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Purification and Characterization of Fruit Bat (*Eidolon helvum*, Kerr) Liver Arginase

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Abstract: The study of the physicochemical and catalytic properties of arginase (L-arginine amidohydrolase, EC 3.5.3.1) from the liver of fruit bat (*Eidolon helvum*, Kerr), a flying mammal, was investigated for the purposes of biochemical and evolutionary comparison with its extensively studied terrestrial mammalian, ureotelic and uricotelic forms. Arginase was isolated from the liver of the fruit bat and purified to apparent homogeneity. The purification procedure involved two ion-exchange chromatography steps on DEAE-Cellulose and CM-Sephadex columns followed by a gel filtration step on Bio-Gel P-100. The specific activity of the enzyme was estimated to be 36.7 U mg^{-1}. The enzyme exhibited Michaelis-Menten kinetics with a K_m of 17 mM for arginine and V_{max} of 1.39 μmol mL^{-1} min^{-1}. The apparent molecular weight estimated on a Sephadex G-200 column was 80,000. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed two subunits with molecular weights of 34,000 and 52,000. The enzyme probably exists as two dissimilar subunits or as multimer of the 34,000 dalton protein. Fruit bat liver arginase showed slight thermostability with optimum temperature of 60°C and a pH optimum of 9.0. The enzyme was inhibited from 10-40% by the chloride salts of barium, cobalt, iron, magnesium and nickel at 1.0 and 1.5 mM. From its molecular weight and kinetic parameters, it can be concluded that fruit bat hepatic arginase is similar to the enzyme from ureotelic animals.

Key words: Arginase, fruit bat, liver, ureotelic, uricotelic

INTRODUCTION

Arginase (L-arginine ureahydrolase, or amidohydrolase, EC 3.5.3.1) is a primordial and ubiquitous enzyme that catalyses the hydrolysis of L-arginine to urea and L-ornithine. It was first detected in mammalian livers as the enzyme involved in the terminal cytosolic reaction step of the six enzymes of the urea cycle (Greenberg, 1960; Mora *et al*., 1965a,b; Kaysen and Strecker, 1973; Jenkinson *et al*., 1996). The isolation and properties of arginase in a wide range of ureotelic, uricotelic, ammonotelic and ureogenic organisms have been investigated (Brown and Cohen, 1960; Lisowska-Myjak *et al*., 1978; Boutin, 1982; Green *et al*., 1990; Kang and Cho, 1990; Jenkinson *et al*., 1996). As a result of its wide distribution throughout the evolutionary spectrum, a variety of fundamental metabolic and physiological roles have been shown to be mediated by the enzyme. These include nitrogen metabolism in the livers of ureotelic animals, production of ornithine for polyamines for cellular proliferation (Yip and Knox, 1972; Tabor and Tabor, 1984; Jenkinson and Grigor, 1994; Jaimshidzadeh *et al*., 2001; Colleluori and Ash, 2001), proline and glutamate biosynthesis (Porta *et al*., 1976; Baranczyk-Kuzma *et al*., 1981; Baranczyk-Kuzma and Porembska, 2003) and regulation of nitric oxide synthesis by modulating the availability of arginine for nitric oxide synthase (Jenkinson *et al*., 1996; Colleluori and Ash, 2001).

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There are two mammalian types; Type I (cytosolic hepatic arginases that function as the final enzyme of the urea cycle) and Type II (arginases found in the mitochondria of extrahepatic tissues-kidneys, small intestine, lactating mammary gland, penile corpus cavernosum and they are involved in reactions other than nitrotelism but which are basically the regulation of L-arginine or L-ornithine pools for other biosynthetic transformations) (Bach and Killip, 1958; 1961; Kaysen and Strecker, 1973; Herzfeld and Raper, 1976; Jenkinson *et al*., 1996; Colleluori and Ash, 2001; Baranczyk-Kuzma and Porembska, 2003).

