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Properties of Arginase from the Hepatopancreas of Giant Freshwater Prawn (*Macrobrachium rosenbergii,* de Man)

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ABSTRACT
We describe the hepatopancreas arginase activity of freshwater prawn (*Macrobrachium rosenbergii*). The enzyme was isolated using reactive blue 2- agarose affinity chromatography and gel filtration on Sephadex G-150. The enzyme had a specific activity of 5.70 µmol/min/mg of protein. The enzyme exhibited a maximal activity at pH 8.5 and K_m of 12.5 mM. The enzyme was capable of hydrolysing L-arginine and to a lesser extent, L-arginine monohydrochlorate and L-arginine monohydrate. The optimum temperature of the enzyme was 35 ^{0}C. The molecular weight as determined by gel filtration was approximately 160,000 dalton and SDS-PAGE, was 22,000 dalton. The different amino acids (L-lysine, L-cysteine, L-valine, L-proline, L-aspartic acid, L-glutamic acid and L-serine) and metal ions (Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+}) did not show any inhibition on the enzyme activity. The enzyme was activated with Mn^{2+} and different concentration of Mn^{2+} had no effect on the enzyme activity. EDTA, citrate and urea showed considerable inhibition on the enzyme activity.
Key words: Freshwater prawn; arginase; uricotelism; invertebrates; hepatopancreas

INTRODUCTION

Arginase (L-arginine ureahydrolase, or amidinohydrolase, EC 3.5.3.1) is the terminal enzyme of the urea cycle among the six enzymes [1]. The enzyme was found to exist in two forms and has a broad tissue distribution [1,2]. The arginase type I form is highly expressed in the liver or hepatic cells and is important in ureogenesis. Extra-hepatic arginase type II form is thought to be involved in the biosynthesis of polyamines, the amino acids ornithine, proline and glutamate and in the inflammatory process [1]. The function of arginase in microbes and invertebrates is mostly unknown. It is speculated that the urea cycle evolved from a biosynthetic pathway for L-arginine and appeared for the first time in amphibians as an adaptation to air-breathing in a terrestrial environment [3]. Many invertebrates are uricotelic organisms and eliminate excess nitrogen in the form of solid uric acid [1]. Nevertheless, a large number of uricotelic organisms possess arginase. It is thought that their generation of L-ornithine by arginase feeds into the production of L-proline, L-glutamate and polyamines used for collagen synthesis, energy metabolism and cell proliferation [1].

Nitrogen excretion had been studied extensively in vertebrates while 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 [1]. Most studies have concentrated on mammalian liver arginases [1, 4]. In addition to urea synthesis in the liver of ureotelic species, arginase is also involved in biosynthesis of polyamines and proline [5], conversion of arginine into α-ketoglutarate for oxidation in the Krebs cycle [6], adaptive responses to anoxia in some invertebrates [7] and production of urea for osmoregulatory purposes [8]. It should be noted that arginase is found in various tissues of non-ureotelic organisms, including liver, but is not part of a functional urea cycle [9]. It had previously been thought that there were significant kinetic and structural differences between ureotelic and non-ureotelic arginases [10], but many studies suggested that the characteristics of arginases are not consistent with a particular mode of nitrotelism [11, 12]. Non-ureotelic arginases are generally similar to ureotelic arginases but can be distinguished immunologically [12,13].

In uricotelic organisms, that include bacteria, fungi, invertebrates, reptiles and birds [1, 14,] and

ammonotelic organisms such as fish and amphibians (at early stage of development) [1,15,16], complete urea cycle enzymes are lacking. Thus, the function of arginase in these organisms is the production of ornithine which is channelled into metabolic pathways associated with proline and glutamate production [1]. Ammonia is the main nitrogenous excretion product of freshwater Teleost fishes while urea excretions in teleost make up the secondary but significant component of total nitrogen excretion [17]. This work therefore is to determine the arginase activity in hepatopancreas of freshwater prawn and possibly assign a role for the enzyme in the organism.

