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BIOCHEMICAL CHARACTERIZATION OF A PURIFIED ARGINASE FROM THE GUT OF *Oryctes rhinoceros* LARVAE

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ABSTRACT

Arginase acts as a mediator in the final phase of the urea cycle, protecting against excessive ammonia under homeostatic conditions by producing L-ornithine and urea. In this study, the physicochemical properties of *Oryctes rhinoceros* Larva arginase were investigated for biochemical comparison with its well-studied terrestrial mammalian. *Oryctes rhinoceros* larva arginase was isolated and purified to homogeneity. The purification procedure involved ion-exchange chromatography on DEAE-cellulose and affinity chromatography on reactive Blue 2-agarose. The pure enzyme had a specific activity of 38.7 U/mg, a purification fold of 63.4, and a percentage yield of 16.5%. *O. rhinoceros* gut arginase had a native and subunit molecular weight of 82 and 45 kDa, respectively and the K_m and V_{max} were 11.25 mM and 13.055 μmmol/min/mL, respectively. The activity was optimum at 60 °C (pH 8). The enzyme retained more than 60% of its activity at 50 °C for 60 min. The inhibition study on the enzyme showed that cations salts ($CaCl_2$, $BaCl_2$, $HgCl_2$, and $SnCl_2$) enhanced the enzyme activity at 1 mM concentration except for $SnCl_2$. EDTA, a chelating compound, strongly inhibited the activity of the enzyme. The effect of different amino acids on activity showed that L-valine, L-serine, L- aspartic acid, and L-glutamic acid had a moderate inhibitory (60%, 63%, 65.4%, 69.1%, respectively) effect on the enzyme activity. The study concluded that there were similarities between *O. rhinoceros* larva arginase and those of other ureotelic animals in terms of kinetics and physicochemical properties.

Keywords: Arginase, *Oryctes rhinoceros*, Larvae, Gut, Ornithine.

INTRODUCTION

Arginase is a manganese-dependent enzyme found practically in all living things (Jenkinson *et al.*, 1996). It catalyses the conversion of L-arginine to urea and L-ornithine, which is the last step in the Urea cycle. It plays an essential role in nitrogen metabolism and ammonium removal from the body (Greengard *et al.,* 1970; Ikemoto *et al.*, 1990; Ash, 2004). The ability of arginase to control arginine availability has been widely documented. It has been shown to influence the formation of nitric oxide (NO), polyamines, arginine, and glutamate (Benson *et al.*, 2011). Some studies have shown arginase to be a metal-activated enzyme, i.e., a metalloenzyme (Ehigie *et al.,* 2010; Rath *et al.,* 2014; Al-saad *et al.*, 2021). Bivalent metal ions such as cobalt, nickel, manganese, and iron can activate arginase (Li *et al.,* 2006). Arginase has also been identified in some insects, worms, molluscs, fishes, bacteria, fungi, yeast, actinomycetes, algae, and plants, including mammalian tissues.

Oryctes rhinoceros are commonly called coconut beetle, rhinoceros beetle, palm rhinoceros beetle and asiatic rhinoceros beetle. It is of economic importance because it is a known pest of palm trees. It has a life cycle that can range from 4 to 9 months. More than one generation can occur per year (Giblin-Davies, 2001). Arginase plays a crucial role in the urea cycle of ureotelic organisms. It also occurs in the extrahepatic tissues of uricotelic (e.g., insects, birds) and ammonotelic (e.g., fishes, amphibians) organisms with no functioning urea cycle. The function of arginase in these organisms is the production of ornithine, proline, and glutamate. There is a need to provide information on the physicochemical and catalytic properties of arginase from uricotelic organisms, e.g., *O. rhinoceros*, thus allowing for comparison between ureotelic and non-ureotelic arginases.

METHODS

Sample collection

The *Oryctes rhinoceros* larva was sourced from Itokin, Ogun State, Nigeria, and identified at the

Department of Zoology, Obafemi Awolowo University, Ile-Ife, Osun State. The gut of the larva was removed and stored at -4 °C until required.

Enzyme extraction

One hundred grams (100 g) of the gut was homogenized in three volumes of 0.01 M Tris-HCl buffer pH 7.5 containing 0.25 M sucrose and 0.01 M $MnCl_2$. The resulting mixture was centrifuged at 6000 rpm for 30 min at 10 °C. The pellet was discarded and the supernatant obtained was taken as the crude enzyme which was assayed for arginase activity and protein concentration.