It is interesting to note that the invaluable roles of arginase in the elucidation of key metabolic pathways and concepts are emerging. One of these concepts is the reciprocal activities of arginase and nitric oxide synthase which led to the study of arginase inhibitors in smooth muscle contraction, sexual functions and ultimately in the rational drug design for sexual arousal dysfunction (Colleluori and Ash, 2001; Cama *et al*., 2003). Moreover, the long recognized relationship between arginine and cancer on one hand and that between arginase and cancer on the other, has led to the consideration of arginase as index of malignancy in cancerous tissues (Vielle-Breitburd and Orth, 1972; Singh *et al*., 2000; Jaimshidzadeh *et al*., 2001; Wheatley and Campbell, 2002; Baranczyk-Kuzma and Porembska, 2003) and the significance of the amount of the enzyme in blood (Baranczyk-Kuzma *et al*., 1981). We therefore believe that the study of arginase in other tissues and systems would add to the available information that will be required for the purposes of harnessing its full potential for applied uses. To our knowledge, this enzyme has not been investigated in flying mammals such as the fruit bat. The fruit bat is a chordate and a mammal; one would expect very close similarity between the fruit bat enzyme and its mammalian counterparts. Moreover, the properties of this enzyme will be useful in the biochemical comparison of arginases of ureotelic and uricotelic animals whose physical features the fruit bat shares.

MATERIALS AND METHODS

Materials

The experiments were performed at the Enzyme and Protein Laboratory at the Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria between June 2004 and January 2006. Fruit bats were collected at the Botanical Garden of Obafemi Awolowo University, Ile-Ife, Nigeria. Manganous chloride, sucrose, sodium chloride, phosphoric acid, ammonium sulphate, urea, ethanol, methanol and glacial acetic acid were obtained from BDH Chemical Limited, Poole, England. Potassium chloride was obtained from May and Baker, Dapenham, England. Tris (hydroxymethyl) aminomethane (Tris base), bovine serum albumin, sodium dodecyl sulphate (SDS), electrophoresis molecular weight marker (Molecular weight range: 15,000-150,000) and gel filtration molecular weight markers (12,400-200,000) were obtained from Sigma Chemical Company, St. Louis, U.S.A. CM-Sephadex C-50 and Sephadex G-200 were from Pharmacia Fine Chemical Uppsala, Sweden. Bio-Gel P-10 and Bio-Gel P-100 were from Bio-Rad Laboratories, Richmond, California, U.S.A. while DEAE-Cellulose was from Whatman, Maidstone, England. All other reagents were of analytical grades and were obtained from either Sigma or BDH. All solutions were prepared with distilled water.

Methods

Assays

Arginase activity was measured by the rate of urea formation by its colour reaction with Ehrlich's reagent by a modification of the methods of Hagam and Dallam (1968) and Kaysen and Strecker (1973). The reaction was started by adding 50-100 μL of an appropriately diluted enzyme sample to a solution containing, in final concentrations, 1.0 mM arginine and 2.0 mM Tris-HCl buffer (containing

2.0 mM $MnCl_2$), pH 9.5 in a final volume of 1.0 mL. The incubation, termination and estimation of the amount of urea produced were according to Kaysen and Strecker (1973). The protein concentration was measured routinely by the method of Bradford (1976) using bovine serum albumin as the standard.