MATERIALS AND METHODS

Materials

Trizma base, Trizma-HCl, ethylenediamine tetra acetic acid (EDTA), Reactive Blue 2- crosslinked agarose (suspension) and p - dimethylaminobenzaldelyde (Ehrlich reagent) were purchased from Sigma Chemical Company, St. Louis, Mo, USA. Sucrose, sodium chloride, orthophosphoric acid and manganese chloride tetrahydrate where purchased from BDH Chemicals, Poole House, London. All other reagents were of analytical grade. Giant Freshwater Prawn (*Macrobrachium rosenbergii*) were purchased from a local market in Ile-Ife, Nigeria.

Methods

Assay method.

Arginase activity was determined by the measurement of urea produced by the reaction of Ehrlich's reagent according to the method previously described by [5]. The protein concentration was determined by the method of [18] using bovine serum albumin (BSA) as standard.

Purification of Freshwater prawn arginase

Unless otherwise stated all steps were performed at 4–37 ^{0}C using 5 mM Tris–HCl buffer, pH 7.5. The crude extract was prepared by cutting open the shells of the prawns and the hepatopancreas excised and stored in the refrigerator until required.

Reactive Blue 2-Agarose Chromatography

The resin was equilibrated in 5mM Tris-HCl buffer, pH 7.5 containing 5 mM $MnCl_2$ and later packed into 2.5 × 20 cm column. It was then eluted with a linear gradient of 0-1 M KCl. The enzyme solution was layered on the packed column 2.5 × 20 cm and eluted with a 400 ml linear gradient of 0-1 M KCl in 5mM Tris-HCl buffer, pH 7.5. Fractions of 5 ml were collected from the column at a flow rate of 30 ml per hour. Protein profile was monitored spectrophotometrically at 280 nm. The fractions were also assayed for arginase activity. The active fractions were pooled and dialysed against 50% glycerol in 5mM Tris-HCl buffer, pH 7.5.

Gel Filtration on Sephadex G-150

The enzyme fraction from the preceding step was then applied to the Sephadex G-150 column (2.5 × 40 cm) previously equilibrated with 5mM Tris-HCl buffer, pH 7.5. Fractions of 4 ml were collected at a flow rate of 10 ml per hour. The active fractions were pooled and dialysed against 50 % glycerol in 5mM Tris-HCl buffer, pH 7.5. The pooled enzyme was pure as judged by the presence of only one band of protein after SDS and non-SDS polyacrylamide gel electrophoresis.

Molecular Weight Determination

The native molecular weight was determined on a Biogel P-200 column (2.5 X 90 cm). The column was equilibrated with the following standard proteins: ovalbumin (M_r 45,000; 3 mg/ml), bovine serum albumin (M_r 67,000; 5 mg/ml), creatinine phosphokinase (M_r 88,000; 5 mg/ml), γ-globulin (M_r 150,000; 5 mg/ml) and pyruvate kinase (M_r 230,000; 5 mg/ml). The void volume of the column was determined by the elution volume of Blue Dextran. The subunit molecular weight of the enzyme was determined by SDS polyacrylamide gel electrophoresis as described by [19]. Standard proteins were as contained in Sigma Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis (Daltons Mark VII-L, Molecular Weight Marker Range 14,000-70,000).

Determination of Kinetic Parameters and Substrate Specificity

The kinetic parameters of the enzyme were determined as described by [5] in 2mM Tris-HCl buffer, pH 9.5 containing 0.01 mM $MnCl_2$. The kinetic parameters were estimated from the double reciprocal plot [20]. The substrate specificity study was carried out using arginine monohydrochloride and arginine monohydride.

Effects of Amino Acid on Enzyme Activity

The effects of amino acids (L-lysine, L-cysteine, L-valine, L-proline, L-aspartic acid, L-glutamic acid and L-serine) on the activity of prawn arginase were studied. The amino acids (25 mM) were

used as substrates, while all other conditions of the assay remained the same.

Effects of Metal ions on Enzyme Activity

The method of [5] was used to study the effects of the following salts of cations on the activity of arginase: $CoCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$ at a final concentration of 1 mM and 1.5 mM in a typical enzyme assay. Also the effect of further concentrations of $MnCl_2$ (1 mM-5mM) on the enzyme activity was also studied.