Enzyme assay

The modified method of Kaysen and Strecker (1973) was adopted. The reaction mixture contained 0.6 mL of 50 mM sodium carbonate buffer (pH 10) containing 1 mM $MnCl_2$, 0.3 mL of 0.33M arginine, and 100 µL of the enzyme in a final volume of 1 mL. The mixture was incubated at 37°C for 10 min and terminated with 2.5 mL of Ehrlich reagent (2.0 g of p-dimethylaminobenz aldehyde in 20 mL of concentrated hydrochloric acid and made up to 100 mL with distilled water). The absorbance was taken at 450 nm after 20 min. The urea produced was estimated from a standard urea curve prepared by varying concentrations of urea. Protein concentration was determined by the modified method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Enzyme purification

The supernatant of the crude homogenate obtained was brought to 80% ammonium sulphate saturation. The mixture was left overnight at 4°C, followed by centrifugation at 4,000 rpm for 10 min at 10ºC. The precipitate was dialysed and centrifuged at 4,000 rpm (10ºC) for 10 min to obtain a clear supernatant (enzyme solution). A 6 mL volume of the enzyme solution was layered on a pre-treated DEAE-cellulose anion exchanger column and fractions of 3 mL were collected from the column at a rate of 25 mL per hour. The fractions were assayed for arginase activity and protein was monitored spectrophotometrically at 280 nm. The active fractions were pooled and concentrated using 50% glycerol in 0.04 M Tris-HCl buffer, pH 7.5. A 2 mL volume of the active fraction was layered on the Reactive Blue 2-Agarose Affinity column. Fractions of 2 mL were collected from the column at a rate of 12 mL per hour. The active fractions from the column were pooled and dialyzed against 50% glycerol. The dialyzed fractions were assayed for arginase activity and protein content.

Determination of native molecular weight

The native molecular weight of *O. rhinoceros*' arginase was determined on a Sephadex G-100 column (2.5 x 90 cm). The column was calibrated using prepared 10 mg/mL (in 40 mM Tris-HCl buffer pH 7.5) of bovine serum albumin (M_r 66000 Dalton), ovalbumin (M_r 45000 Dalton), alpha globulin (M_r 150,000 Dalton), α-chymotrypsinogen (M_r 25,000 Dalton) and lysozyme (M_r 14,500 Dalton). Thereafter, 10 mL of the protein markers were applied to the column and eluted with Tris-HCl buffer pH 7.5. Fractions of 5 mL were collected, and protein determination was carried out as earlier described. The void volume (V_o) of the column was determined by the elution volume (V_e) of Blue dextran (5 mg/mL). A 10 mL aliquot of the enzyme solution was then applied to the same column and the elution volume of the arginase was determined.

Determination of subunit molecular weight

The subunit molecular weight of the pure enzyme was determined by SDS polyacrylamide gel electrophoresis following the procedure of Weber and Osborn (1975) on a 10% rod gel. Standard proteins were as indicated in the Sigma Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis.

Determination of kinetic parameters

The kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying the concentration of arginine between 20 mM and 250 mM in 2 mM Tris-HCl (pH 8), in the presence of 1 mM $MnCl_2$. The kinetic parameters were obtained from the double reciprocal plot (Lineweaver and Burk, 1934).

Effect of pH and temperature on enzyme activity

The effect of pH on arginase activity was studied

by assaying the enzyme at pH 3 to 11 using different buffer solutions (5mM citrate buffer (pH 3-6), 5mM phosphate buffer (pH 7) 5mM Tris-HCl buffer (pH 8 -9) and 5mM sodium carbonate buffer (pH 10-11). To determine the effect of temperature on the activity of the enzyme, arginase was assayed at a temperature between 30 °C and 100 °C. The assay mixture was first incubated at the indicated temperature for 10 min before the reaction was initiated by the addition of 100 µL of the enzyme that had been equilibrated at the same temperature.

Heat stability of the enzyme

A 1.2 mL aliquot of the enzyme solution was incubated at 30 °C, 40 °C, 50 °C and 60 °C respectively for 1 h. Also, 100 µL of the enzyme was withdrawn and assayed for residual activity at the incubation time. A plot of percentage residual activity against incubation time was made to determine the stability of the enzyme.

Effect of metal ions on arginase activity

A modified method of Kaysen and Strecker (1973) was employed to study the effect of the following salts on the activity of arginase: Hg^{2+}, Ba^{2+}, Sn^{2+}, and Ca^{2+} at a concentration of 1 mM, 5 mM, and 10 mM in a typical arginase assay mixture. The assay mixture without the salts was taken as a control with 100% activity.

