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Inhibition and Kinetic Studies of Tortoise (*Kinixys erosa*) Liver arginase.

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Abstract

The effect of amino acid on tortoise liver arginase showed that L-lysine, L-valine, L-serine, L-aspartic acid and L aspartic acid had significant inhibitory effect on the enzyme but proline and glutamic acid showed slight inhibition. Ethylenediaminetetraacetic acid (EDTA), citrate, ascorbic acid, boric acid and sodium borate completely inactivated tortoise liver arginase. Inhibition studies on the enzyme with a number of cations showed decreased arginase activity. While Mn^{2+} satisfies the metal ion requirement of tortoise arginase, Sn^{2+}, Hg^{2+}, Ba^{2+} and Co^{2+}, to a lesser extent inhibited the enzyme. The enzyme was markedly sensitive to inhibition by Zn^{2+}, Ni^{2+} and Mg^{2+}. The enzyme was not inactivated by thiol compounds: reduced glutathione (GSH), cysteine and 2-mercaptoethanol.

Key words: Tortoise liver arginase, substrate specificity, kinetics, inhibition studies

INTRODUCTION

There are two distinct isoforms of mammalian arginase (arginase Type I and Type II), which are encoded by different genes and differ in molecular and immunological properties, tissue distribution, subcellular location and regulation of expression [3,5]. Arginase Type I is highly expressed in the liver of ureotelics and to a much lesser extent in a few other cell types, whereas expression of arginase II is widespread [3]. Gasiorowska *et al.* [6] found four differently charged isozymes of arginase in different rat tissues (all with about the same K_m and native molecular weight), one of which had a pH optimum of 7.5. A minor arginase component was recorded in beef liver with an optimal pH of 7.0 [7]. Mammalian liver arginases tend to have basic isoelectric point (pI) values of about 8.8-9.4 [3].

Arginases have been classified into ureotelic and uricotelic forms on the basis of their physical (molecular weight) and kinetic (K_m values for arginine) differences that were found between birds and mammalian liver arginases [1-3]. The ureotelic arginase have molecular weight of approximate 130,000 and a K_m value for arginine that is lower than 10 mM while the uricotelic arginases have molecular weights of approximate 280,000 and a K_m for the substrate that is at the order of 100 mM [2]. Kadowaki and Nesheim [4] showed that dietary and hormonal stimulations causes the development of a ureotelic arginase in chicken liver that is clearly different from the pre-existing uricotelic arginase.

The occurrence of at least two forms of arginases in mammalian tissues, one in liver and another in kidney and mammary gland has been reported [8-10]. The two forms differed in stability with respect to treatment with acetone, substrate specificity and incubation with Mn^{2+} [6,8,9], heating at temperatures between 40-65 °C and alkaline pH [8], in their interaction with antisera [8-9] and their electrophoretic mobilities on starch [11] or polyacrylamide gels [10]. Reddi *et al.* [10] showed that kidney, intestine and mammary gland contained an isoenzyme that migrates towards the anode which is absent from liver and submaxillary gland. Arginase reaction in rat tissues was shown to be catalyzed by three isoenzymes which can be separated by bidirectional electrophoresis on polyacrylamide gels.

There are significant differences between hepatic and extrahepatic arginases concerning their subcellular location, kinetics, physicochemical properties and immunological cross-reactivity [12-13]. This research will

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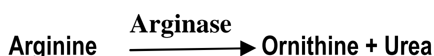
therefore, provide kinetic information on arginase from a reptile, tortoise, which will further demonstrate the difference between the ureotelic (mammalian type) and uricotelic arginase.

MATERIALS AND METHODS

Trizma base, Trizma-HCl, ethylenediamine tetraacetic acid (EDTA), DEAE Cellulose and p-dimethylaminobenzaldelyde (Ehrlich reagent) were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Sucrose, manganese chloride tetrahydrate and urea were purchased from BDH Chemical Limited, Poole England. All other reagents were of analytical grade and were obtained from either Sigma or BDH. Tortoises were purchased from a local market in Ile-Ife, Nigeria.