Effects of Chelating compounds (EDTA, citrate and urea) on enzyme activity

The effects of metal chelating compounds on the activity of freshwater prawn hepatopancreas arginase were studied. Enzyme preparation was incubated with different concentrations (10 mM - 50 mM) of the required chelating compound at pH 7.5 at 37 ^{0}C for 30 min. An aliquot was tested for arginase activity.

Effect of Temperature on the Enzyme Activity

The activity of arginase was assayed at temperatures between 30 ^{0}C and 100 ^{0}C to determine the optimum temperature.

Effect of pH on the Enzyme Activity

The effect of pH on arginase was studied by assaying the enzyme using the following buffers of different pH values: 5 mM citrate buffer (pH 5.0-6.5), 5 mM phosphate (pH 6.5-8.0), 5 mM Tris-HCl buffer (pH 8.0-11.0). One ml of the reaction mixture contained 2.5 mM of the appropriate buffer, 0.1 M arginine and 20 µl enzyme preparations.

RESULTS

Purification of Arginase

The results of the purification of arginase from freshwater prawn hepatopancreas are summarized in Table 1. The elution profiles after reactive blue 2-agarose affinity chromatography and gel filtration on Sephadex G-150 are shown in Figures 1and 2 respectively.

Molecular Weight Determination

The native molecular weight was found to be 160,000 dalton on Biogel P-200 while the subunit molecular weight estimated from SDS-PAGE was approximately 22,000 dalton (Figure 3-4).

Kinetic Parameters

Figures 5-7 shows the Lineweaver-Burk plots of the different substrates. The K_m values of arginine, arginine monohydrochloride and arginine monohydride are presented in Table 2.

Effects of Chelating compounds (EDTA, citrate and urea) on enzyme activity

Figure 8 shows the effects of the different chelating compounds on the enzyme activity.

Effects of temperature and pH on Enzyme Activity

The maximum activity of the enzyme was obtained at pH of 8.5 was observed (Figure 9). While optimum temperature was 35 ^{0}C (Figure 10).

Effects of metal ions on enzyme activity

Table 3 shows the results of the effect of different cations on arginase activity. The enzyme was activated by the metals investigated. The enzyme was activated by Mn^{2+} and different concentration of Mn^{2+} had little or no effect on the enzyme activity (Table 4).

Effects of amino acids on Enzyme Activity

The effects of amino acids on the activity of the enzyme is presented in Table 5. The effectiveness of these amino acids as inhibitors of the enzyme activity was found to be similar.

Table 1: Purification profile of freshwater prawn (*M. rosenbergii*) hepatopancreas arginase

	Total protein(mg)	Total activity(U)	Specific activity(U/mg)	Purification fold	% Yield
Crude	19,893.60	34,217.00	1.72	1.00	100.00
Affinity chromatography	12,922.50	29,561.43	6.71	3.90	64.96
Sephadex G-150	2004.50	19,307.34	9.63	5.60	10.00

Each step was carried out as described in the text. Activity was measured by monitoring the urea produced in the reaction. Protein was determined by the method of [18]. A unit of activity is the amount of the enzyme that will catalyse the hydrolysis of micromole of arginine per minute under the condition described in the text.