Purification Procedures

All the procedures were carried out at temperatures between 0-10°C and all buffers contained 2 mM $MnCl_2$ unless otherwise stated. The fruit bats were rendered unconscious by hitting them on the back of their heads and the livers were quickly removed and stored in the refrigerator. A number of previously described methods were adapted for the isolation and purification procedures (Veille-Breitburd and Orth, 1972; Porta *et al*., 1976). The frozen liver was homogenized in 9 volumes of 0.01 M Tris-HCl buffer, pH 7.5 containing 0.01 M $MnCl_2$ and 0.05 M sucrose. The homogenate was centrifuged for 20 min at 10,000 rpm in a Beckman Model TJ-6 Cold Centrifuge. The pellet was resuspended in 2 volumes of the same buffer and extracted as described above. The combined supernatant was subjected to heat treatment at 60°C for 5 min, cooled rapidly in an ice-bath and the pellet that was collected by centrifugation at 10,000 rpm for 20 min was discarded. The supernatant was brought to 70% ammonium sulphate saturation. The suspension was allowed to stand in the refrigerator for 12 h, centrifuged at 10,000 rpm for 30 min and the supernatant was discarded. The precipitate was suspended in a small amount of 2 mM Tris HCl buffer, pH 7.2. The fraction above was dialyzed against four changes of 2 mM TrisHCl buffer pH 7.2. The dialysate was clarified by centrifugation at 10,000 rpm for 20 min and then mixed with 50 mL slurry of pretreated (following the Whatman manual) DEAE-Cellulose already equilibrated with 2 mM Tris-HCl buffer, pH 7.2 and allowed to stand for 1 h with occasional stirring. The mixture was then packed into a column (2.5×40 cm) and eluted batch-wise with 2 mM Tris-HCl buffer, pH 7.2. The column was eluted further with the buffer containing 1 M KCl and this batch was discarded as it contained no arginase activity. The enzyme solution from the preceding step was applied to a CM-Sephadex A-50 column (2.5×40 cm) that had been equilibrated with 2 mM Tris-HCl buffer pH 7.2. The column was washed with 160 mL of the same buffer at a flow rate of 30 mL h^{-1} followed by elution with a 200 mL linear gradient of 0-1.0 M KCl in the same buffer. The active fractions were pooled and precipitated in 70% ammonium sulphate.

The precipitate was desalted on a column of Bio-Gel P-10 and then filtered through a Bio-Gel P-100 column (1.5×40 cm) eluted with 2 mM Tris-HCl buffe, pH 7.2. Fractions containing arginase activity were pooled and brought to 70% ammonium sulphate precipitation.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the absence of SDS was carried out on 7.5% gels according to the Pharmacia manual (Polyacrylamide gel electrophoresis laboratory techniques. Revised Edition. February, 1983) in order to ascertain the purity of the preparation. The electrophoresis was performed in phosphate buffer, pH 7.2 with a current of 8 mA/gel for 5 h.

Investigation of the Properties of Arginase from Fruit Bat Liver

The subunit molecular weight of the pure enzyme preparation was determined by SDS polyacrylamide gel electrophoresis according to Weber and Osborn (1975) on 10% rod gel using the phosphate buffer system. Standard proteins kit was the Sigma's Molecular Weight Standard Recombinant Mixture for SDS-PAGE and Western Blotting (Molecular weight range 15,000-150,000).

The molecular weight of the native enzyme was determined by gel filtration on a Sephadex G-200 column (2.5×40 cm) in 2 mM Tris-HCl buffer, pH 7.2 (containing 2 mM $MnCl_2$) based on the method of Andrews (1964). Bovine erythrocyte carbonic anhydrase (M_r 29,000; 3 mg mL^{-1}), bovine serum

albumin (M_r 66,000; 10 mg mL^{-1}), yeast alcohol dehydrogenase (M_r 150,000; 5 mg mL^{-1}) and β-amylase (M_r 200,000; 4 mg mL^{-1}) were used as molecular weight standards.

The kinetic parameters (K_m and V_{max}) of the enzyme were determined according to Lineweaver and Burk (1934). The concentration of arginine was varied between 25 mM and 125 mM and arginase.

The activity of arginase was assayed at temperatures between 10 and 70°C to investigate the effect of temperature on the activity of the enzyme. The assay mixture was first incubated at the indicated temperature for 10 min before initiating reaction by the addition of an aliquot of the enzyme which had been equilibrated at the same temperature.

The effect of pH on arginase activity was performed by assaying the enzyme at pH between 7 and 11 using 0.1 M Tris HCl buffer after the method of Reyero and Dorner (1975), Porta *et al*. (1976) or Baranczyk-Kuzma and Porembska (1981).