Assay method.

Arginase activity was determined according to the method of Kaysen and Strecker [9]. The reaction mixture contained 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 min at 37 °C. The reaction was terminated by the addition of 2.5 ml Erhlich reagent. The optical density reading was taken after 20 min at 450 nm. The urea produced was estimated from the urea curved prepared by varying the concentration of urea between 0.1 µmol and 1.0 µmol against the respective optical density at 450 nm. The unit of activity of arginase is defined as the amount of enzyme that will produce one µmol of urea per min at 37°C.



The protein concentration was routinely determined by the method of Bradford [14].

Extraction and Purification

The enzyme was purified to homogeneity in earlier work [15] through ion exchange chromatography, affinity chromatography and Biogel P-200 gel filtration chromatography.

Amino Acid Inhibition Study

The inhibitory effects of amino acids on the activity of arginase were investigated on the enzyme with arginine as substrate. The effects of eight amino acids on the activity of tortoise liver arginase were studied. The amino acids used for the study includes L-lysine, L-valine, L-proline, L-aspartic acid, L-glutamic acid, L-serine, L-arginine, and DL-aspartic acid. The amino acids (25 mM) were used as inhibitors with the routine assay procedure.

Effects of Metal Chelating Compounds and Boric acid

The effects of chelating compounds and boric acid were investigated to determine the involvement of Mn^{2+} in catalytic reaction of tortoise liver arginase. The compounds include EDTA, citrate and ascorbic acid. The inhibitory effects of boric acid and sodium borate were also investigated. The effect of the chelating compound was investigated according to the method of Baranzyk-Kuzma *et al.* [16]. Enzyme preparation was incubated with 50 mM of the required chelating compound at pH 7.5 at 37°C for 30 min and aliquot tested for arginase activity. While 10 mM of boric acid and sodium borate where added to the reaction mixture in separate reactions.

The kinetics of EDTA and citrate inhibitions were also studied according to Dixon [17]. From the coordinate of the points of intersection of the Dixon plots ($1/v_i$ versus concentration of added inhibitor at two different concentrations of the substrate), the type of inhibition can be deduced and the inhibition constant (K_i values) determined. The concentrations of the inhibitors were varied between 0.1 mM and 0.3 mM. All other conditions of the assay remained the same.

Effects of Thiol Compounds

The effects of cysteine, reduced glutathione (GSH) and 3-mercaptoethanol on the activity of tortoise liver arginase were investigated according to the method of O'Malley and Terwilliger [18] to ascertain the presence of critical sulphydryl group on the tortoise liver arginase catalytic region. 5 µM each of the thiol compounds was added to 0.1 ml of the enzyme solution and incubated for 1 h. Aliquot was

taken out for arginase assay. Blank was prepared in the presence of the same amount of thiol compound. This was followed by the determination of the effect of incubating the enzyme at different time intervals. The effect of varying concentration of the thiol compounds between 10 µM and 100 µM on arginase was also investigated.

Effects of Metal Ions on Enzyme Activity

Arginase is a metalloenzyme in which manganese acts at cofactor as well as activator [3,9,19]. It has also been shown that arginase can be activated by other metals [3,20].

(i) The method of Kaysen and Strecker [9] was used to study the effects of the following salts on the activity of arginase: $HgCl_2$, $BaCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, $SnCl_2$, NaCl, $(NH_4)_2SO_4$ and $ZnCl_2$ at a final concentration of 1 mM and 1.5 mM in a typical enzyme assay.

(ii) The effect of different concentrations of $MnCl_2$ (1 mM-5 mM) on the enzyme activity was studied.