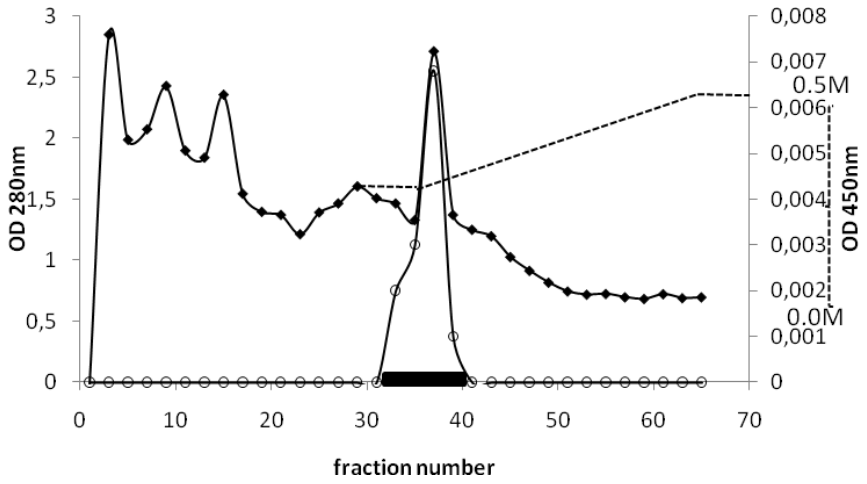

Figure 1: Reactive Blue 2 crosslinked agarose affinity chromatography column (2.5 × 20 cm) of freshwater prawn hepatopancreas arginase. The enzyme solution was layered on the packed column and eluted with a linear gradient of 0.05 M KCl in 5mM Tris-HCl buffer, pH 7.2. ○-○-○ activity, ■-■-■ protein and ▬▬ pooled fractions.

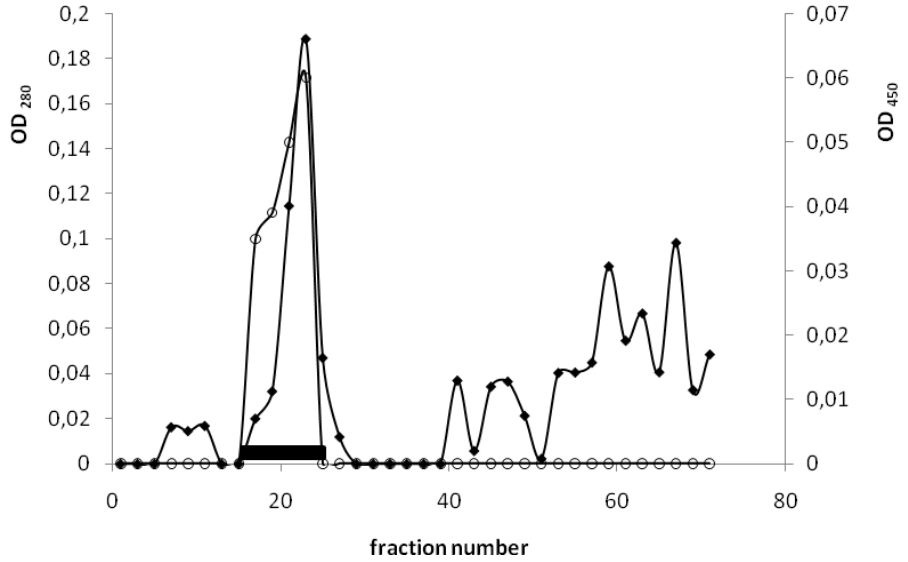

Figure 2 Elution profile of freshwater prawn (*M. rosenbergii*) hepatopancreas arginase after Sephadex G-150 chromatography (column 2.5 × 40 cm) of freshwater prawn hepatopancreas arginase. The eluant was 5mM Tris-HCl buffer, pH 7.5. ○-○-○ activity, ▲-▲-▲ protein and ▬▬ pooled fractions.

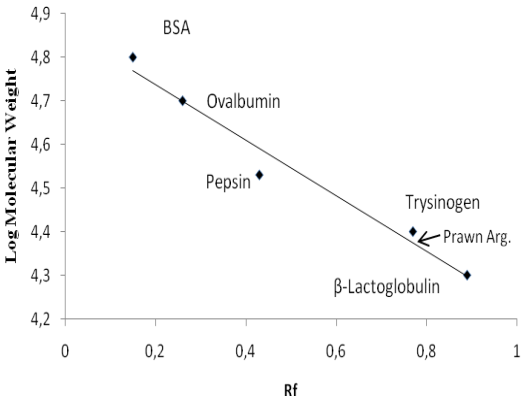

Figure 3: Calibration curve on 10% SDS-PAGE for subunit molecular weight determination of freshwater prawn (*M. rosenbergii*) hepatopancreas arginase. The separating gel was a 10% rod gel. The running buffer was 0.1M phosphate, pH 7.2. Other conditions were as described in the text. The standard proteins were as contained in Sigma Molecular Weight Markers Calibration Kit: Bovine serum albumin (BSA) (M_r 67,000 dalton), ovalbumin (45,000 dalton), pepsin (37,000 dalton), tryptinogen (24, 000 dalton) and β-lactoglobulin (14,000).