Inhibition studies

Effect of metal-chelating compounds and amino acids

The inhibitory effects of chelating compounds and boric acid were investigated to determine if Mn^{2+} is involved in the catalytic reaction of *Oryctes rhinoceros* larva gut arginase. The compounds used include EDTA, boric acid, sodium borate, and ascorbic acid. The inhibitory effects of amino acids on the enzyme activity using arginine as a substrate were investigated. The amino acids used for the study included L-proline, L-glutamic acid, L-valine, L-aspartic acid, and L-serine. The different amino acids (25 mM) were used as inhibitors in a routine arginase assay procedure.

Substrate specificity

The ability of the *O. rhinoceros* gut arginase to utilize different substrates was investigated by testing its activity towards structurally similar compounds, i.e., L-arginine, L-arginine monohydrochloride, and guadinine chloride. The enzyme activity was measured as described earlier. The solutions of the above compounds (30 mM) were prepared in 2 mM Tris-HCl buffer (pH 7.2) and assayed routinely.

Statistical analysis

All experimental assays were conducted in triplicate. The standard deviation values were estimated using GraphPad software ($P<0.05$).

RESULTS

Purification of *O. rhinoceros* gut arginase

The summary of the purification steps of arginase isolated from the gut of *Oryctes rhinoceros* is shown in Table 1. The elution profiles after ion exchange chromatography and reactive blue-2 affinity chromatography are shown in Figures 2 and 3, respectively. The results showed a purification fold of 63.4, a yield of 16.5 and a specific activity of 38.7 U/mg.

Table 1: Summary of the purification of arginase from the gut of *Oryctes rhinoceros* larva.

Purification steps	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	% Yield	Purification fold
Crude Enzyme	125	3,352.5	2,036.25	0.61	100	1
80% ammonium Sulphate	68	925	1309.50	1.42	64.31	2.3
DEAE-Cellulose Ion exchange chromatography	15	58.5	600	10.3	29.47	16.9
Reactive Blue 2 agarose affinity chromatography	11	8.68	335.9	38.7	16.5	63.4

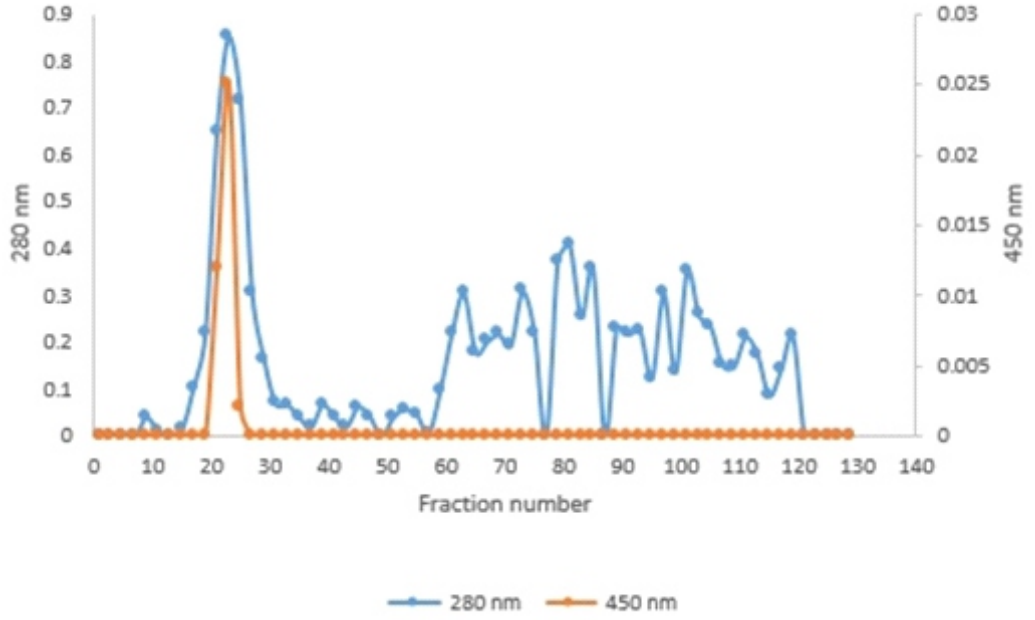

Figure 1: Elution profile of arginase from the gut of *Oryctes rhinoceros* on DEAE Cellulose Ion-Exchange Chromatography.

