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Physicochemical Properties of Liver Arginase from *Heterotis niloticus* (Cuvier 1829)

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Abstract
We investigated the activity of arginase in the liver of *Heterotis niloticus*. The enzyme was partially purified and characterized. The native molecular weight was estimated to be 178,000 dalton with Biogel P-200. The enzyme acted on not only L-arginine, but also L-arginine monohydrochloride and L-arginine monohydrate with preference for L-arginine. The enzyme had a specific activity of 6.18 µmol/min/mg of protein. The enzyme exhibited a maximal activity at pH 5.5 and K_M of 11.6 mM. The optimum temperature was found to be 35^0C. The enzyme showed increased activated with Mn^{2+}, Zn^{2+}, Mg^{2+}, Hg^{2+}, Co^{2+}, and Na^+ while the other metals Sn^{2+}, Ni^{2+} and Ba^{2+} showed slight inhibition. The order of effectiveness of amino acids as inhibitors of enzyme was found to be proline> lysine>cysteine>valine>serine>aspartate with 56%, 46%, 42%, 42%, 42% and 30% inhibition, respectively.

Key words: *Heterotis niloticus*; freshwater habitat; arginase; ureotelism; uricotelism.

Introduction

The L-arginine metabolism is closely related to the ureogenic activity in animals and excretion of urea, probably a prerequisite for the life in terrestrial environments, which display intermittent disposability of water [1-2]. Arginase (EC 3.5.3.1) catalyzes the hydrolysis of L-arginine to L-ornithine and urea. It is the terminal enzyme of the urea cycle among the six enzymes [2, 3]. 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 [4]. Many invertebrates are uricotelic organisms and eliminate excess nitrogen in the form of solid uric acid [2, 5].

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Nevertheless, a large number of uricotelic organisms possess arginase. It has been reported that their generation of L-ornithine by arginase feeds into the production of L-proline, glutamate and polyamines used for collagen synthesis, energy metabolism and cell proliferation [2]. The enzymatic activity of arginase from a number of sources has been shown to be manganese-dependent [6, 7]. Arginase is ubiquitous in fish tissues, with highest activities found in liver and kidney tissue [2, 8-10]. Despite this, only a handful of adult teleost species is known to have a functional Ornithine Urea Cycle (OUC) [11-13], although the OUC is present in the more ancient fish lineages (i.e. elasmobranchs and the coelacanth) [14]. Most teleosts are ammoniotelic and only very few species rely on the energetically costly *de novo* synthesis of urea as a route for removing excess 'nitrogen' in a non-toxic form. Regardless of the ultimate role of urea, these ureogenic fishes (with the exception of the lungfishes) use an identical metabolic pathway for their hepatic urea synthesis [1].

Most studies have concentrated on mammalian liver arginases [2, 15-16]. In addition to urea synthesis in the liver of ureotelic species, arginase is also involved in biosynthesis of polyamines and proline [17],

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conversion of arginine into a-ketoglutarate for oxidation in the Krebs cycle [18], adaptive responses to anoxia in some invertebrates [19] and production of urea for osmoregulatory purposes [20]. 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 [21]. It had previously been thought that there were significant kinetic and structural differences between ureotelic and non-ureotelic arginases [22-23], but many studies suggested that the characteristics of arginases are not consistent with a particular mode of nitrotelism [24-25]. Non-ureotelic arginases are generally similar to ureotelic arginases but can be distinguished immunologically [24, 26].

