonstrated (Wilkie et al., 2004).

Arginase (L-arginine ureahydrolase, or amidinohydrolase, EC 3.5.3.1) is a protein widely distributed in the biosphere, found throughout the evolutionary spectrum in organisms as diverse as bacteria, yeasts, plants, invertebrates and vertebrates. Most studies have concentrated on mammalian liver arginases (Jenkinson et al., 1996; Lavulo et al., 2001). In addition to urea synthesis in the liver of ureotelic species, arginase is also involved in biosynthesis of polyamines and proline (Kaysen and Strecker, 1973), conversion of arginine into α-ketoglutarate for oxidation in the Krebs cycle (Portugal and Aksnes, 1983), adaptive responses to anoxia in some invertebrates (Carvajal et al., 1988) and production of urea for osmoregulatory purposes (Yancey and Somero, 1980). It should be noted that arginase is found in various tissues of nonureotelic organisms, including liver, but is not part of a functional urea cycle (Jackson et al.,

1986). It had previously been thought that there were significant kinetic and structural differences between ureotelic and nonureotelic arginases (Brown and Cohen, 1960; Mora et al., 1966), but many studies suggested that the characteristics of arginases are not consistent with a particular mode of nitrotelism (Reddy and Campbell, 1968; Carvajal et al., 1984). Nonureotelic arginases are generally similar to ureotelic arginases but can be distinguished immunologically (Reddy and Campbell, 1968; Grody et al., 1987). Very little information was reported about the presence of urea cycle in sheep liver F. gigantica. The results presented herein reveal evidence that the urea cycle occurs in the F. gigantica. The properties of arginase, a key enzyme of urea cycle, were compared with those of arginase from other sources.

2. Materials and methods

2.1. Fasciola material

F. gigantica was obtained from sheep liver in the Cairo slaughter house. F. gigantica was saved directly in an ice box during transportation to the Lab, lyophilized and stored at -20 °C.

2.2. Determination of urea cycle enzymes

2.2.1. Preparation of crude extract

Enzyme extract was prepared according to Brown and Cohen (1959), by homogenization of tick material using 50 mM Tris–HCl buffer, pH 7.2 containing 0.1% (w/v) cetyltrimethyl ammonium bromide at 4 °C. The homogenate was centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was designated as crude extract, and used for enzymatic assays. For arginase extraction, 50 mM $MnSO_4$ was added to the homogenate buffer (Reddy and Campbell, 1969).

2.3. Carbamoyl phosphate synthetase assay

Carbamoyl phosphate synthetase (ATP: carbamate phosphotransferase, EC. 2.7.2.5) was assayed as described by Brown and Cohen (1959). Assay reaction mixture contained in 1 mL: 100 μmol ammonium carbonate, pH 7.0, 10 μmol adenosine triphosphate (ATP), 10 μmol N-acetyl glutamic acid, 10 μmol L-ornithine, 20 μmol magnesium sulphate and 0.2 mL of crude extract. The reaction mixture was incubated at 37 °C for 1 h. The citrulline formed was measured by the method of Archibald (1944).

2.4. Ornithine carbamoyltransferase assay

Ornithine carbamoyltransferase (carbamoyl phosphate: L-ornithine carbamoyltransferase, EC. 2.1.3.3) was assayed as described by Brown and Cohen (1959). Assay reaction

mixture contained in 1 mL: 100 µmol glycyl–glycine, pH 8.3, 20 µmol L-ornithine, 20 µmol carbamoyl phosphate and 0.2 mL of crude extract. The reaction mixture was incubated at 37 °C for 1 h. The citrulline formed was measured by method of Archibald (1944).

2.5. Argininosuccinate synthetase assay

The activity of argininosuccinate synthetase (L-citrulline: L-aspartate ligase, EC 6.3.4.5) was determined as described by Ratner (1955). The reaction mixture contained in 1 mL: 50 µmol KH_2PO_4, pH 7.0, 3 µmol L-citrulline, 5 µmol L-aspartic acid, 8.75 µmol $MgSO_4$, 5 µmol ATP and 0.2 mL of crude extract. The reaction mixture was incubated at 37 °C for 1 h. Decrease in citrulline was measured by the method of Archibald (1944).

