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PROPERTIES OF THIAMINASE IN FRESHWATER PRAWN *MACHROBRACHIUM ROSENBERGII*

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ABSTRACT

Thiaminase (EC 2.5.1.2) catalyzes the displacement of the thiazole moiety of thiamine (vitamin B1) by a wide variety of nucleophiles. The presence of thiaminase in the diet has been associated with the occurrence of thiamine deficiency symptoms among mammals, including humans and fish. Here, we reported the properties of thiaminase in the hepatopancreas of fresh water prawn (*Machrobrachium rosenbregii*) from Asejire Lake in Southwestern Nigeria. The enzyme was partially purified with ammonium sulphate precipitation and affinity chromatography. The specific activity of the thiaminase was 0.2 µmol/min/mg of protein. The Michealis-Menton (K_m) constant was 0.5 mM as compared to 1 mM of aniline. The optimum pH was 5.0, while the optimum temperature was 50^0C. Reduced glutathione (GSH), 2 – mercapto ethanol (MCPE), Ethylene diamine tetra acetic acid (EDTA) and ascorbate significantly inhibited the enzyme but citrate showed activation of the enzyme activity at 97%. The amino acids: serine, lysine, valine, cysteine, proline, and aspartate also showed significant inhibition. The cations: Ca^{2+}, Mg^{2+} Mn^{2+} and Sn^{+} showed complete inhibition on the enzyme. Hg^{2+}, showed a slight inhibition on the enzyme. The thiaminase activity was significantly enhanced by Ni^{2+} while Zn^{2+} had no activity on the enzyme.

KEYWORDS: Thiaminase, Fresh Water Prawn, Hepatopancreas, Physicochemical, Properties

INTRODUCTION

Thiaminase (EC 2.5.1.2) is an enzyme found in various aquatic foods, including some fish, molluscs and crustaceans, many of which form the diets of turtles and other exotic pets (Fujita, 1954; Fujita, 1972; Murata, 1982). Fundamentally, Thiaminase breaks down and inactivates thiamine (Vitamin B1). The presence of thiaminase in the diet has been associated with the occurrence of thiamine deficiency symptoms among mammals, including humans (Green and Evans 1940; Evans 1975; Chick *et al.* 1989; Earl and McCleary 1994; Nishimune *et al.* 2000), and fish (Saunders and Henderson 1974; Fisher *et al.* 1996, 1998a, 1998b; Fitzsimons and Brown 1998; Honeyfield *et al.*, 2005).

Vitamin B1 is essential for normal nervous system and immune system function, as well as generating energy (in the form of glucose) from carbohydrates in the diet. This means that animals which are fed a large amount of thiaminase-containing foods and develop a Vitamin B1 deficiency often show symptoms of muscle and sensory disorders, susceptibility to disease and infection, and lethargy/lack of appetite. The important role of thiamine (vitamin B1) in human

and animal health has been long-recognized, but a broad appreciation of its role in environmental health has developed more slowly (Balk *et al*; 2009). In animal husbandry, instances of mortality from thiamine deficiency were first described in the 1940s in mink and foxes raised for fur production (Green and Evans 1940; Stout *et al*; 1963), then later in cattle, sheep and goats (Edwin and Jackman 1970; Thomas *et al*; 1987; Ramos *et al*; 2003).

Japanese scientists pursued extensive studies during and following World War II linking thiamine deficiency in humans to thiamine decomposing bacteria (Kimura, 1965). Thiamine deficiencies in wild populations of predatory fish were first recognized in the 1990s as responsible for a widespread mortality syndrome observed for decades in valuable Baltic Sea and Laurentian Great Lakes fisheries (Fisher *et al*; 1995). One common dietary link in thiamine-deficiency mortality syndromes observed in commercial fisheries, animal husbandry and humans has been the consumption of food with high thiaminase activity. Thiaminase activity occurs among cyanobacteria (Arsan and Malyarevskaya 1969), plants, bacteria (Fujita 1954), invertebrates (Birger *et al.*, 1973; Nishimune *et al.*, 2000), shellfish, and fish (Melnick *et al.*, 1945; Harris 1951; Fujita 1954; Greigh and Gnaedinger 1971). Thiaminase catalyzes the displacement of the thiazole moiety of thiamine (vitamin B1) by a wide variety of nucleophiles: aromatic primary amines, heterocyclic compounds, and sulfhydryl compounds (i.e., co-substrates) (Fujita 1954). The physiological significance of thiaminase in species that contain the enzyme is not known (Harris 1951; Fujita 1954; Evans 1975; Sato *et al.*, 1994; Costello et al. 1996; Bos´ and Kozik 2000; Onozuka *et al.*, 2007) and consequently there is a lack of knowledge concerning the possible agents or factors that cause the wide variation in thiaminase activity observed within organisms.