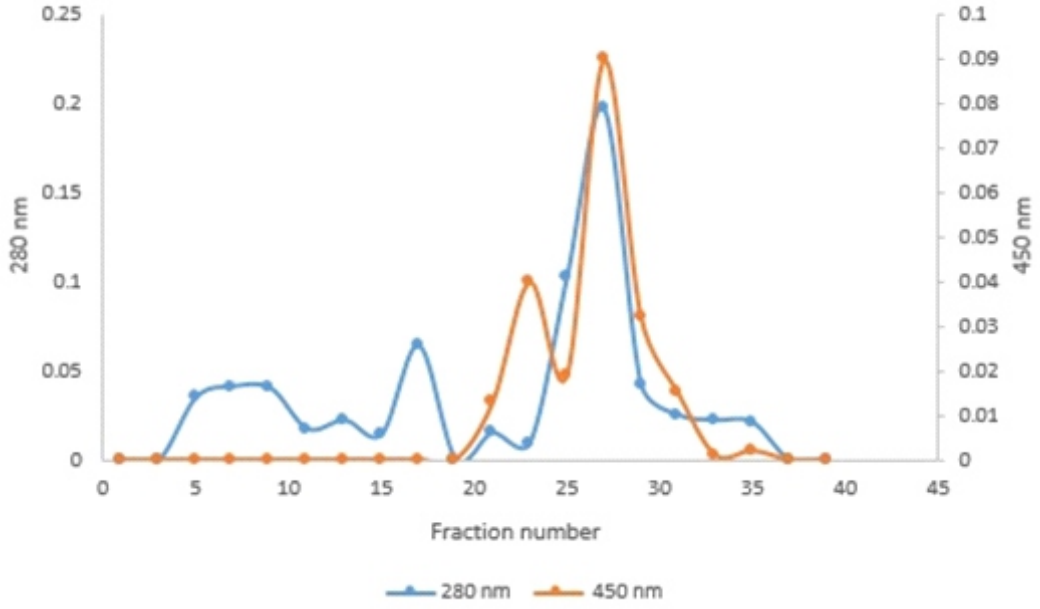

Figure 2: Elution profile of arginase from the gut of *Oryctes rhinoceros* on Blue 2-agarose affinity chromatography.

Molecular weights

Gel filtration on Sephadex G-100 resulted in an estimated molecular weight of 82,000 daltons. The calibration curve for the determination of the native molecular weight of the enzyme is presented in Figure 3. The homogeneity of the enzyme was ascertained by performing a 10% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A single band was obtained with a molecular weight of 45 kDa (Figure 4).

Kinetic parameters

The K_m and V_{max} obtained for *Oryctes rhinoceros* larva gut arginase was 11.25 mM and 13.055 μmol/ml/min.

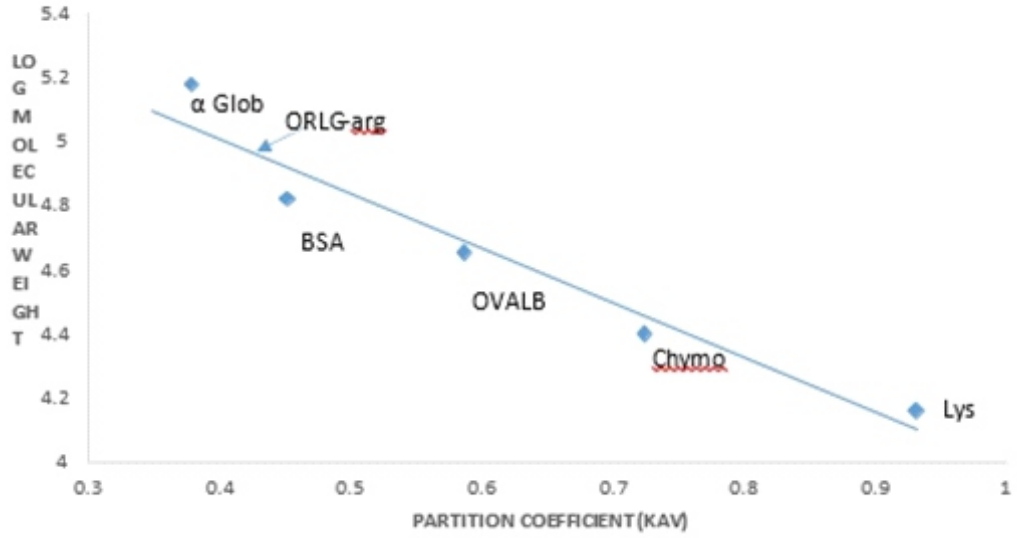

Figure 3: Calibration curve for the determination of the native molecular weight of *Oryctes rhinoceros* larva gut arginase (ORLG-arg).