2.6. Argininosuccinate lyase assay

Argininosuccinate lyase (L-Argininosuccinate arginine-lyase, EC. 4.3.2.1) assay based on Brown and Cohen (1959). The reaction mixture contained in 1 mL: 50 µmol KH_2PO_4 buffer, pH 7.3, 2 µmol L-argininosuccinate and 0.2 mL of crude extract. The reaction mixture was incubated at 37 °C for 1 h. Urea formed was measured according to Marsh et al. (1965) using diacetyl monoxime and read at 520 nm.

2.7. Arginase assay

Arginase (L-Arginine amido hydrolase, EC. 3.5.3.1) assay was based on Campbell (1961). Assay reaction mixture contained in 1 mL: 50 µmol $NaHCO_3$ buffer, pH 9.5, 20 µmol arginine, 0.5 µmol $MnCl_2$ and 0.2 mL crude extract. The reaction mixture was incubated at 37 °C for 1 h. The reaction was stopped with 1 mL of 0.5 M $HClO_4$ followed by centrifugation at 5000 g for 5 min, and the urea formed was determined as above.

2.8. Purification of F. gigantica arginase

Unless otherwise stated all steps were performed at 4–7 °C using 50 mM Tris–HCl buffer, pH 7.2.

2.9. Crude extract

Crude extract was prepared by homogenizing 200 mg of lyophilized *F. gigantica* in 3 mL of 50 mM Tris–HCl buffer, pH 7.2 containing 50 mM $MnSO_4$ and 0.1% CTAB. The homogenate was centrifuged at 10,000 $\times g$ for 20 min at 4–7 °C and supernatant was designated as crude extract. The crude extract was dialyzed overnight against 50 mM Tris–HCl buffer, pH 7.2 to remove $MnSO_4$ and CTAB from the crude extract. The presence of $MnSO_4$ and CTAB in the enzyme extract prevented the adsorption of the enzyme on DEAE-Sepharose.

2.10. Chromatography on DEAE-Sepharose

The dialyzed crude extract was applied on the top of DEAE-Sepharose column (6×1 cm i.d.) previously equilibrated with the same buffer. The exchanged material was eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.4 M prepared in the same buffer at a flow rate of 40 mL/h and 3 mL fractions were collected. The major protein contamination with minor arginase activity was eluted by 0.0 M NaCl and designated Arg I. Protein fractions exhibiting major arginase activity were eluted at 0.3 M NaCl and designated Arg II.

2.11. Chromatography on Sephacryl S-200

Arg II pooled fractions were concentrated by sucrose and applied on a Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with the same buffer and developed at a flow rate of 20 mL/h and 3 mL fractions were collected. The enzyme was eluted with the same buffer.

2.12. Protein determination

Protein was quantified by the method of Bradford (1976). Bovine serum albumin was used as the protein standard.

2.13. Polyacrylamide gel electrophoresis

Electrophoresis under nondenaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of Davis (1964) using a Tris–glycine buffer, pH 8.3. Protein bands were stained with Coomassie Brilliant Blue R-250.

2.14. Molecular weight determination

Molecular weight was determined by gel filtration using Sephacryl S-200. The column (90×1.6 cm i.d.) was calibrated with cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (V_o). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). SDS-denatured phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,200) were used for the calibration curve.

3. Results and discussion

3.1. Urea cycle enzyme activities

Urea cycle enzyme activities, carbamoyl phosphate synthetase, arginase, ornithine carbamoyltransferase, argini-

Table 1
The activity of enzymes of the urea cycle in *F. gigantica*

Enzyme	Units[a]/g tissues
Carbamoyl phosphate synthetase	4 ± 0.23
Ornithine carbamoyltransferase	5 ± 0.3
Arginosuccinate synthetase	17 ± 0.9
Arginosuccinate lyase	47 ± 3.1
Arginase	680 ± 22

Each value is the mean $\pm$ SE for three determinations.

[a] Units expressed as micromoles of citrulline or urea/gram tissues.