In uricotelic organisms, that includes bacteria, fungi, invertebrates, reptiles and birds [2] and ammonotelic organisms such as fish and amphibians (at early stage of development) [2, 22, 27], 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 [2]. 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 [28-29]. Arginase activity in the freshwater teleostean fish, *Clarias batrachus* was spectrophotometrically measured in brain, liver, heart, kidney, spleen, gills, ovary and testis [8], and the enzyme was found to be highest in the liver, kidney and heart in that order representing the tissues of high metabolic activities. The argininolytic activity in Antarctic fish was found to be high in the kidney's distal portion and liver than other tissues of the fish [30]. Also, [9] observed high levels of arginase in kidney than in liver of the different species of teleost fish. *H. niloticus* (African arowana) is a primitive fish and member of the group Osteoglossomorpha, the most primitive subdivision of Teleost. *H. niloticus* have air-breathing organs on its branchiae, enabling them to survive in oxygen-depleted water and they are widespread throughout Africa. This work therefore is to determine the physico-chemical properties of *Heterotis niloticus* arginase activity in metabolically competent liver tissue with the aim of obtaining a better understanding of the nitrogen metabolism of the fish.

Materials and Methods

Materials

Trizma base, Trizma-HCl, ethylenediamine tetraacetic 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. All other reagents were of analytical grade. *Heterotis niloticus* (Cuvier 1829), African Arowana were caught from Osinmo Reservoir in Ara town, South-Western Nigeria.

Methods

Assay Method

Arginase activity was determined according to the modified method of [17]. The reaction mixture contained, in final concentration, 1.0 mM Tris-HCl buffer, pH 9.5 containing 1.0 mM $MnCl_2$, 0.1 M arginine and 50 µl of the enzyme preparation was added in a final volume of 1.0 ml. The mixture was incubated for 10 minutes at 37 °C. The reaction was terminated by the addition of 2.5 ml Erhlich reagent (that contains 2.0 g of p-dimethylaminobenzaldelyde in 20 ml of concentrated hydrochloric acid and made up to 100 ml by adding distilled water). Optical density reading was taken after 20 minutes at 450nm. The calibration curve was prepared with increasing amounts of urea between 0.1 µmol and 1.0 µmol. One unit (U) of enzymatic activity is defined as the amount of enzyme that catalyses the formation of 1 µmol urea/min at 37^0C. The protein concentration was determined by the method of [31] using bovine serum albumin (BSA) as standard.

Purification of *Heterotis niloticus*

Unless otherwise stated all steps were performed at 4–37 ^{o}C using 5 mM Tris–HCl buffer, pH 7.5. The crude extract was prepared by homogenising the liver in 9 volume of homogenisation buffer (5 mM Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose and 5 mM $MnCl_2$). This was centrifuged at 4500 rpm for 30 min using a refrigerated centrifuged. The supernatant was collected and the residue rehomogenised in one volume of the same buffer. After centrifuging, the resulting supernatant was combined with the former. The combined supernatant was adjusted to 70% saturation with solid ammonium sulphate $(NH_4)_2S0_4$ and stirred for 4 hours. The solution was then centrifuged and the pellet was retained. The pellet was resuspended in 5 mM Tris-HCl buffer, pH 7.5. The resuspended precipitate was dialysed against 50% glycerol in 5 mM Tris-HCl buffer, pH 7.5. The dialysate was used for the next step of purification.

Reactive Blue 2-Agarose Chromatography

The reactive blue 2-agarose resin was first equilibrated in 5 mM Tris-HCl buffer, pH 7.5 containing 5 mM $MnCl_2$ and later packed into (2.5 × 20 cm) column. 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 5 mM 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 5 mM Tris-HCl buffer, pH 7.5.

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 [32]. 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 [17] in 2 mM Tris-HCl buffer, pH 9.5 containing 0.01 mM $MnCl_2$. The kinetic parameters were determined from the double reciprocal plot [33]. The substrate specificity study was carried out using arginine monohydrochloride and argininemonohydrate

Effect of Temperature on the Enzyme Activity

The activity of arginase was assayed at temperatures between 30°C and 100°C to determine the optimum temperature and thermal stability of the enzyme.

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) in a typical assay mixture.