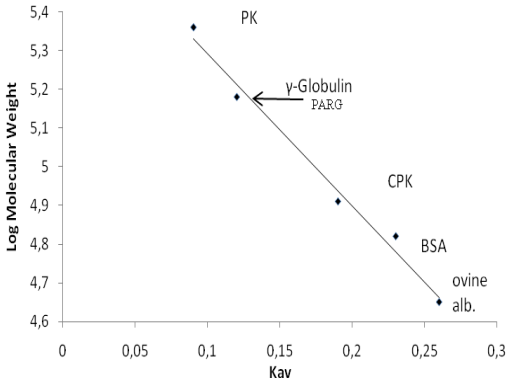

Figure 4: Calibration curve on Biogel P-200 for native molecular weight determination of freshwater prawn (*M. rosenbergii*) hepatopancreas arginase. The native molecular weight was determined on a Biogel P-200 column (2.5 × 90 cm). The standard proteins as indicated on the curve are ovalbumin, bovine serum albumin (BSA), creatinine phosphokinase (CPK), γ-globulin and pyruvate kinase (PK). Freshwater prawn hepatopancreas arginase (PARG) is indicated by the arrow.

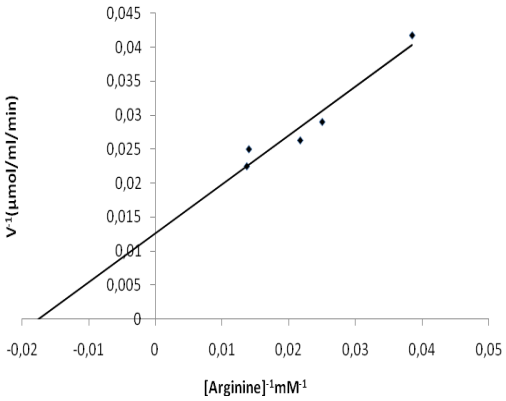

Figure 5: The Lineweaver-Burk plot showing the effect of varying concentrations of arginine on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase on the initial reaction velocity at pH 9.5. The reaction mixture is as described in the text. The lines through the points were drawn by the method of regression. Enzyme activity is expressed in micromole per min.

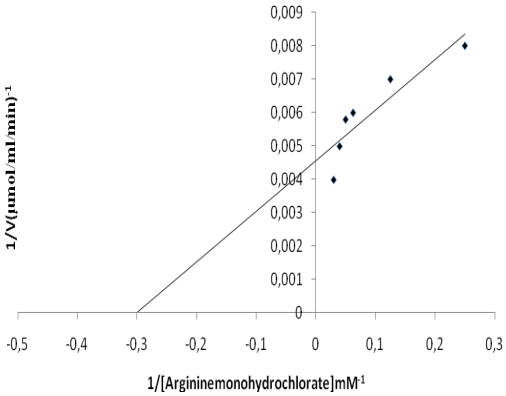

Figure 6: The Lineweaver-Burk plot showing the effect of varying concentrations of arginine monohydrochlorate on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase on the initial reaction velocity at pH 9.5. The reaction mixture of 1 ml contained 1 mM Tris-HCL buffer, pH 9.5, 1 mM $MnCl_2$, 50 µl of enzyme and arginine concentration between 25 mM and 250 mM.