Table 2
Purification scheme for *F. gigantica* arginase

Step	Total activity (Units[a])	Total protein (mg)	S.A. (Units/mg protein)	Fold purification	Recovery %
Crude extract	108	22.5	4.8	–	100
DEAE-Sepharose					
0.0M NaCl Arg I	15	6	2.5	0.52	13.8
0.3 M NaCl Arg II	66	0.68	97	20.2	61
Sephacryl S-200					
Arg II	32	0.22	145	30.3	29.6

[a] One unit of arginase activity was defined as the amount of enzyme that liberated 1 μmol urea per hour under standard assay conditions.

nosuccinate synthetase, and argininosuccinate lyase, of *F. gigantica* are presented in Table 1. Arginase had a very high activity (680 units/g tissues) compared to the other enzymes. Carbamoyl phosphate synthetase (4 units/g tissues) and ornithine carbamoyltransferase (7 units/g tissues) had very low activity. A moderate enzymatic activity was recorded for argininosuccinate synthetase (17 units/g tissues) and argininosuccinate lyase (47 units/g tissues). In liver fluke, carbamoyl phosphate synthetase, ornithine carbamoyltransferase (Kurelec, 1972) and argininosuccinate synthetase (Janssens and Bryant, 1969) were not detected. *F. hepatica* possessed a very active arginase (Kurelec, 1975). Two of the five enzymes of urea cycle, ornithine carbamoyltransferase and arginase, were present in *Ascaris lumbricoides* (Paltridge and Janssens, 1971). The distribution of ornithine carbamoyltransferase and argininosuccinate synthetase suggested that arginine synthesis is a property of both the chloragog tissue and the intestinal epithelium of earth worm *Lumbricus terrestris* (Prentø, 1989). The presence of appreciable activity of urea cycle enzymes in the tissues of *Sarcophaga ruficornis* suggested the possibility of the operation of the Krebs–Henseleit urea cycle in this carnivorous insect (Pant and Kumar, 1978). Ornithine carbamoyltransferase and arginase, but not arginase synthetase, were detected in the body wall and gut tissues of the leech (Natesan et al., 1992). A low to moderate activity levels of four urea cycle enzymes were determined during embryogenesis of *H. dromedarii*, ornithine carbamoyltransferase (1.25–23.75 units/g eggs), argininosuccinate synthetase (13.9–29.5 units/g eggs), argininosuccinate lyase (1.25–23.75 units/g eggs) and arginase (13.6–71.3 units/g eggs) (Fahmy et al., 1994a). The *F. gigantica* arginase value (680 units/g tissues) was near to the values of mammalian arginases, where rat arginase values fall between 159–2550 units/g tissues (Jenkinson et al., 1996). In conclusion, the low levels of *F. gigantica* urea cycle enzymes except to the arginase suggest the urea cycle is operative but its role is of a minor important. The high level of arginase activity may benefit for the hydrolysis of the exogenous arginine to ornithine and urea.

4. Purification of arginase from *F. gigantica*

Since one of the objectives of this study was the preparation of pure homogeneous arginase from *F. gigantica*

for further characterization, a simple reproducible method was established. The method of purification involved extraction, chromatography on DEAE-Sepharose column, followed by Sephacryl S-200 column. The purification of enzyme was summarized in Table 2. By chromatography on DEAE-Sepharose column (Fig. 1), two arginases Arg I and Arg II were separated, which was similar to enzyme preparation from land snails *Helix pomatia* (Baret et al., 1969), *Helix aspersa* (Baret et al., 1972) and *H. dromedarii* (Fahmy et al., 1994b). Due to the low activity level of Arg I, further purification was restricted to Arg II which contained the highest level of activity (61% of total activity) and the lowest level of protein (3% of total protein). This step is very important because it removed about 97% of contaminated proteins. The eluate from the Sephacryl S-200 column (Fig. 2) yielded a single activity peak and the protein was electrophoretically homogenous with a single band on native and SDS/PAGE (Fig. 3a,b).

4.1. Molecular weight

The molecular weight of purified *F. gigantica* Arg II was estimated to be 92,000 by gel filtration and SDS-PAGE and appeared as monomer. Invertebrate arginases occur as large
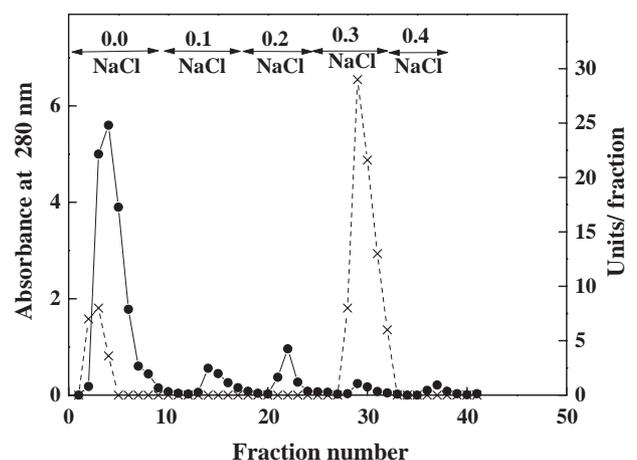