Thiaminase is linked to fish reproductive failure and animal mortality, consequently it has been the focus of studies investigating syndromes associated with enzymatic thiamine degradation (Shimazono and Katsura 1965; Evans, 1975; Honeyfield *et al*; 2005). Predatory salmonine fishes suffering from thiamine deficiency in North America and Europe prey upon clupeid fishes with high thiaminase I activity (Wistbacka *et al*; 2002; Brown *et al*; 2005). Feeding experiments showed that mink and foxes died from thiamine deficiency when fed raw fish that contained thiaminase (Green and Evans 1940; Stout *et al*; 1963). Domestic animals such as horses and cattle regularly die from thiamine deficiency after feeding on bracken fern that contains high thiaminase I activity (Evans, 1976).

Freshwater Prawns (*Macrobrachium rosenbergii*) is a species of freshwater shrimp native to the Indo-Pacific region, northern Australia and Southeast Asia. This species (as well as other *Macrobrachium*) is commercially important for its value as a food source (Wynne; 2000). *M. rosenbergii* have been reported to contain significant amount of thiaminase, along with other digestive enzymes (Chuang *et al.*, 1985). The digestive enzymes activities reflect the feeding habits of the aquatic animals and reflect their ability to utilize different feed ingredients. This study reports the properties of thiaminase in the hepatopancreas of fresh water prawn (*Machrobrachium rosenbregii*).

MATERIALS

Enzyme Source

All reagents and chemicals used were analytical grade and were purchased from Sigma Chemical Company. Giant fresh water prawn (Machrobrachium rosenbergii) was harvested from Asejire lake (07[degrees] 21’N 04[degrees] 05’E), Osun State, Nigeria.

METHODS

Tissue Extraction and Purification

The enzyme was isolated at room temperature unless otherwise stated using extensive modifications of the procedure of Wittliff and Airth (1968).All operations were carried out at temperatures between 0 and 36°C. Centrifugations were done at 10°C using IEC-DPR Cold Centrifuge. The hepatopancreas of *Machrobrachium rosenbergii* were quickly excised and stored in the refrigerator until required. The frozen tissue (hepatopancreas) were allowed to thaw at room temperature and weighed. A total of 50.0g (wet weight) of hepatopancreas was used for this preparation. The weighed tissue was minced and homogenized in a Warring Blender for 10 min in four volumes (w/v) of the homogenization buffer containing 0.2 M Sodium phosphate buffer, pH 6.5. The homogenate obtained was stirred, occasionally, for one hour and then subjected to centrifugation at 6,000 rpm at 10°C for 30 min. The supernatant was filtered through a double layer of cheesecloth. The cellular debris was resuspended in one volume of the homogenization buffer, homogenized in the Warring Blender and centrifuged under the same condition. The supernatant obtained was combined with that from the first centrifugation.

Thiaminase and Protein Assay

Thiaminase activity was determined according to the modified method of Nishimune *et al*. (2000). The enzyme sample was incubated at 37°C in 0.1 M Tris-HCl buffer, pH 8.0, with 10^{-5} M thiamine and 4×10^{-3} M aniline and incubated for 30 min. The remaining thiamine was oxidized with the addition of 1.0 ml of 200 g/l NaOH and assayed spectrophotometrically. Absorbance was read at 411 nm immediately. One unit of enzyme activity is that amount of enzyme which catalyses the formation of one micromole of heteropyrithiamine in 30 minutes.

The protein concentration was routinely determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Ammonium Sulphate Precipitation

The homogenate was brought to 70% saturation by the addition of solid ammonium sulphate and stirred for 1 hr. This was stored at 4^{o}C in a refrigerator 18 hrs before centrifugation at 4000 rpm for 30 min. The ammonium sulphate was removed by dialysis in 5mM Tris Hcl elution buffer; pH 7.5.

Affinity Chromatography

The dialysate was loaded directly into a reactive blue agarose dye affinity column (2.5 x 10cm) which had been equilibrated in 5mM Tris Hcl elution buffer; pH 7.5. The thiaminase was eluted from the column using 1 mM solution of sodium chloride in the elution buffer at a flow rate of 0.8 ml/min. 5 ml fractions were collected and the activity and protein concentrations of the various fractions were determined.