RESULTS

The effect of amino acid on tortoise liver arginase showed that L-lysine, L-valine, L-serine, L-aspartic acid and L-aspartic acid had significant inhibitory effect on the enzyme but proline and glutamic acid showed slight inhibition (Table 1). The enzyme was markedly inhibited by lysine and also by branched chain amino acids, especially valine. The effect of chelating compounds is shown in Table 2. Ethylenediaminetetraacetic acid (EDTA), citrate, ascorbic acid, boric acid and sodium borate showed complete inactivation of tortoise liver arginase.

With ascorbic acid there was about 96% inactivation but when the enzyme was treated with boric acid and sodium borate, complete inactivation of the enzyme was observed at pH 9.5.

The results obtained with varying the concentration of (i) EDTA and (ii) citrate, at two different concentrations of the substrate (arginine) is illustrated according to Dixon [17] and shown in Figures 1 and 2 respectively. The nature of the inhibition is competitive and non-competitive respectively. The inhibition constant (K_i) for EDTA and citrate obtained from Dixon plot were 0.15 mM and 0.03 mM respectively.

Table 1: Effects of amino acids on activity of tortoise liver arginase

Inhibitors (100 µM)	(%) Residual Activity
Arginine	100
L-Proline	39
L-Lysine	19
L-Glutamic acid	35
L-Aspartic acid	24
DL-Aspartic acid	24
L-Serine	24
L-Valine	24

The reaction mixture (final volume of 1.0 ml) contained 1.0 mM Tris HCl, pH 9.5, 1.0 mM $MnCl_2$, 0.1 M arginine and 25 mM of the indicated amino acid as substrate and 50 µl of the enzyme.

Table 2: Effects of metal chelating compounds, boric acid and sodium borate on tortoise liver arginase

Inhibitors (100 µM)	% Residual activity
EDTA	29
Citrate	26
Ascorbic acid	04
Boric acid	00
Sodium borate (borax)	00

The reaction mixture (final volume of 1.0 ml) contained 1.0 mM Tris HCl, pH 9.5, 1.0 mM $MnCl_2$, 0.1 mM of the indicated compound, 0.33 M arginine and 50 µl of the enzyme.

The treatment of the enzyme with 5 µM of reduced glutathione (GSH) and 2-mercaptoethanol did not inactivate the enzyme except for cysteine that showed about 87% inactivation of the enzyme.

The effect of thiol compounds on tortoise liver arginase was further investigated by incubating the liver arginase with the thiol compounds at different time intervals. The result showed that the enzyme was inhibited with increase in incubation time (Fig. 3). Also the effect of different concentrations (between 10 µM and 100 µM) of 2-mercaptoethanol and GSH on the liver arginase showed considerably inhibition of the enzyme by the two thiol compounds (Figures 4 and 5).

Inhibition studies on the enzyme with a number of salts showed decreased arginase activity. Mn^{2+} satisfied the metal ion requirement of tortoise liver enzyme. Treatment with 100 µM concentrations of the various metal ions (Sn^{2+}, NH4+, Hg^{2+}, Ba^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+} and Hg^{2+}) showed slight inhibition of the enzyme by Sn^{2+}, NH_4^+, Hg^{2+}, Ba^{2+} and Co^{2+}, but was markedly sensitive to inhibition by Zn^{2+}, Ni^{2+}, and Mg^{2+}. The enzyme was also inactivated by Ni^{2+}, Hg^{2+}, Co^{2+} and Mg^{2+} when treated with 250 µM concentrations of the metal ions, except Zn^{2+}, Ba^{2+}, Sn^{2+}, and NH_4^+ that showed about 50% inactivation. At 500 µM, the enzyme was completely inactivated by all the metal ions used (Table 3). It was observed that increase in the concentration of Mn^{2+} did not change the enzyme activity (Table 4).