Fig. 1. A typical elution profile for the chromatography of *F. gigantica* arginase on DEAE-Sepharose column (6 × 1 cm i.d.) previously equilibrated with 50 mM Tris–HCl buffer, pH 7.2 at a flow rate of 40 mL/h and 3 mL fractions, absorbance at 280 nm (● —– ●), arginase activity (x —– x).
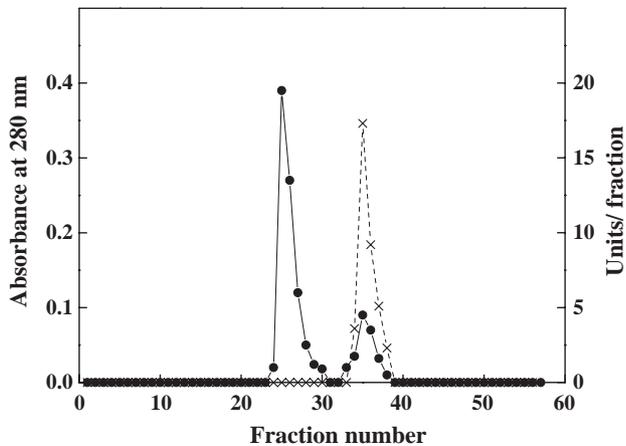
Fig. 2. A typical elution profile for *F. gigantica* arginase DEAE-Sepharose fraction on Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with 50 mM Tris–HCl buffer, pH 7.2 at a flow rate of 20 mL/h and 3 mL fractions, absorbance at 280 nm (● —– ●), arginase activity (x —– x).

multimers and perhaps also as monomers. Octomeric arginases of more than 220,000 Da molecular mass have been reportedly found in the land planarian, *Bipalium kewense*, (ureotele), and in the hepatopancreas of the land snail, *H. aspersa,* and the silkmoth *Hyalophora gloveri*, both uricoteles (Reddy and Campbell, 1969; Porembska, 1973). Large multimeric arginases of a similar molecular mass have been reported in a polychaete annelid and a crayfish, both ammonoteles (Porembska, 1973; O'Malley and Terwilliger, 1974). Arginase from human malaria parasite *Plasmodium falciparum* is a homotrimer of 160,000 Da. Dialysis of the arginase against EDTA results in monomers of approximately 48,000 Da (Muller et al., 2005). In contrast, small monomeric arginases of about 27,000 Da have been encountered in the gut of the earthworms *L. terrestris* and *Pheretima communissima*, both ureoteles, and in the gills of the marine mollusk

Concholepas concholepas, an ammonotele (Reddy and Campbell, 1968; Carvajal et al., 1984; Iino and Shimadate, 1986). Large monomeric arginases from the gills of the bivalve *Semele solida* (12,000 Da) (Carvajal et al., 1994), silkworm *Bombyx mori* (60,000–100,000 Da) (Davtyan et al., 1981) and mollusk *Chiton latus* (79,000 Da) (Carvajal et al., 1988) have been described.