The method of Kaysen and Strecker (1973) was used to study the effect of various cations on the activity of fruit bat arginase at 1 and 1.5 mM final concentration. The salt used include manganese chloride ($MnCl_2$), magnesium chloride ($MgCl_2$), Iron II chloride ($FeCl_2$), Nickel chloride ($NiCl_2$) and barium chloride ($BaCl_2$).

RESULTS

Purification

A typical result of the purification protocol for fruit bat liver arginase produced about 40 mg of pure protein starting with 55.0 g of liver tissue with a specific activity of 36.70 U mg^{-1}. The purification procedure is summarized in Table 1. Figure 1 shows the elution profile after the CM-Sephadex C-50 ion exchange chromatography step and Fig. 2 shows the elution profile after gel filtration on Bio-Gel P-100. In the two chromatographies, only one major activity peak were obtained. The preparation was adjudged pure by the presence of a single band of protein after electrophoresis in the absence of SDS (Fig. 3).

Properties

The molecular weight of the native enzyme was estimated to be 80,000 by gel filtration on Sephadex G-200. Although, electrophoresis under non-denaturing conditions showed only a single protein band, SDS-PAGE showed two bands whose molecular weights were estimated to be 34,000 and 52,000 (Fig. 3). The enzyme followed Michealis-Menten kinetics with a K_m for arginine of 17 mM and V_{max} of 1.39 µmol mL^{-1} min^{-1} estimated from the double-reciprocal plot. The optimum temperature for arginase activity was 60°C as shown in Fig. 4a. The influence of pH on the rate of arginase activity is shown in Fig. 4b with an optimum pH of 9.0. The result of the effect of various cations on the activity of fruit bat liver arginase is shown in Table 2.

Table 1: Purification of fruit bat liver arginase

Fraction	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)
Crude homogenate	408	10200	61200	6	100
Heat step (60°C)	401	6019	48120	8	79
70% Ammonium sulphate	92	723	18492	26	30
DEAE-Cellulose	59	165	4950	30	8
CM-Sephadex A-50	30	78	2580	33	4
Bio-Gel P-100	18	43	1584	37	3

All procedures were carried out at temperatures between 0-10°C either in a cold room or ice bucket. All buffers contained 2.0 mM $MnCl_2$ to stabilize the enzyme. In this typical report, 55.0 g of liver tissue was used. A unit of arginase activity is defined as the amount of enzyme that produces 1.0 µmol of urea per min at 37°C i.e., µmol mL^{-1} min^{-1}. Specific activity is expressed in enzyme units per mg of protein. Protein was measured by the procedure of Bradford (1976) with bovine serum albumin as a standard

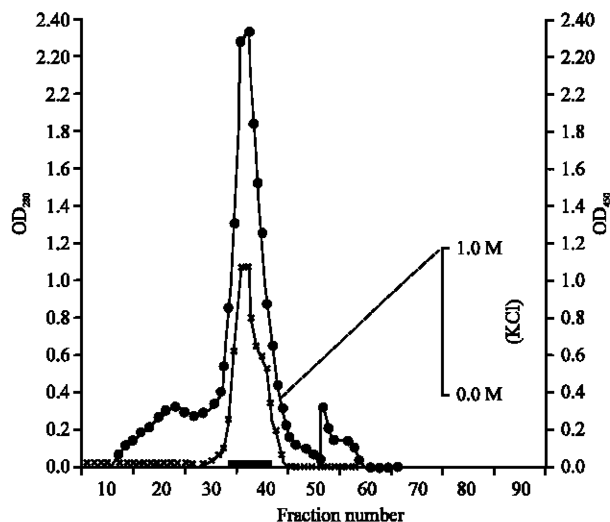

Fig. 1: Elution profile after chromatography on CM-Sephadex A-50. The column was first washed with 160 mL of 2 mM Tris HCl, pH 7.2 containng 2 mM $MnCl_2$ before been eluted with a linear salt gradient of 0-1.0 M KCl, (×-×-×) absorbance at 450nm, (●-●-●) absorbance at 280 nm, (▬) pooled fraction, (– – –) salt gradient