Effects of Metal ions and Amino Acids on the Enzyme Activity

The effect of metal ions on the activity of arginase from *H. niloticus* was investigated using the following metal ions: Zn^{2+}, Mg^{2+}, Sn^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, H^{2+}, Na^{+} and NH_4^{+}. One ml of the reaction mixture contained 0.05 ml 100 µM of the appropriate metal ion in a typical assay mixture.

The effect of amino acids on the activity of arginase from *H. niloticus* was also investigated with the following amino acids: $_L$-lysine, $_L$-cysteine, $_L$-serine, $_L$-proline, $_L$-glutamate, $_L$-aspartate and $_L$-valine. One ml of the reaction mixture contained 0.05 ml of the appropriate amino acid in a typical assay mixture.

Results

Purification of H. niloticus Arginase

The results of ammonium sulphate and affinity chromatography purification of arginase from liver of *H. niloticus* are summarized in Table 1. The elution profiles after reactive Blue 2-Agarose Affinity chromatography is shown in Figure 1.

Molecular Weight Determination

The native molecular weight of *H. niloticus* liver arginase was found to be 178,000 dalton on Biogel P-200 (Fig. 2).

Kinetic Parameters

Figure 3 shows the Lineweaver-Burk plots of arginine as substrate. The result of specificity studies are shown in Figures 4 and 5. The K_m values of arginine, argininemonohydro-chloride and argininemonohydrate are presented in Table 2.

Effects of Temperature and pH on Enzyme Activity

The maximum activity of the enzyme was obtained at 35 ^{0}C (Fig. 6). While an optimum pH of 5.5 was observed (Fig. 7).

Effects of Metal ions and Amino Acids on the Enzyme Activity

Tables 3 and 4 shows the effects of some metal ions and amino acid on the activity of *H. niloticus* liver arginase

Table 1: Purification Profile of *H. niloticus* Arginase

	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification fold
Crude	7694.94	6847.66	0.89	1.00
70% Ammonium Sulphate Precipitation	3214.13	5912.01	1.84	1.12
Affinity chromatography	625.21	3865.01	6.18	6.90