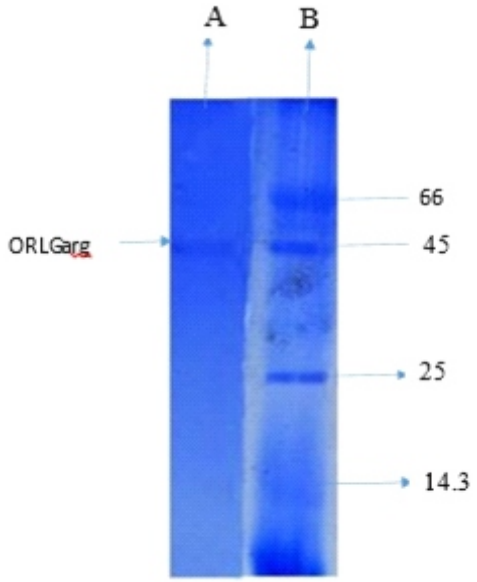

Figure 4: SDS-PAGE for the determination of subunit molecular weight of arginase from the gut of *Oryctes rhinoceros* Larva. Lane A = Purified arginase from the gut of *Oryctes rhinoceros* larva (ORLG-arg). Lane B = Standard Proteins: Lysozyme (14.3 kDa), α Chymotrypsinogen (25 kDa), (45 kDa), and Bovine Serum Albumin (66 kDa).

Effect of pH, Temperature on the enzyme activity and Stability

Arginase activity was found to be optimum at pH 8.0 (Figure 5). The assay to investigate the effect of temperature on the arginase activity showed the enzyme to have its optimum temperature at 60°C (Figure 6). Also, heat stability studies indicated that the *Oryctes rhinoceros* gut arginase retained more than 60% of its activity at 50°C for 60 min (Figure 7).

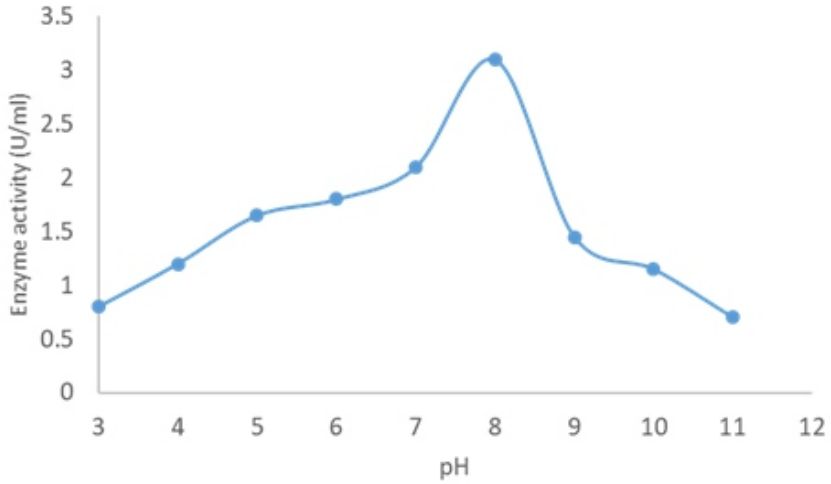

Figure 5: Effect of pH on arginase activity from the gut of *Oryctes rhinoceros* larvae.

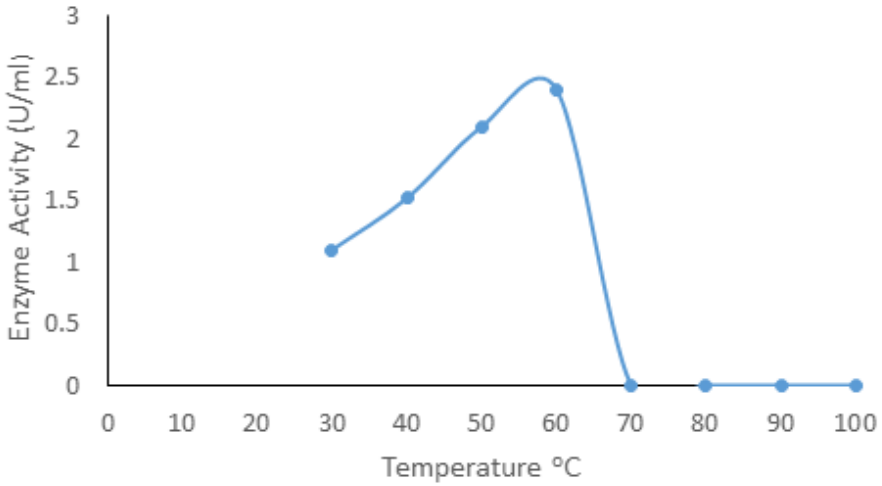

Figure 6: Effect of temperature on arginase activity from the gut of *Oryctes rhinoceros* larvae.