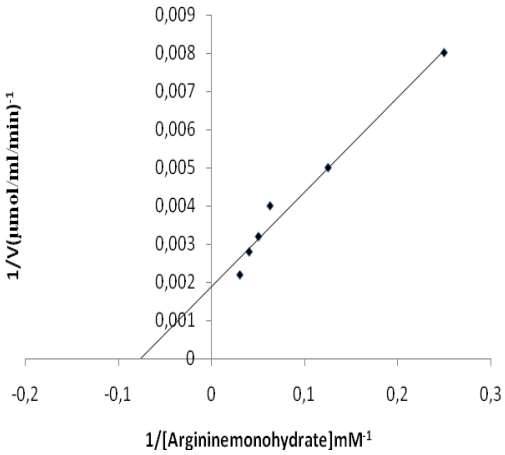

Figure 7: The Lineweaver-Burk plot showing the effect of varying concentrations of arginine monohydrate on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase on the initial reaction velocity at pH 9.5. The reaction mixture of 1 ml contained 1 mM Tris-HCL buffer, pH 9.5, 1 mM $MnCl_2$, 50 µl of enzyme and arginine monohydrate concentration between 25 mM and 250 mM.

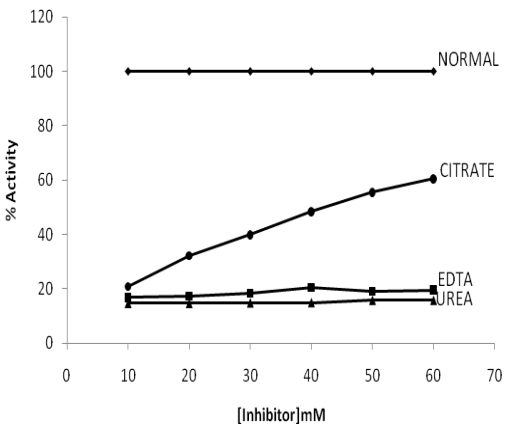

Figure 8: Effect of varying concentration of citrate, EDTA, and urea on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase

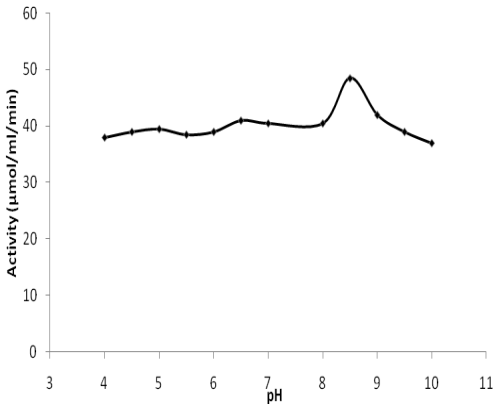

Figure 9: Effect of pH on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase. One ml of the reaction mixture contained 2.5 mM of the appropriate buffer, 0.1 M arginine and 0.02 ml enzyme preparation. The following buffers of different pH values were used for the pH determination: 5 mM citrate buffer (pH 6.0-6.5), 5 mM phosphate (pH 6.5-8.0), 5 mM Tris-HCl buffer (pH 8.0-11.0).

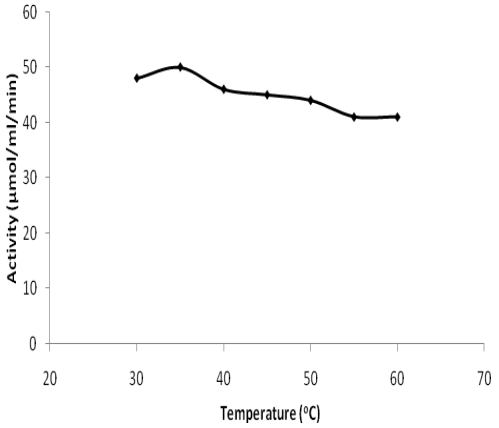

Figure 10: Effect of temperature on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase activity. The assay mixture containing 50µl was first incubated at the indicated temperature for 10 min before initiating the reaction by the addition of an aliquot of the enzyme.

Table 2: Kinetic parameters of arginine and alternate substrates of arginase the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase

Substrate	K_m (mM)	V_{max} (µmol/ml/min)
Arginine	12.5	76.9
Arginine monohydrate	12.8	555.6
Arginine monohydrochlorate	33.0	217.4

Purified enzyme was incubated for 30 min in the assay system except that the Mn^{2+} concentration was varied as indicated.