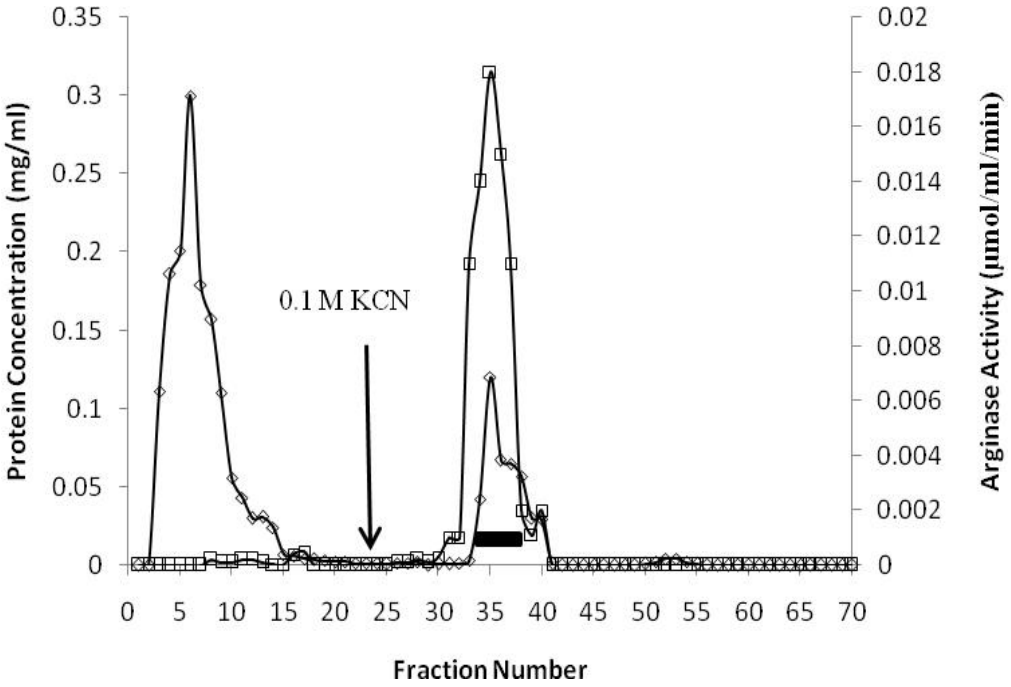

Fig. 1: Reactive blue 2 crosslinked agarose affinity chromatography column (2.5 × 20 cm) of *H. niloticus* liver arginase. -Δ-Δ-Δ- protein profile (OD 280); -□-□-□- activity profile (OD 460); ▬▬ pooled fractions.

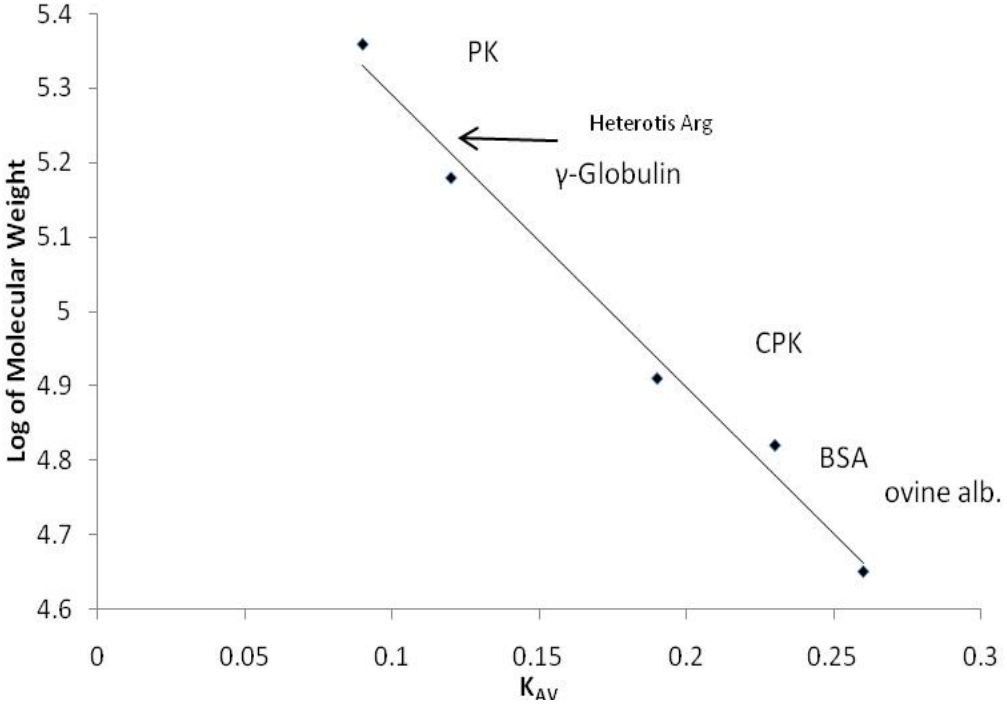

Fig. 2: Calibration curve on Biogel P-200 for native molecular weight determination of *H. niloticus* liver arginase.