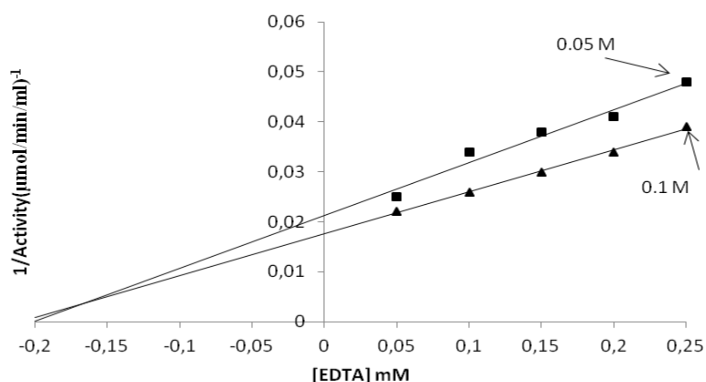


Figure 1: Dixon plot of the inhibition of arginase reaction by EDTA.
The plots were drawn following the method of Dixon [17] with EDTA as varied inhibitor and arginine as the varied substrate at 0.05 M and 0.1 M. Each reaction mixture (final volume of 1.0 ml) contained 1.0 mM Tris HCl, pH 9.5, 1.0 mM $MnCl_2$, indicated amount of arginine and EDTA from 0.1-0.25 mM of the require compound and 50 µl of the enzyme.

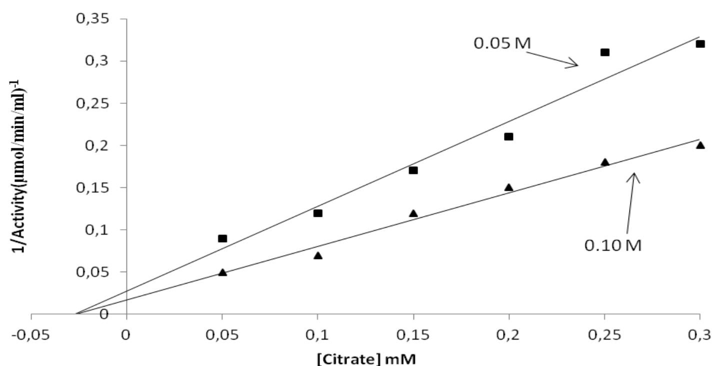


Figure 2: Dixon plot of the inhibition of arginase reaction by added citrate.
The plots were drawn following the method of Dixon [17] with citrate as varied inhibitor and arginine as the varied substrate at 0.05 M and 0.1 M. Each reaction mixture (final volume of 1.0 ml) contained 1.0 mM Tris HCl, pH 9.5, 1.0 mM $MnCl_2$, indicated amount of arginine and citrate from 0.1-0.3 mM of the require compound and 50 µl of the enzyme.

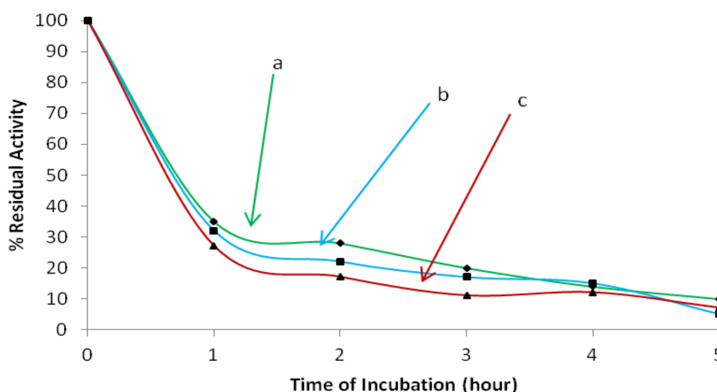


Figure 3: Effects of Thiol Compounds on the Activity of Arginase at Different Time Interval. Enzyme assay was carried out using the standard assay mixture. The enzyme was first incubated with a thiol compound at the indicated time before initiating the reaction. Green colour (a: Reduced Glutathione)**,** Blue colour **(**b: 2-Mercatoethanol) and Red (c: Cysteine)