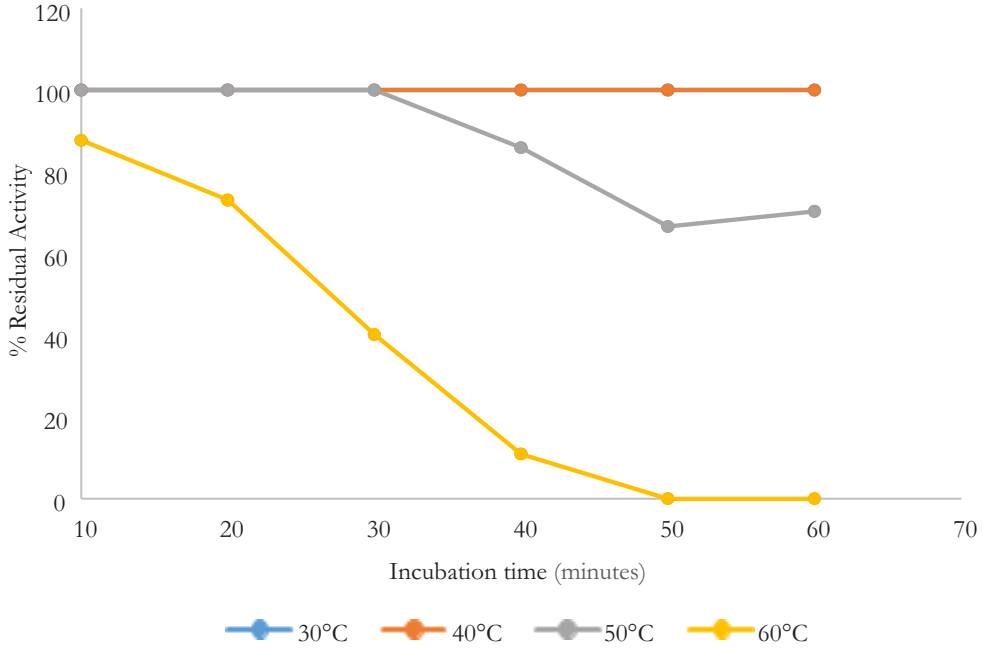

Figure 7: Thermal stability of arginase from the gut of *Oryctes rhinoceros* larvae

Inhibitory effect of some compounds and metal ions on the activity.

The effect of divalent metal ions (Ca^{2+}, Ba^{2+}, $Ag^{2+}$and Sn^{2+}) at 1 mM, 5 mM, and 10 mM concentrations is described in Figure 8. The effect of chelating compounds was investigated on arginase activity. The enzyme was significantly inhibited by all the chelating compounds (Figure 9). A decrease in residual activity was observed in the presence of L-proline (93%), L-serine (63%), L-valine (60%), L-aspartic acid (65%), and L-glutamic acid (69%).

Substrate Specificity

The substrate specificity of *Oryctes rhinoceros* larva gut arginase was investigated by assaying the enzyme with other guanidine compounds. *O. rhinoceros* larva gut arginase showed a preference for arginine (100%), while other substrates (arginine monohydrochloride and guanidine chloride) had a residual activity of 96.4% and 88.8%, respectively.