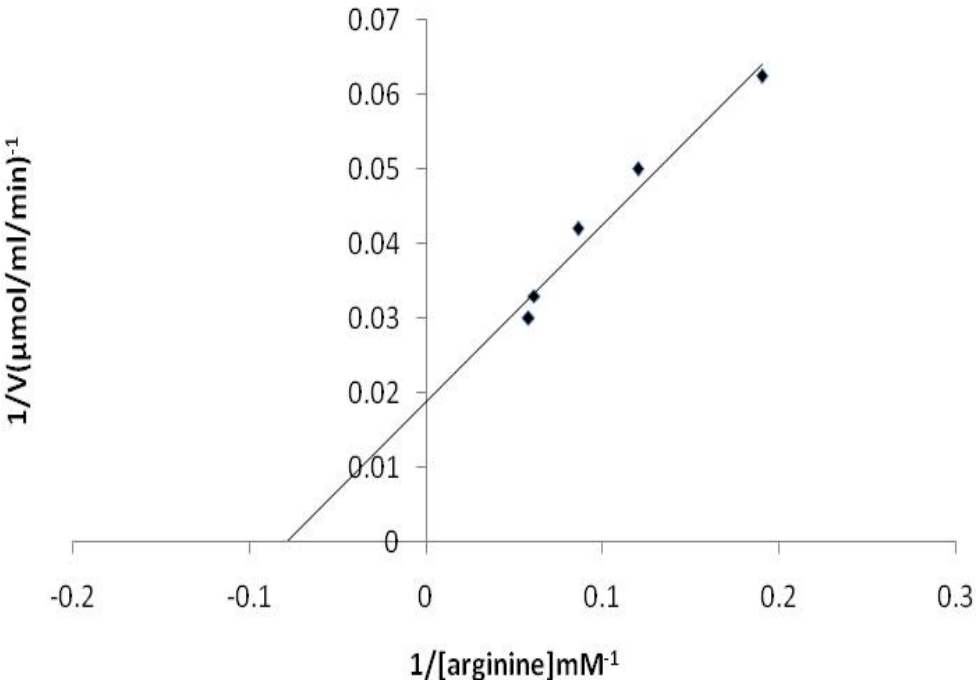

Fig. 3: The Lineweaver-Burk plots showing the effect of varying concentrations of arginine on the initial reaction velocity at pH 9.5.

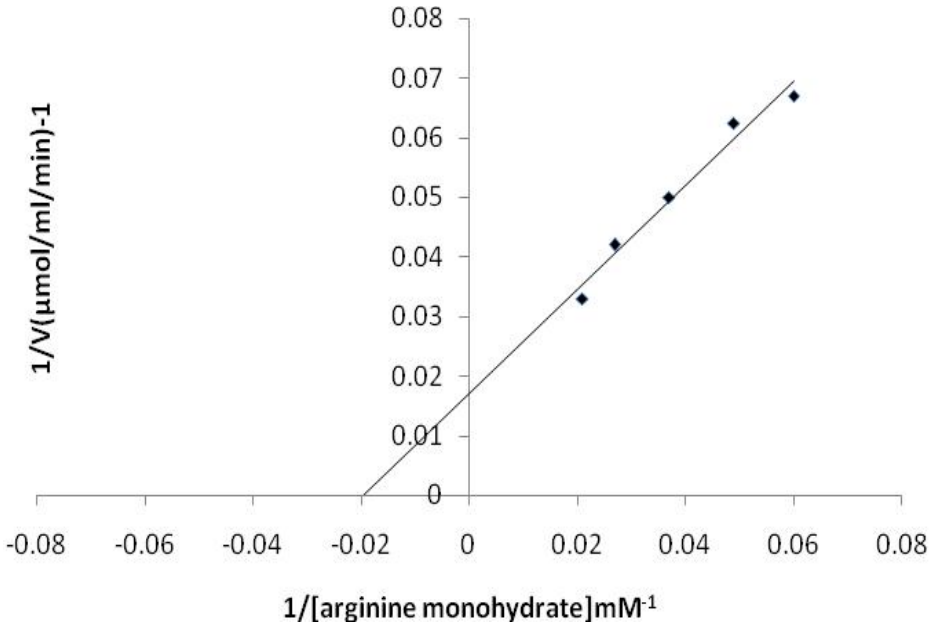

Fig. 4: The Lineweaver-Burk plot showing the effect of varying concentrations of arginine monohydrate on the initial reaction velocity at pH 9.5.