Determination of Kinetic Parameters

The kinetic parameters (K_m and V_{max}) of the enzyme were determined according to the modified method of Nishimune *et al.* (2000). The K_m of thiamine and aniline were determined by varying the concentrations of thiamine and aniline between 40 mM and 150 mM in 0.1 M Tris-HCl buffer pH 8.0 respectively. The kinetic parameters were determined from the double reciprocal plot of Lineweaver and Burk (1934).

Effect of Amino Acids on Thiaminase Activity

The effect of amino acids on thiaminase activity was studied using the following amino acids (0.005M); proline, cysteine, histidine, valine, and lysine. The enzyme was assayed in a typical enzyme assay. A reaction mixture of 1 ml contained 0.4 ml of buffer, 0.2 ml of thiamine, 0.2 ml of aniline, 0.1 ml distilled water and 0.1 ml of enzyme solution.

Effect of pH on Thiaminase Activity

The effect of pH on thiaminase was studied by assaying the enzyme at different pH values: citrate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0-8.0), A reaction mixture of 1 ml contained 0.4 ml of buffer, 0.2 ml of thiamine, 0.2 ml of aniline, 0.1 ml distilled water and 0.1 ml of enzyme solution.

Effect of Some Inhibitors on Thiaminase Activity

The effects of reduced glutathione (GSH), 2-mercaptoethanol, ethylene diamine tetra acetic acid (EDTA), ascorbate, and citrate on the activity of *Machrobrachium rosenbergii* thiaminase were investigated. 0.5 mM of each of the compounds was used in the typical assay reactions.

Effect of Temperature

The enzyme was assayed at different temperatures between 30°C and 100°C to investigate the effect of temperature on the activity of the enzyme and to determine the optimum temperature of the enzyme. The assay mixture was first incubated at the indicated temperature for 30 min before initiating reaction by the addition of an aliquot of the enzyme which had been equilibrated. The residual enzyme was then assayed at 30 minutes interval.

Effect of Cations on Thiaminase Activity

The effect of cations on thiaminase activity was studied using the following cations Zn^{2+}, Ni^{2+}, Hg^{2+}, Mn^{2+}, Sn^{2+}, Ca^{2+}, and Mg^{2+}. The enzyme was assayed in a typical enzyme reaction using 0.1 mM metal concentrations.

RESULTS

Enzyme Purification

The purification of *Machrobrachium rosenbergii* hepatopancreas tissue thiaminases was carried out as described in the materials and methods. The elution profile of the Reactive blue 2-crosslinked agarose affinity chromatography is as shown in Figure 1.

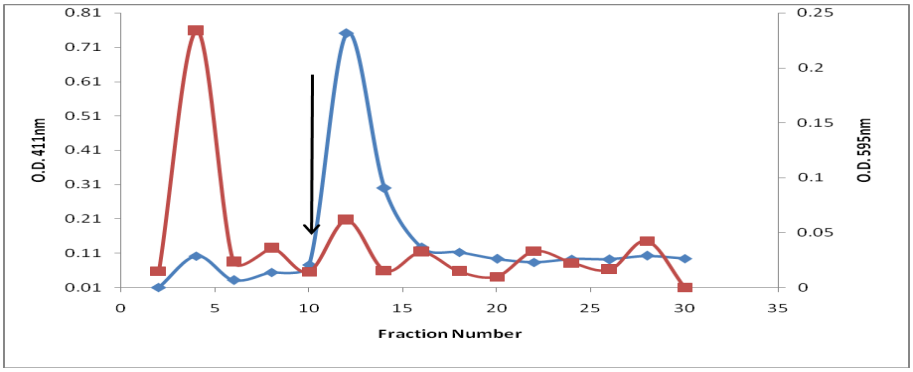

Figure 1: Reactive Blue 2-Crosslinked Agarose on Affinity Chromatography

The column was first washed with 100 ml 5 mM Tris-HCl buffer, pH 7.5. The enzyme solution was layered on the packed column and eluted with a 200 ml linear gradient of 0.1 M NaCl in 5m M Tris- HCl buffer, pH 7.5. Fractions of 1 ml were collected from the column. -■-■-■- activity profile; -■-■-■- Protein profile.

Kinetic Parameters

The Line weaver-Burk plot of thiaminase using thiamine as substrate is shown in Figure 2 while Figure 3 shows that of aniline. The overall results indicated that thiaminase from *Machrobrachium rosenbergii* shows more affinity towards thiamine as substrate with a K_m value of 0.5 mM as compared to 1 mM of aniline.