Table 3: Effects of metal ions on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase activity

Metal ions	% Residua 100µM	200µM	500µM
Ni^{2+}	93%	98%	100%
Co^{2+}	96%	105.8%	96%
Zn^{2+}	96%	96%	92%
Mn^{2+}	100%	105.8%	105.8%
Mg^{2+}	90.8%	92%	92%

Enzyme assay was carried out using the standard assay mixture containing 0.05 ml of each salt at the final concentrations of 100 µM, 250 µM, and 500 µM. The values are mean of triplicate determinations

Table 4: Effect of varying concentration of manganese chloride on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase activity

$[MnCl_2]$ mM	% Activity
5	103.5%
10	107.0%
15	107.9%
20	107.9%
25	123.0%
30	124.2%

Table 5: Effects of different amino acids on the freshwater prawn (*M. rosenbergii*) hepatopancreas arginase

	% Residual Activity	
Amino acid	0.05M	0.1M
Proline	108.3%	110.4%
Lysine	104.2%	106.7%
Valine	102.1%	104.2%
Serine	137.5%	143.8%
Glutamate	91.7%	102.1%
Aspartate	110.8%	102.1%
Cysteine	101.7%	113.3%

The reaction mixture is as described in the text. The values are mean of triplicate determination

DISCUSSION

Hepatopancreas arginase was isolated from freshwater prawn (*Macrobrachium rosenbergii*, de Man) using affinity chromatography with Reactive Blue 2- Agarose and Biogel P-200 gel filtration chromatography. Quantitative details of the enzyme preparation are presented in Table 1. The procedure yielded an arginase with specific activity of 5.7 µmole/min/mg (unit/mg) of protein representing about 10- fold over that found in the crude homogenate. [21] had reported a purification fold of about 25, 340 and a yield of 11 % for *Iris hollandica* bulb arginase. Also, [22] used Matrex Green and arginine-linked Sepharose 4B for loblolly pine (*Pinus taeda* L.) arginase with a purification fold of 148.

This specific activity value was found to be lower than that reported for most ureotelic and uricotelic arginases. The arginase of rat kidney had a specific activity of 23.9 unit/mg [5], human blood serum: 0.19 units/mg [23], and human full-term placenta: 90 units/mg [24]. [25] obtained 213.0 units/mg from rat fibrosarcoma. The high level of arginase in *Iris hollandia* bulb and some germinating plants is consistent with elevated activity in germinating plants [21, 26, 27].

The molecular weight (M_r) of the native enzyme was estimated to be 160,000 dalton by gel filtration on Biogel P-200 and a subunit molecular weight of 22,000 dalton obtained by sodium dodecyl sulphate gel electrophoresis. Ureotelic

arginases have been shown to have molecular weight range of 110,000-140,000 dalton and uricotelic arginases (including the ammonotelic) having molecular weight range of 180,00-280,000 dalton [1, 15, 16]. Some plants species (*Evernia prunastri* cotyledon arginase) have been found to have exceptionally very high molecular weight above 320,000 dalton. Most of the ureotelic liver enzymes have been found to be trimeric in structure [1]. Some are reported to be tetrameric and dimeric in structure. Human full-term placenta arginase was found to be 70,000 dalton [24]. The uricotelic arginases have been reported to be hexameric [1, 15, 16]. In comparison, the cumulative molecular weight evidences supports this view that most eukaryotic arginases are probably tetramers (mice liver arginase has a molecular weight of 127,000 and a sub unit molecular weight of 38,500 [29]. It is apparent that the molecular-weight values obtained for this enzyme indicate an oligomeric protein with possible octameric structure.