4.2. K_m and substrate specificity

The K_m value for *F. gigantica* Arg II was determined according to the method of Lineweaver and Burk (1934), where the K_m and V_{max} were 6 mM arginine and 1.17 µmol urea, respectively (Fig. 4). This value was in the range of the K_ms of invertebrate arginases which vary widely between about 2 mM in silkmoths and crayfish up to about 158 mM in a polychaete annelid (Porembska, 1973; O'Malley and Terwilliger, 1974). The K_m value for arginase from human malaria parasite *P. falciparum* was 13 mM arginine (Muller et al., 2005).The K_m values for mammalian arginases exhibited wide variation, with values of 1–20 mM for rat liver arginase and 4–45 mM for other rat tissues (Jenkinson et al., 1996). The K_m's of arginases from embryologically different nephron segments of the *Meriones shawi* kidney ranged from 1.6 to 18.8 mM arginine (Hus-Citharel and Levillain, 1999). L-Canavanine was tested for its capacity to function as substrate for *F. gigantica* Arg II. The enzyme showed high specificity for arginine, but hydrolyzed L-canavanine to a low extent with an arginine: canavanine ratios above 10. The arginase present in the livers of uricotelic and ureotelic animals has similar substrate specificity. All hydrolyze L-arginine and to some extent L-canavanine (Mora et al., 1965). Arginase/canavanase ratios (>10) have been reported from rat liver (Mora et al., 1966), silkmoth (Reddy and Campbell, 1969), polychaete annelid
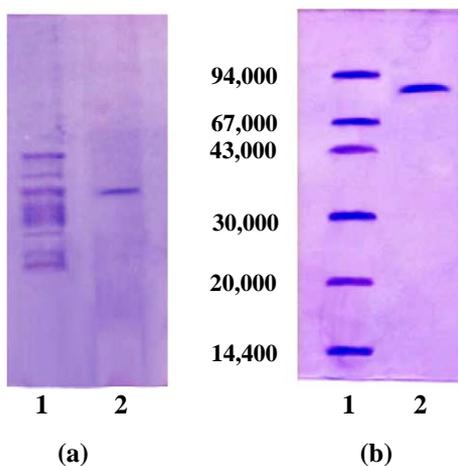

Fig. 3. (a) Polyacrylamide gel electrophoresis for *F. gigantica* arginase during purification steps. 1— Crude extract; 2— Sephacryl S-200 Arg II. (b) SDS-PAGE for molecular weight determination of *F. gigantica* arginase. 1— Standard proteins; 2— Sephacryl S-200 Arg II.
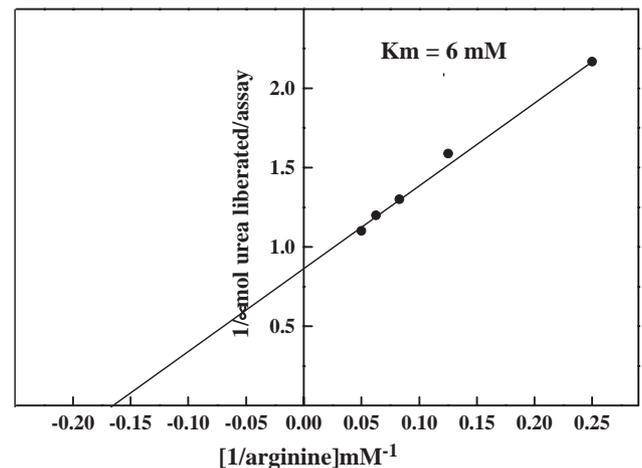

Fig. 4. Lineweaver–Burk plot relating *F. gigantica* Arg II, reaction velocities to arginine concentration. The reaction mixture contained in 1.0 mL: 50 mM glycine buffer, pH 9.5, 1 mM MnCl$_2$, a suitable amount of enzyme preparation and different concentrations of arginine ranged from 4 to 20 mM. Each point represents the average of two experiments.
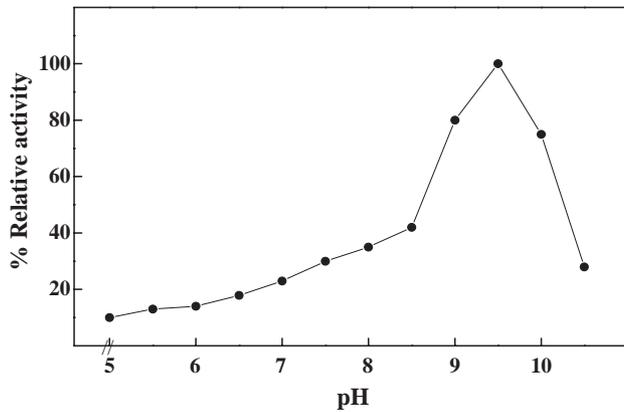
Fig. 5. pH optimum of *F. gigantica* Arg II. The reaction mixture contained in 1.0 mL: 20 mM arginine, 1.0 mM MnCl$_2$, a suitable amount of enzyme preparation and 50 mM acetate buffer (pH 5.0–5.5), sodium phosphate buffer (pH 6.0–7.5), Tris–HCl (pH 8–8.5), glycine buffer (pH 9.0–9.5) and sodium bicarbonate buffer (pH 10–10.5). Each point represents the average of two experiments.

(O'Malley and Terwilliger, 1974) and *H. dromedarii* (Fahmy et al., 1994a). Earthworm *P. communissima* arginase differs from many other arginases by lacking canavanase activity (Iino and Shimadate, 1986).