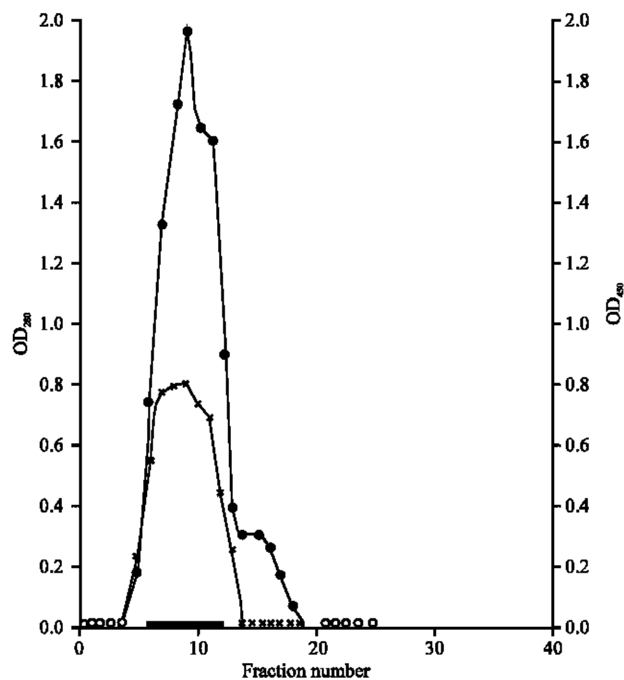

Fig. 2: Gel Filtration on Bio-Gel P-100. The ammonium sulphate precipitate from the CM-Sephadex ion-exchange step was desalted by passing it through a small column of Bio-Gel P-10 and then layered on the Bio-Gel P-100 column. Three milliliter fractions were collected at a flow rate of 12 mL h^{-1}, (×-×-×) absorbance at 280 nm, (●-●-●) absorbance at 450 nm, (▬) pooled fraction

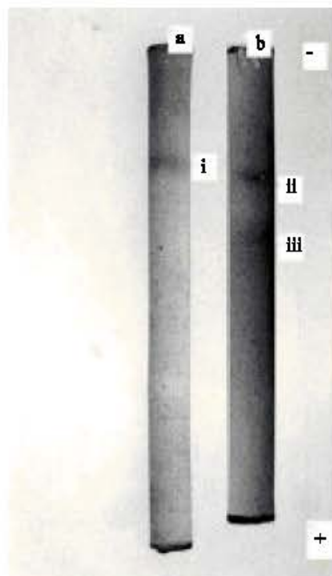

Fig. 3: Photograph of the result of non-SDS Polyacrylamide Gel electrophoresis (a) shown only one protein band (I). Inset is the photograph of the SDS-PAGE (b) with two protein bands (ii and iii)