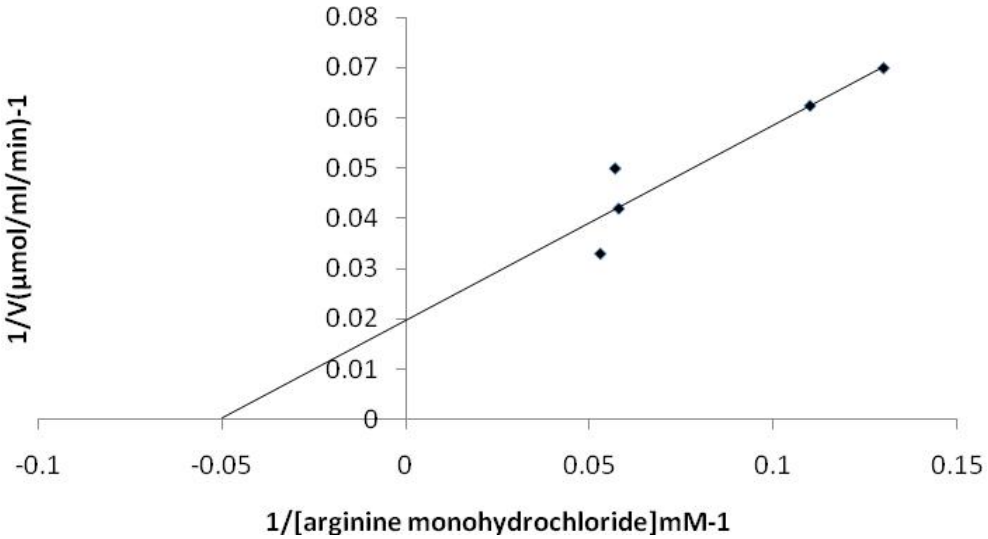

Fig. 5: The Lineweaver-Burk plot showing the effect of varying concentrations of arginine monohydrochloride on the initial reaction velocity at pH 9.5.

Table 2: Substrate Specificity Studies of *H. niloticus* liver arginase activity

Substrate	K_M (mM)	V_{max} (µmol/min)
Arginine	11.6	52.6
Arginine Monohydrochloride	20.0	50.0
Arginine Monohydrate	50.1	58.8

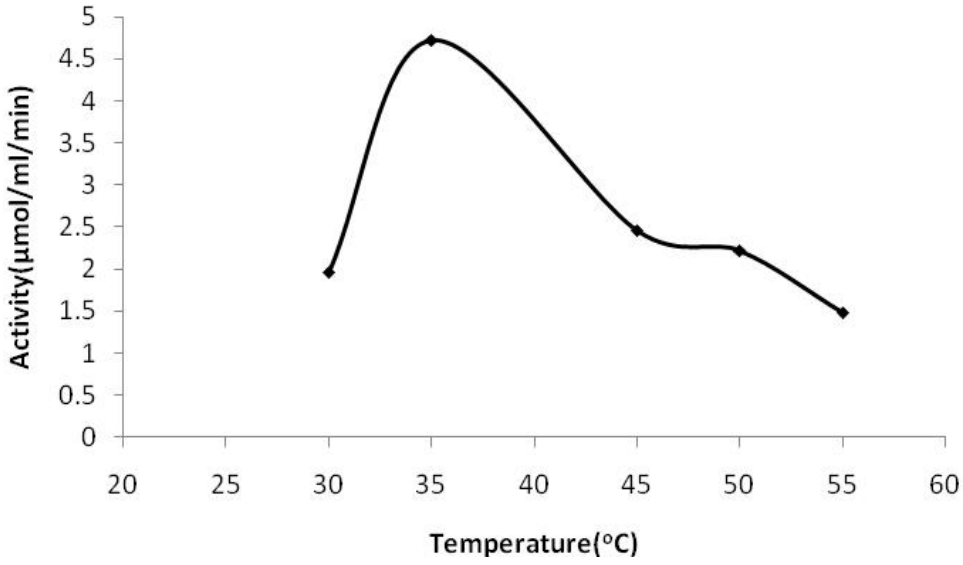

Fig. 6: Effect of temperature on *H. niloticus* arginase activity.