The k_m values of the freshwater prawn hepatopancreas arginase for arginine and other substrates is shown in Table 2. This value (12.5 mM) 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 [30, 31]. The k_m values of ureotelic organisms' falls between 1-20 mM [16] while that of the uricotelic ranges from 40-200 mM [1]. *M. rosenbergii* shows ureotelic k_m value. The uricotelic enzymes are found in other parts of the body where they perform different functions in these organisms and also in the liver [16]. Moreover, low k_m values have been reported for some other uricotelic organisms [32-34]. Furthermore, in some ureotelic organisms, k_m values obtained from extrahepatic tissues showed different properties that makes it difficult for classifying these arginases into type I (liver enzymes) and type II (extrahepatic enzymes). Some investigators have further shown that these organisms do not possess the complete urea cycle enzymes, therefore, the arginase present in them must be for the production of ornithine which is channelled into other metabolic pathways [1, 5, 35].

Different guanidino compounds were tested as alternative substrates for the hepatopancreas arginase of prawn. The k_m of prawn hepatopancreas arginase for L-arginine was found to be a slightly lower compared to that of L-arginine monohydrate and L-arginine monohydrochloride (Table 2), an indication that the enzyme from prawn hepatopancreas has preference for L-arginine as substrate. Different workers have shown in their reports that L-arginine is the preferred substrate for arginase (both arginases; type I and type II) [1, 21, 23, 36, 37].

Chelating compounds (EDTA, urea and citrate) strongly inhibited freshwater prawn hepatopancreas arginase activity. EDTA has been reported to completely inactivate arginase [23]. [5] showed that incubation of rat kidney arginase in a solution of EDTA did not affect the enzyme activity significantly. [38] working on *Saccharomyces cerevisiae,* observed strong inhibition of the enzyme with EDTA and other chelating compound. The enzyme was also strongly inhibited by citrate at a very low concentration of 10 mM (Figure 8).

The requirement for Mn^{2+} as a metallic cofactor seems to be common for most reported arginases, although, other divalent cations, e.g. Co^{2+}, Fe^{2+} or Ni^{2+} have been reported to be activators [39, 40]. The effects of Mn^{2+} concentration was investigated on prawn hepatopancreas arginase (Table 4). The result revealed that prawn hepatopancreas arginase was not affected by increase in Mn^{2+} concentration. Similar result was observed by [31] with *P. pacifica* arginase. The effects of the different cations (Ni^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+}) on the activity of prawn hepatopancreas arginase showed no inhibition. Similarly, [15, 16] have reported the divalent cations (Co^{2+} and Ni^{2+}) requirement of some arginases and in some instances by Fe^{2+}, Vo^{2+}, and Cd^{2+} [41, 42]. Also, the amino acids (L-lysine, L-cysteine, L-valine, L-proline, L-aspartic acid, L-glutamic acid and L-serine) did not inhibit the enzyme.

An optimum temperature of 35 ^{0}C was obtained for freshwater prawn hepatopancreas arginase. The arginase from the *S. salmon* was reported to have an optimum temperature of 45 ^{0}C [43]. The temperature optimum for *P. pacifera* was about 60 ^{0}C [31]. The maximum activity for *V. catjang* cotyledon arginase and buffalo liver arginase were at 35 ^{0}C and 45 ^{0}C respectively [27].

The effect of pH on arginase activity has been studied extensively by many investigators. The influence of pH on freshwater prawn hepatopancreas arginase was studied and an optimum pH of 8.5 was obtained. This pH value is

in consonance with the optimum pH range of 9.0-9.5 for buffalo liver arginase [27] and human blood serum [23]. Also an optimum pH of 9.7-9.8 was reported for chicken kidney [44]. Some exceptions have been noted. [45] have found four different iso-enzymes of arginases in rat tissues, one of which had a pH optimum of 7.5. [24] obtained an optimum pH of 9.1 from human full-term placenta arginase. Optimum pH of 10.0 was reported for arginases of rat fibrosarcoma [25] and bovine liver [36], cotyledon arginase [27].

In conclusion, freshwater prawns, like all crustaceans, have a hard outer skeleton that must be shed (molting) regularly for growth to occur. Increases in body weight and length of the prawn principally occur soon after completion of each molt. Furthermore, freshwater prawn excretes ammonia as its nitrogen waste. The role of arginase in these organisms could be the synthesis of ornithine and proline which are essential in the growth of the exoskeleton.

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