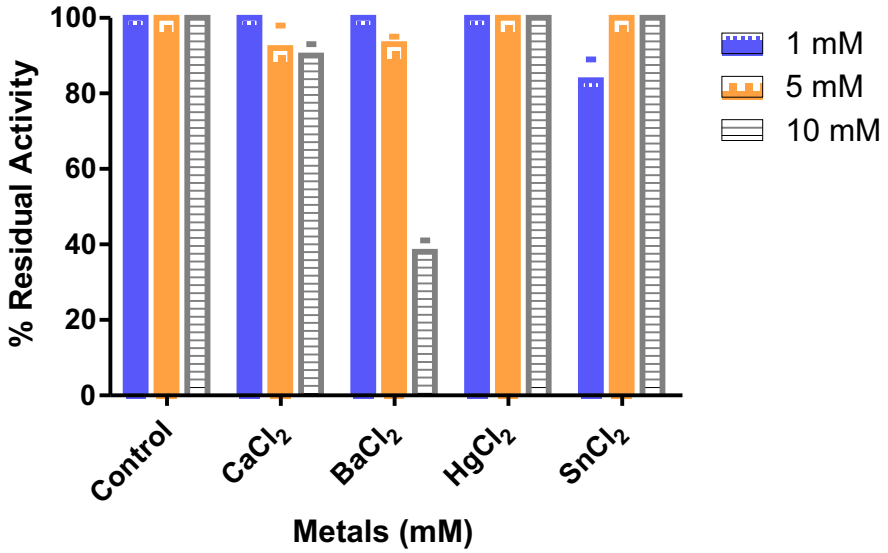

Figure 8: Effect of Ca^{2+}, Ba^{2+}, Hg^{2+}, and Sn^{2+} on activity of *Oryctes rhinoceros* larva gut arginase.

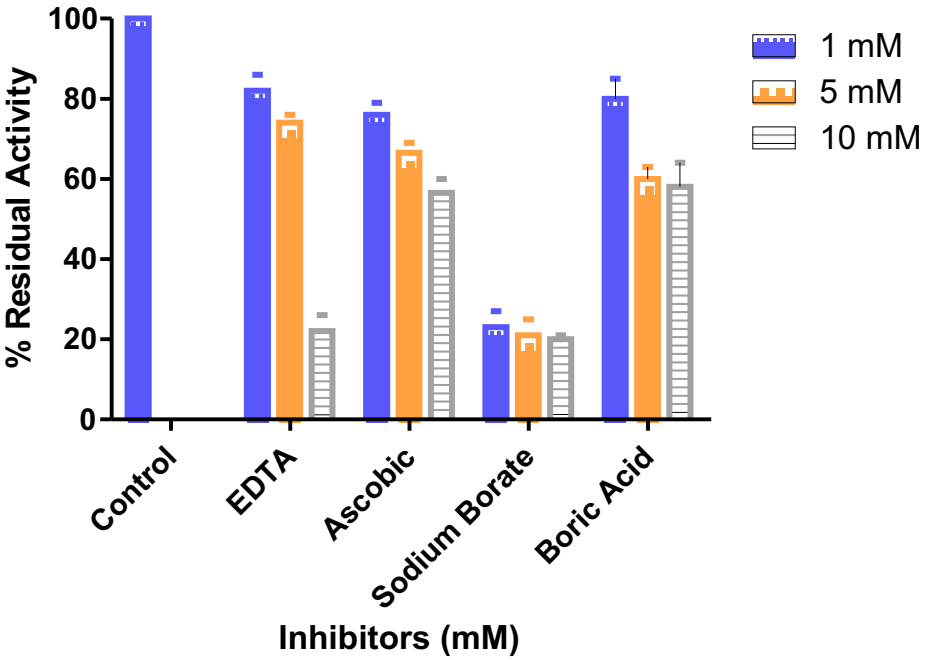

Figure 9: The inhibitory effect of some compounds on the activity of *Oryctes rhinoceros* larva gut arginase.

DISCUSSION

Arginase has been reported in many organisms from bacteria to animals (Dabir *et al*., 2005; Zhang *et al.,* 2011; Hwangbo *et al.,* 2019; Al-Saad *et al.,* 2021). In this study, *Oryctes rhinoceros* larva arginase was purified with a specific activity of 38.7 U/mg, yield of 16.5%, and purification fold of 63.4. The specific activity of arginase as determined by different researchers varied from 5.21 to 40 U/mg protein (Okonji *et al*., 2011; Al-Saad *et al.,* 2021). Okonji *et al.* (2013) reported a specific activity of 3.7 U/mg of protein for grasshopper gut arginase. The specific activity reported for freshwater prawn hepatopancreas arginase was 5.7 µmol/min per mg of protein (Ehigie *et al*., 2010). The specific activity obtained in this study is higher than the one reported for some ureotelic and uricotelic arginases.