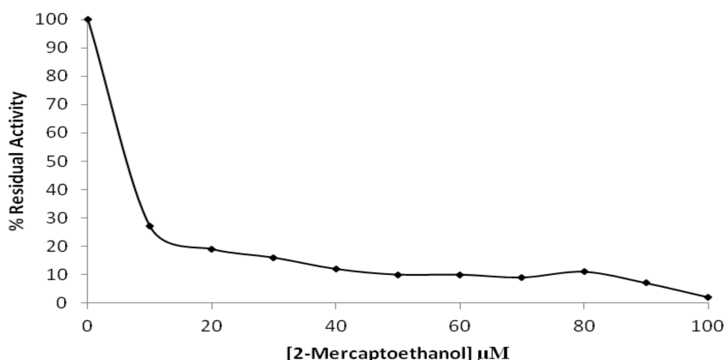


Figure 4: Effects of varying concentration of 2-Mercaptoethanol on Arginase Activity.
Enzyme assay was carried out using the standard assay mixture containing varying concentrations of 2-mercaptoethanol between 10 µM and 100 µM. The values are mean of triplicate determinations.

DISCUSSION

Inhibition studies on arginase involving the use of amino acid have been studied extensively. The liver arginase of teleost fish *Gerupterus maculatus* was inhibited competitively by lysine, but proline, leucine, valine and isoleucine caused a non-competitive inhibition [21]. The liver arginase of teleost fish *Marluccius gayi* liver was also found to be markedly inhibited by branched chain amino acid and especially by isoleucine, leucine, valine, lysine and proline where they all caused 50% inhibition [22]. In another report, Carvajal *et al.* [23] showed the inhibition of arginase from the gills tissue of bivalve *Semele solida* by lysine and proline. The inhibition was found to be competitive but the branched amino acids showed a non-competitive inhibition with the enzyme. The effect of 16 amino acids was reported by Carvajal *et al.* [22] on sheep brain arginase. It was observed that leucine, valine and lysine inhibited the enzyme significantly and the other amino acid (aspartic acid, histidine, proline, isoleucine, etc.) tested did not

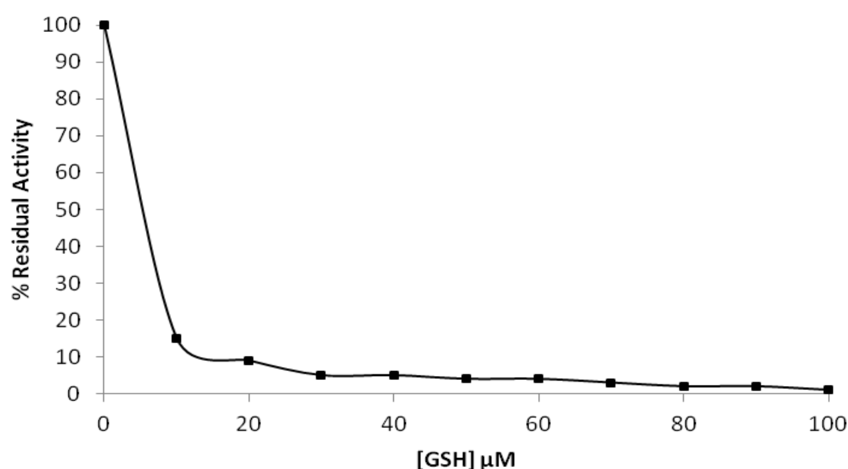


Figure 5: Effects of varying concentration of Reduced Glutathione on Arginase Activity.
Enzyme assay was carried out using the standard assay mixture containing varying concentrations of reduced Glutathione between 10 µM and 100 µM. The values are mean of triplicate determinations.

show significant influence on the enzyme. They related the inhibition of the sheep brain arginase to the carbon length of the amino acid. Aspartic acid and histidine showed a slight stimulation of the sheep brain arginase. The effect of amino acid on tortoise liver arginase showed that L-lysine had significant inhibitory (19 %) effect on the enzyme followed by L-valine, L-serine, L-aspartic acid and L aspartic acid (Table 1). L-proline and glutamic acid were observed to show slight inhibition ((34-35% inhibition). This result compares very well with the results obtain for other arginases. The amino acids have been reported to cause inhibition in rat liver [25] and rat lactating mammary gland [26]. The amino acid, L-arginine, which is the substrate for the enzyme has six carbon atoms.