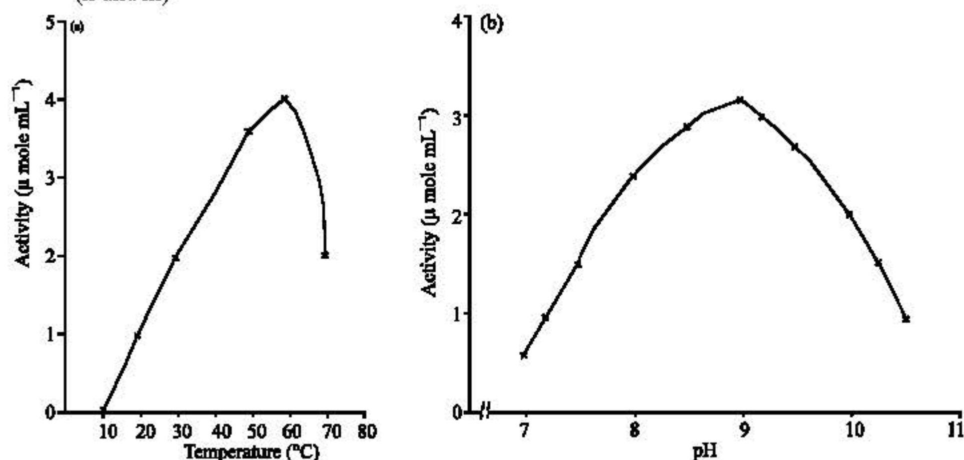

Fig. 4: Effect of (a) temperature and (b) pH on fruit bat liver arginase. The enzyme was assayed at temperatures between 10 and 70°C and pHs between 7 and 11 in 0.2 M Tris-HCl buffer

Table 2: Effects of cations on fruit bat liver Arginase

	Residual enzyme activity (%)	
Salt	1.0 mM	1.5 mM
Control	100	100
$CoCl_2$	96	84
$FeCl_2$	93	78
$NiCl_2$	92	83
$MgCl_2$	91	64
$BaCl_2$	81	80

The salts used were barium chloride ($BaCl_2$), cobalt chloride ($CoCl_2$), iron II chloride ($FeCl_2$), magnesium chloride ($MgCl_2$) and nickel chloride ($NiCl_2$) at final concentrations of 1.0 and 1.5 mM in the assay mixture. The salts were dissolved in the routine assay buffer. The control was assayed with the same buffer containing 1.0 mM $MnCl_2$

DISCUSSION

Arginase has been detected and studied in many organisms especially in mammalian livers (Jenkinson *et al*., 1996). These studies have been enhanced by the fact that ureotelic liver arginases are cytosolic enzymes. A yield of 39% of 7.4 mg of protein was obtained by Veille-Breitburd and Orth (1972) from the liver of a rabbit while Grazi and Magri (1972) obtained a yield of 18% from 59 g of chicken liver. The specific activity is, however, low compared with that obtained for chicken liver (810 U mg^{-1}) (Grazi and Magri, 1972) and rabbit liver (2,460 U mg^{-1}) (Veille-Breitburd and Orth, 1972).

The results of the chromatographic separation methods as well the polyacrylamide gel electrophoresis did not suggest the presence of isoenzymic forms of the fruit bat enzyme. The activity had appeared in a single homogenous peak from each of the column chromatographic steps. Also, the enzyme migrated as a single protein band in disc gel electrophoresis when stained with Coomasie blue. Gasiorowska *et al*. (1970) reported four arginase isoenzymes in various rat tissues including the liver. Turkoglu and Ozer (1991) reported three arginase isoenzymes in bovine liver. Two human liver arginase isoenzymes have been described (Bascur *et al*., 1966) while the rechromatography of a pure preparation of rabbit liver arginase revealed multiple active forms of the enzyme (Vielle-Breitburd and Orth, 1972).

The apparent molecular weight of 80,000 is slightly lower than, but in the same magnitude, with the values of 100,000 to 140,000 estimated for native arginases from ureotelic species (Jenkinson *et al*., 1996). Human liver arginase has a molecular weight of 98,000 (Spector *et al*., 1982; Brusdeilins *et al*., 1985). The molecular weights of rabbit, rat and pig liver arginases were 110,000, 118,000 and 130, 000 respectively (Hirsch-Kolb *et al*., 1970; Veille-Breitburd and Orth, 1972; Brusdeilins *et al*., 1985). The low molecular weight of the fruit bat enzyme, presumably, may be due to changes in the quaternary structure due to disaggregation into subunits and the possible reassociation into smaller aggregates during the purification procedures as proposed by Porta *et al*. (1976) and Venkatakrishnan and Reddy (1993). O'Malley and Terwilliger (1974) also reported an active monomer of the *Pista pacifica* arginase. Uricotelic arginases have relatively higher molecular weight of between 250,000-276,000 (Jenkinson *et al*., 1996). The subunit molecular weight of 34,000 shows that arginase from fruit bat liver is an oligomeric protein and probably trimeric. However, the presence of a higher subunit of 52,000 is probably due to the occurrence of arginases from bacteria, invertebrates and higher organisms as large multimers and perhaps also as monomers (Porembska, 1973; O'Malley and Terwilliger, 1974; Jenkinson *et al*., 1996). Alternatively, the enzyme probably exists as a dimeric protein of dissimilar subunits. The value of 34,000 compares very well with the estimated values for some ureotelic species; rat liver arginase (30,000-34,700) (Hirsch-Kolb and Greenberg, 1968; Kawamoto *et al*., 1986; 1987), human liver arginase (30,000-34,700) (Carvajal *et al*., 1971; Spector *et al*., 1982; Haraguchi *et al*., 1987) and beef liver (30,000-37,300) (Hirsch-Kolb *et al*., 1970; Harell and Sokolovsky, 1972).