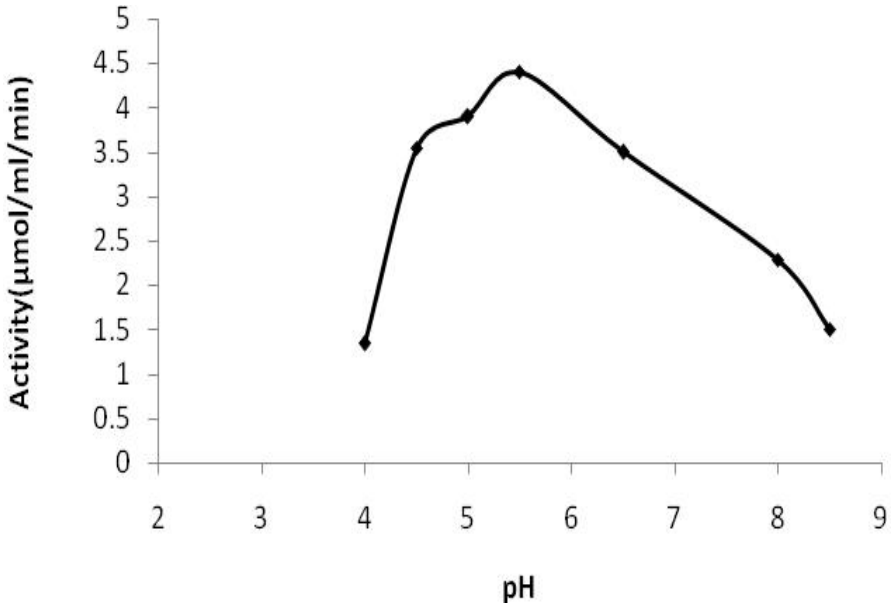

Fig. 7: Effect of pH on *H. niloticus* liver arginase activity.

Table 3: Effects of Metal ions on *H. niloticus* liver arginase activity

Metal ions	% Residual Activity 100 µM
Mn^{2+}	100
Zn^{2+}	103
Mg^{2+}	96
Sn^{2+}	60
Ni^{2+}	87
Co^{2+}	107
Ba^{2+}	60
Hg^{2+}	107
Na^{+}	125

Table 4: Effects of amino acids on *H. niloticus* liver arginase Activity

Amino acids	% Residual Activity 100 µM
$_{L}$-Arginine	100
$_{L}$-Cysteine	58
$_{L}$-Lysine	54
$_{L}$-Valine	58
$_{L}$-Serine	58
$_{L}$-Aspartate	70
$_{L}$-Proline	44

Discussion

Arginase activity was examined in the liver of African arowana fish (*Heterotis niloticus*). The specific activity was 6.18 µmol/min per mg protein (Table 1) with a yield of 8.12 %. The K_M of 11.6 mM for l-arginine by *H. niloticus* liver arginase (Table 2) is similar to the values described for most ureotelics [2, 22]. However, this value is also 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 [34-35]. 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 [36]. [8] obtained a K_M of 26.32 mM for l-arginine for *Clarias batrachus* liver arginase. [37] found values of K_M of 9.1 mM at pH 7.6 and 22.5 mM at pH 9.6 at 37 ^oC in a partially purified liver arginase preparation from the teleost fish *Genypterus maculatus*. In liver arginase preparation of *Merlucius gayl,* Carvajal *et al.* [38] found K_m values of 1.7 and 10.3mmol/l at pH 9.5 and 7.5, respectively. Studies by [39] on the comparative aspects of L-arginine metabolism carried out with the Antarctic fishes *N. neglecta* and *N. rossii*, and the tropical fish (*Epinephelus marginatus*) showed that arginase K_M assayed in both mitochondrial suspensions and mitochondrial extracts gave lower values in tropical fishes preparations than the ones found in the Antarctic ones. The arginase present in the livers of uricotelic and ureotelic animals has similar substrate specificity; all hydrolyses l-arginine [2, 40]. *Helix pomatia* and *Helix aspersa* also hydrolyses homoarginine [41]. Table 2 shows the specificity of *H. niloticus* liver arginase.