The amino acid L-lysine and L-valine are monocarboxylic acyclic amino acid with five or more carbon atoms. Hence, it is possible to assume that carbon chain length is critical for effective inhibition of arginase at the catalytic site of the enzyme molecule [24]. Though, it has been reported that proline, isoleucine and leucine also inhibit lactating rat mammary gland arginase to a lesser extent [26], in this study, the inhibition of proline was not too significant. This may be due to arginase involvement in the synthesis of proline in the liver of tortoise (a uricotelic organism). Some amino acids [e.g L-valine and L-lysine] have been shown to inhibit arginase activity by switching off the electron paramagnetic resonance (EPR) signal of the bimolecular centre probably by removing a bridging ligand or by increasing the inter-manganese separation which are required for catalysis [27,28].

Table 3: Effects of Salts on Enzyme Activity.

	% Residual Activity		
Cations	**100 µM**	**250 µM**	**500 µM**
Mn^{2+}	100	100	100
Co^{2+}	82	46	21
Zn^{2+}	26	57	24
Ba^{2+}	95	59	26
Hg^{2+}	91	35	14
Sn^{2+}	90	73	40
Mg^{2+}	47	46	20
Ni^{2+}	4	32	13
NH_4^+	78	51	14

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.

Tortoise liver arginase was strongly inhibited by the chelating compounds. EDTA was reported to show complete inactivation of arginase [16]. In another report, incubation of rat kidney arginase in a solution of EDTA did not affect the enzyme activity significantly [9]. Green *et al.* [29] working on *Saccharomyces cerevisiae,* observed strong inhibition of the enzyme with EDTA. Earlier work by Eisenstein *et al.* [30] and Carvajal *et al.* [31] showed that incubation of arginase with EDTA separated the enzyme into monomer units. Cavalli *et al.* [32] showed that preincubation of arginase

with EDTA did not alter the kinetic constant and the activity of the enzyme. A similar observation was reported by Vielle-Breitburd and Orth [33] on the effect of EDTA on rabbit liver arginase, but prolong incubation almost completely inactivated the enzyme without altering its immunoprecipitation pattern.

Table 4: Effect of varying concentration of MnCl

Mn^{2+} Concentration (mM)	Activity (µmol/min)
1.0	21
1.5	22
2.0	24
2.5	22
3.0	22
4.0	22
4.5	24
5.0	24

Purified enzyme was incubated for 30 min in the assay system with the Mn^{+} salt concentration as indicated.

A different observation was reported by Reczkowski and Ash [34]. There report showed that, assaying a fully Mn^{2+} activated enzyme in the presence of 10 mM EDTA, or 10 mM o-phenanthioline (a chelating compound) at room temperature did not affect the activity of the enzyme. However, preincubating the enzyme with 10 mM citrate and 10 mM EDTA at 60°C for 10 min prior to assay at room temperature resulted in 1 % of the activity remaining when compared to control sample that was heated at 60 °C for 10 min in the absence of the chelating compound.