4.3. pH optimum

The pH optimum of *F. gigantica* Arg II was 9.5 (Fig. 5). This is close to those for several other arginases that range from 9.3 to 10.5 (Campbell, 1966; Carvajal et al., 1984; Fahmy et al., 1994a). Mammalian arginases appear to have basic pH optima of 9.5–10.5, although some exceptions have been noted (Jenkinson et al., 1996).The optimum pH of human vitreous humor arginase was 8.8 (Gursu, 2001). One rat arginase isoenzyme had a pH optimum of 7.5 (Gasiorowska et al., 1970) and a minor arginase component was recorded in beef liver with an optimal pH of 7.0 (Robbins and Shields, 1956). Remarkably, the purified arginase from *Heliobacter pylori* had an acidic pH optimum of 6.1 (McGee et al., 2004).
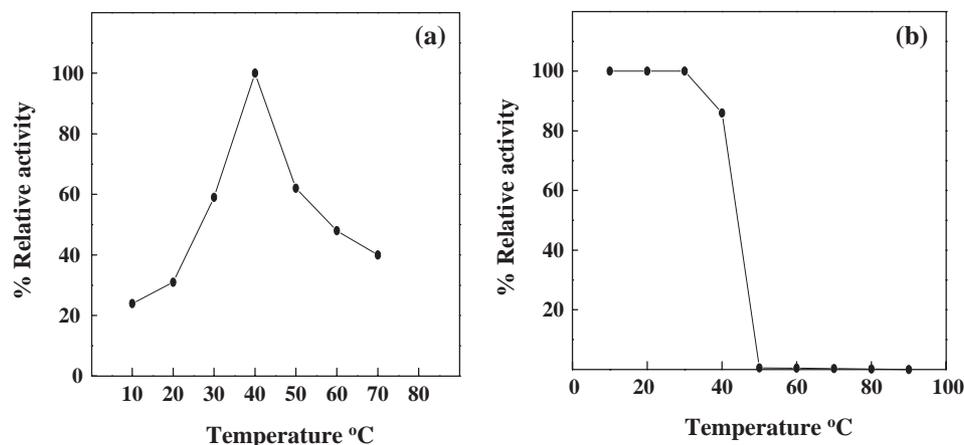
Table 3
Inhibitory effects of proline and branched chain amino acids (5 mM) on *F. gigantica* Arg II at pH 9.5

Amino acid	% Relative activity
Lysine	33
Isoleucine	57
Ornithine	69
Valine	75
Leucine	77
Proline	85

The arginase activity was measured in the presence of the amino acid listed. Activity in absence of amino acids was taken as 100%.

4.4. Effect of temperature

The optimum temperature of *F. gigantica* Arg II was recorded after by measuring the enzyme activity under standard assay conditions at various temperatures, where it detected at 40 °C (Fig. 6a). The thermal stability was investigated by measuring the residual activity of the enzyme after 15 min of incubation at different temperatures prior to substrate addition. The enzyme was stable up to 30 °C and retained 80% of its activity after incubation at 40 °C for 15 min and lost all of its activity at 50 °C (Fig. 6b). *H. dromedarii* arginases A and B showed no loss in their activities up to 70 °C, whereas a loss of 53.84% and 62.5% was recorded at 80 °C, respectively (Fahmy et al., 1994a). However, arginase from fresh water teleostean fish *Clarias batrachus* was found to be stable between 10 and 37 °C, its activity was decreased at higher temperature and showed no activity at 80 °C (Singh and Singh, 1988). For mammals, the optimum temperature for human vitreous humor was 40 °C (Gursu, 2001).

4.5. Effect of amino acids

Branched chain amino acids are well known inhibitors of arginase from several species and tissues. Of the branched chain amino acids and proline tested at concentration of 5 mM, those indicated in Table 3 were inhibitory to the Arg II

Fig. 6. Temperature optimum (a) and temperature stability (b) of *F. gigantica* Arg II. Each point represents the average of two experiments.