The native molecular weight was estimated to be 178,000 dalton with Biogel P-200 for the affinity purification step of the enzyme. [42] reported an apparent molecular weight of 200,000 dalton for purified iris bulbs, similar apparent molecular weight of 200,000 dalton was obtained with arginase from crude extract and DEAE chromatography step (first step purification) of the plant. The molecular weight of purified *Fasciola gigantica* arginase was estimated to be 92,000 dalton by gel filtration and SDS-PAGE [40]. Arginases with molecular weights between 118,000 dalton and 160,000 dalton have been found primarily among ureotelic species [35]. Molecular weights ranging from 228,000 to 276,000 dalton have been found for arginases from uricotelic species [43]. However, a ureotelic land planarian shows an arginase with a molecular weight of 238000 [43-44] and a uricotelic seagull has a liver arginase with a molecular weight of 134000 [43].

The arginase of the sockeye salmon has a temperature optimum of 45°C [45], *Helix pomatia and Helix aspersa* between 60^{o}C and 65°C [41]. The temperature optimum of *Heterotis niloticus* is about 35 °C (Fig. 6). However, arginase from fresh water teleostean fish *Clarias batrachus* was found to be stable between 10 and 37 ^oC, its activity was decreased at higher temperature and showed no activity at 80 ^oC [8]. The pH optimum of *H. niloticus* was 5.5 (Fig. 7). The purified arginase from *Helicobacter pylori* had an acidic pH optimum of 6.1 [46]. One rat arginase isoenzyme had a pH optimum of 7.5 [47] and a minor arginase component was recorded in beef liver with an optimal pH of 7.0 [48]. Arginase from *Sockeye salmon* liver revealed a maximum activity at pH 9.5 [8]. Mammalian arginases appear to have basic pH optima of 9.5–10.5, although some exceptions have been noted [2]. The optimum pH of human vitreous humor arginase was 8.8 [49].

The requirement for Mn^{2+} as a metallic cofactor seems to be common for most reported arginases although other bivalent cations may also be activators [2, 40, 50-51]. Some investigators have found that binding of Mn^{2+} to the enzyme seems to vary for arginases isolated from different species. [44] recorded activation by Mn^{2+}, Cd^{2+} or Ni^{2+} and not Co^{2+} for rat liver arginase. The effects of

divalent cations on *H. niloticus* arginase at different concentrations are shown in Table 3. The enzyme was activated with Mn^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Na^{+} and Mg^{2+}, where the other metals, Ba^{2+}, Sn^{2+} and Ni^{2+} showed slight inhibitory effects. [17 reported similar findings where Hg^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} decreased enzyme activity of rat kidney. The effect of amino acids on the liver arginase of *H. niloticus* is presented in Table 4. Proline, lysine, cysteine, valine and serine strongly inhibited the liver arginase of *H. niloticus*. [52] investigated the effects of proline, ornithine, lysine and certain branched-chain amino acids on lactating rat mammary gland arginase activity, their results showed that lysine, ornithine and valine and to a lesser extent by proline, isoleucine and leucine competitively inhibited the enzyme. [53] also reported partial effects of leucine, isoleucine and valine on kidney arginase of human and a more sensitive inhibition to proline. They further reported that the branched-chain amino acids caused a significant inhibition of liver arginase while proline showed minor effects on liver arginase.

In conclusion, the information presented in this paper on arginase from *Heterotis niloticus*, together with other data [2, 54], further emphasizes the fact that none of the characteristics of arginase observed to date is consistent with a particular mode of nitrotelism.

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