Furthermore, tortoise liver arginase was completely inactivated by borate. The nature of borate inhibition has been proposed to arise from chelation of Mn^{2+} in the binuclear centre thus displacing a metal-bound water molecule that is responsible for nucleophilic attack on the guanidinium carbon [20,27,34,35]. Xie *et al.* [28] further explained the inhibition mechanism by borate through the production of changes in the Electron Paramagnetic Resonance (EPR) spectral, resulting in simplification of the spectrum by conversion to species with a relatively narrow distribution of Mn-Mn separation. Thiol compounds have been reported to have variable effects on arginase from different sources, although, experimental conditions may partially account for the different results. The effects of thiol (cysteine, 2-mercaptoethanol and GSH) groups on tortoise liver showed significant inhibition at the various times of incubation, with inhibition increasing with time. Similar result was obtained when the enzyme was reacted with different concentrations of the thiol compounds. O'Malley and Terwilliger [18] reported a different observation with *Pista pacifica.* They found that incubating cysteine, GSH and 2-mercaptoethanol with the arginase from *Pista pacifica* enzyme showed no inhibition, instead the enzyme activity was enhanced. Bullfrog and bovine arginases were also stimulated when preincubated with GSH, dithiothreitol, 2-mrcaptothanol or cysteine, but p-chloromercuribenzoate had an inhibitory effect on the enzymes [36]. p-hydromercuribenzoate had a slight inhibition on insect arginase [37]. Chlorohydroxyl mercuribenzoate inhibited cray fish arginase [38], and cockroach body arginase [37]. Reddy and Campbell [37] reported that mercuribenzoate inhibits protein by dissociating transmembrane protein from the peripheral cytoskeletal network.

A common feature of all arginases thus far studied, whether of eukaryotic or prokaryotic origin, is a requirement of divalent cations for activity [3]. Mn^{2+} is the physiologic activator, although the divalent cation requirement for some arginases has been reported to be satisfied by Co^{2+} and Ni^{2+} [2, 20] and in some instances by Fe^{2+}, Co^{2+}, and Cd^{2+} [27,39]. Arginase is a metalloenzyme in which manganese act as a cofactor, activator and a stabilizing molecule [3, 9, 19, 27 ,34]. The effect of cations on the tortoise liver arginase was similar to the result obtain by other investigators. Mn^{2+} satisfies the metal ion requirement of tortoise liver arginase. In addition to Mn^{2+}, tortoise arginase was also activated by Sn^{2+}, Hg^{2+}, Ba^{2+} and Co^{2+}. The enzyme was markedly sensitive to inhibition by Zn^{2+}, Ni^{2+} and Mg^{2+}. Kaysen and Strecker [9] showed that Mn^{2+} and Mg^{2+} enhanced arginase activity after preincubation of rat kidney, but, Fe^{2+}, Hg^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} decreased the enzyme activity. In gill tissue of bivalve *Semele solida*, Mn^{2+}, Ni^{2+}, Cd^{2+} and Co^{2+} satisfy the metal ion requirement of the enzyme but was inhibited by Zn^{2+} [23]. In the liver arginase of *Merluccius gayi*, Mn^{2+} was reported to satisfy its metal ion requirement and to a lesser extent by Cd^{2+} and Co^{2+} [22]. The replacement of Mn^{2+} in the *Vigna catjang* cotyledon arginase with other metal ions such as Mg^{2+} and Co^{2+} restored more than 60 % activity, Ni^{2+} and Fe^{2+} restored about 45 % activity, Ca^{2+}

and Zn^{2+} restored about 35-38 % activity and Cd^{2+} restored only 10 % activity [19]. In the case of buffalo liver enzyme, Mg^{2+} restored almost all the activity while Cd^{2+}, Ni^{2+} and Ca^{2+} restored about 40-50 % of the original activity of the enzyme.

The effect of different Mn^{2+} concentration was investigated on tortoise liver arginase. The result showed that tortoise liver arginase was not affected by increase in Mn^{2+} concentration. Similar result was observed by O'Malley and terwilliger [18] with *Pista pacifica* arginase. The amount of Mn^{2+} require for optimal activity of soybean axis arginase was reported to be 1.0 mM [40], 1-2 mM for human liver [41], 8-10 mM for human erythrocyte [41] and 40 mM for most mammalian liver arginases [42].

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