A wide range of K_m (L-arginine) values have been reported for arginases from different sources. In a comparative review of arginases, Jenkinson *et al*. (1996) have reported values in the range 13-73 mM for arginases from microorganisms and plants (pH 9.1-9.4), 2.0-8.5 mM for invertebrates with the exception of the *Pista pacifica* intestine enzyme which was 158 mM (pH 9.5-10.0), 1.2-11.5 mM for elasmobranches, teleosts and amphibians (pH 9.5-9.8) and 100-138 mM for reptiles and birds except for the chick kidney that was 12-37 mM (pH 9.8). They also reported 6, 10 and 29 mM K_m values for rat liver, kidney and mammary gland arginases, respectively and 5.4 and 7 mM for the human liver and kidney enzymes. Other mammals have K_m values ranging from 1.4-14 mM for their hepatic arginases at pH of 9.5-10 (Porembska, 1973; Brusdeilins *et al*., 1985; Turkoglu and Ozer, 1991). From the aforesaid, the K_m for arginine of the fruit bat liver arginase of 17 mM compares well

with the ones obtained from several mammalian species. It could been seen that K_m for arginine obtained for uricotelic animals are of higher values and it has been opined that ureotelic animals excrete urea and are expected to have more efficient enzymatic system for converting L-arginine to ornithine and urea. Like other ureotelic liver arginases, fruit bat liver arginase is suggested to function in the urea cycle hydrolyzing L-arginine to L-ornithine and urea.

The enzyme is fairly thermostable having an optimum temperature of 60°C. This could be as a result of the protective role of Mn^{2+} against heat denaturation. Horse liver arginase has a temperature optimum of between 45-50°C (Mohammed and Greenberg, 1945). It has been reported that both the liver and kidney arginases from chicken retained full activity at 60°C for 10 min and rat liver arginase for 20 min (Schimke, 1962; 1964; Kaysen and Strecker, 1973). O'Malley and Terwillinger (1974) reported an optimum temperature of 60°C for the *Pista pacifica* arginase with unaltered K_m value over the temperature range of 10-45°C.

The pH optimum of 9.0 for fruit bat liver arginase compares with that from beef, sheep and horse liver arginases with pH optimum of 9.3 (Bach and Killip, 1961). Arginases generally have a highly alkaline pH optimum. For examples, the pH optima of 9.5, 9.9, 10.2, 10.3 and 10.3 have been reported for the bovine, rabbit, horse, pig and dog liver arginases, respectively (Jenkinson *et al*., 1996).

Fruit bat liver arginase was slightly inhibited at 1 and 1.5 mM by Ba^{2+} whereas Fe^{2+}, Ni^{2+}, Co^{2+} while Mg^{2+} had little effect on the activity of the enzyme at 1 mM. Earlier, Middelhoven (1969) had reported that Ba^{2+} inhibited arginase activity. Metal ions, in addition to Mn^{2+}, such as Cd^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} have been found to have activating or stabilizing effect on arginase but the rat liver enzyme was not activated by Co^{2+} (Campell, 1966; Kaysen and Strecker, 1973).

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