from *F. gigantica*. The order of effectiveness of amino acids as inhibitors of enzyme was found to be lysine>isoleucine>ornithine>valine>leucine>proline with 67%, 43%, 31%, 25%, 23% and 15% inhibition, respectively. The same amino acid caused a significant inhibition for arginases from *H. dromedarii* (Fahmy et al., 1994a), earthworm *P. communissima* (Iino and Shimadate, 1986) and the gill tissue of the bivalve *S. solida* (Carvajal et al., 1994). It is known that arginase is a cytosolic enzyme in the liver of ureotelic species (Multhaupt et al., 1987), whereas in some non-hepatic tissues and many nonureotelic species, the enzyme is localized in the mitochondria (Carvajal et al., 1987). It is also known that rat liver arginase is much more sensitive to inhibition by branched chain amino acids than the mitochondrial enzyme from rat kidney, and this was taken as evidence to suggest a regulatory role for these amino acids on arginine hydrolysis in the liver cells (Carvajal and Cederbaum, 1986). L-ornithine and L-lysine caused competitive inhibition for liver (Carvajal et al., 1999) and *Mus booduga* (Gray) (Prasad et al., 1997) arginases. In contrast, the slight activation caused by lysine on purified arginase from *Rhodobacter capsulatus* (Moreno-Vivian et al., 1992), has been observed for the enzyme from other sources.

4.6. Effect of metal ions

One general property of arginase is the requirement of a bivalent metal ion for full activity (Turkoglu and Ozer, 1992; Reczkowski and Ash, 1992; Carvajal et al., 1994) and structural stabilization of the enzyme (Green et al., 1990, 1991). The metal ion requirement of arginase is satisfied, especially by Mn^{2+}, but activation by some other metal ions has also been reported (Hirsch-Kolb et al., 1971). Mammalian arginases I and II require intact binuclear manganese cluster for the hydrolysis of L-arginine to generate L-ornithine and urea (Christianson, 2005). Preincubation of human vitreous humor arginase with Mn^{2+} caused a significant increase (33%) in arginase activity (Gursu, 2001). The effects of different divalent cations at a concentration of 1 mM on *F. gigantica* Arg II, after dialysis against Mn^{2+}-free 0.05 M Tris–HCl buffer, pH 7.2 are shown in Table 4. The enzyme was activated with Mn^{2+}, where the other metals Fe^{2+}, Ca^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+} and Mg^{2+} had inhibitory effects. The arginase from earthworm *P. communissima* was extremely labile, losing 90% of its original activity by dialysis against a Mn^{2+}-free buffer for 4 h. Seventy percent of its original activity was recovered following treatment with 1 mM $MnCl_2$ at 37 °C for 20 min (Iino and Shimadate, 1986). In the same manner, *S. solida* arginase was activated by Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, but not by Mg^{2+} and Ca^{2+}, after inactivated by EDTA (Carvajal et al., 1994). They reported that the K_m value for arginine and the affinity of the enzyme–lysine interaction was not dependent on the type of metal ion bound to arginase from *S. solida*, suggesting that the main function of the metal ion

Table 4
Effect of divalent cations on *F. gigantica* Arg II

Divalent cation	% Relative activity
Fe^{2+}	22
Ca^{2+}	23
Hg^{2+}	29
Ni^{2+}	42
Co^{2+}	54
Mn^{2+}	150
Mg^{2+}	77

The enzyme was dialyzed against Mn^{2+}-free 0.05 M Tris–HCl buffer, pH 7.2 and preincubated for 15 min at 37 °C with 1 mM of listed metal cations prior to substrate addition. Activity in absence of cations was taken as 100%. Each value represents the average of two experiments.

is not in binding of arginine to arginase, but in the hydrolysis of the enzyme-bound substrate. This is particularly interesting because, for several years, it was tacitly assumed, but not proved, that the metal ion serves as a bridge for binding of the substrate to the enzyme (Mildvan, 1970). Therefore, it would be of considerable interest to know if these observations have any general validity for arginase. With the available information, a definitive conclusion cannot be reached. Thus, Maggini et al. (1992) reported experiments in cytosolic fractions of rat liver cells showing that the K_m value for arginine is 2- to 3-fold lower in the presence of added Mn^{2+} than in its absence; this would indicate that substrate binding to rat liver arginase is affected by the metal ion. Arginases I (liver) and II (kidney) from rat were strongly inhibited by cupric and mercuric ions (Tormanen, 2001). *H. pylori* arginase exhibited optimal catalytic activity with cobalt as a cofactor, manganese and nickel were significantly less efficient in catalyzing the hydrolysis arginine (McGee et al., 2004).

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