The molecular weight of arginases varies between the ureotelic and uricotelic organisms. The ureotelic arginases have been reported to have a molecular weight of about 130,000 daltons while the uricotelics arginases possess a molecular weight of about 280,000 daltons. Ezima and Agboola (2007) reported that fruit bat arginase has a native molecular weight of 80,000 daltons. Also, earthworm gut arginase was reported to have a native molecular weight of 26,000 daltons (Lino and Shimadare, 1986). A native molecular weight of 82,000 daltons was obtained for arginase from the gut of *Oryctes rhinoceros* larva. The small molecular weight obtained might be a pre-metamorphosis form of the enzyme which might change in form during metamorphosis. Several K_m values have been reported for arginases from varying sources. The K_m value of arginase from the gut of the *Oryctes rhinoceros* larva for L-arginine was 11.25 mM. The K_m value of arginase in some studies varied from 4 to 40 mM (Mohammed *et al*., 2005; Ehigie *et al.* 2010; Okonji *et al.* 2013).

The highest enzyme activity was obtained at pH 8. This value is consistent with those reported from other sources. For example, grasshopper arginase and hepatopancreas of giant freshwater prawn arginase have higher activity in a pH range of 7-8.5 (Ehigie *et al*., 2010; Okonji *et al*., 2013). Also, the effect of temperature was carried out on arginase activity, and an optimum temperature of 60°C was observed in the presence of Mn^{2+}. The result obtained is similar to some reported by investigators for some arginases. The effect of divalent cations (Ca^{2+}, Ba^{2+} Hg^{2+} , and Sn^{2+}) was carried out on arginase activity and it was observed that each of the cations activated the enzyme at 1 mM concentration to residual activity of 100% except Sn^{2+}. This result obtained showed some similarities with reports by other investigators. Okonji *et al.* (2014) reported that the liver arginase activity from *Heterotis niloticus* showed increased activation with Mn^{2+} Zn^{2+} Mg^{2+} Hg^{2+} Co^{2+} and Na^{2+}, while other metals such as Sn^{2+}, Ni^{2+}, and Ba^{2+} show slight inhibition. The effect of the chelating compound on *Oryctes rhinoceros* gut arginase activity showed that it was strongly inhibited by EDTA. More so, increased inhibition was observed as the concentration of EDTA increases. Green *et al.* (1990) noted that *Saccharomyces cerevisiae* arginase reported a strong inhibition of the enzyme by EDTA. In another report, Ehigie *et al.* (2010) reported that EDTA, citrate, and urea strongly inhibited arginase activity from the hepatopancreas of giant freshwater prawn (*Macrobrachium rosenbergii*). Boronic-based inhibitors are potent inhibitors of arginase activity (Reczkowski and Ash, 1994). *Oryctes rhinoceros* larva gut arginase was significantly inactivated by borate and boric acid which was similar to the reports of some investigators (Marc *et al.,* 2017; Borek *et al.,* 2020).

The effect of amino acid on arginase from the gut of *Oryctes rhinoceros'* larva showed that L-valine, L-serine, L-aspartic acid, and L-glutamic acid had moderate inhibitory (60%, 63%, 65.4%, 69.1%, respectively) effect on the enzyme activity. Okonji *et al.* (2012) also reported that L-proline, L-lysine, L-glutamic acid, L-aspartic acid, L-aspartate, L-serine, and L-valine caused less than 50% inhibition of arginase activity from the liver of *Kinixy erosa* (tortoise). Okonji *et al.* (2013) also reported that the amino acids (proline, lysine, aspartate, and valine) showed no inhibition on *Z. variegates* gut arginase. Also, the liver arginase of *H. nitoticus* was slightly inhibited by L-aspartate and L-proline (Okonji *et al*., 2014).

Arginase activity has been reported to depend on the presence of an intact guanidine group of its substrate (Ash, 2004). Therefore, the study of substrate specificity involved the use of a series of

guanidine compounds as an alternative substrate to L-arginine. For this study, arginine monohydrochloride and guanidine chloride were used as an alternative substrate to arginine and the arginase from the gut of *Oryctes rhinoceros* larva utilized the substrate to residual activity of 96.4% and 88.4 %, respectively. The results obtained compared very well with the results of other works that reported a preference for L-arginine by arginases (both type I and type II) from different sources (Reckowski and Ash, 1994; Jenkinson *et al.,* 1996; Okonji *et al.,* 2014).

CONCLUSION

The properties of *Oryctes rhinoceros* arginase compared very well with other uricotelic arginases in terms of kinetic and physicochemical properties, and the enzyme's physiological role in *O. rhinoceros* is most likely the production of ornithine, proline, and glutamate, which are involved in polyamine biosynthesis.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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