Effect of pH on the Enzyme Activity

The activity of *Machrobrachium rosenbergii* thiaminase was determined in the buffer pH range from 3.0 to 10.0 at 37^0c. The optimum pH was found to be 5.0 (Figure 4).

Effect of Temperature

The activity of thiaminase was assayed at temperatures between 30°C and 100°C. The optimum temperature of the enzyme was found to be 50°C at pH 8.0 (Figure 3).

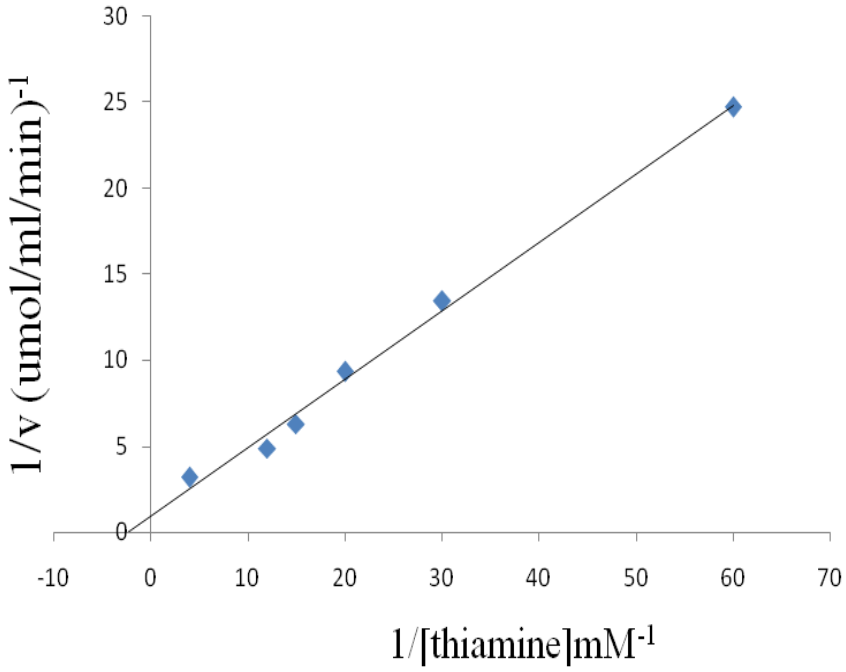

Figure 2

The Lineweaver-Burk plot showing the effect of varying concentrations of thiamine on the initial reaction velocity at pH 8.0. The reaction mixture of 1 ml contained 0.1 mM Tris-HCL buffer, pH 8.0, and varying concentrations of thiamine at fixed concentration of 50 mM aniline.

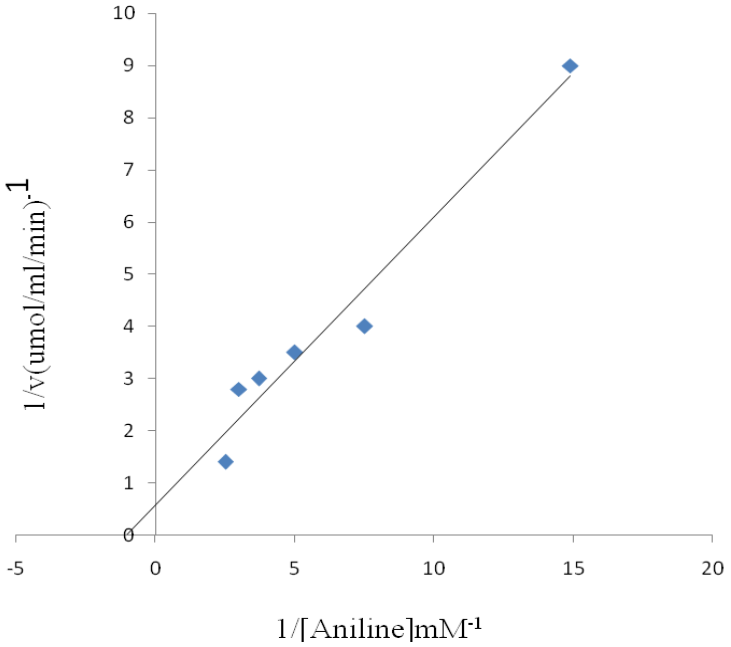

Figure 3: The Line weaver-Burk Plot Showing the Effect of Varying Concentrations of Aniline on the Initial Reaction Velocity at Ph 8.0. The Reaction Mixture of 1 Ml Contained 0.1 Mm Tris-HCL Buffer, Ph 8. 0, and Varying Concentrations of Aniline at Fixed Concentration of 20 Mm Thiamine

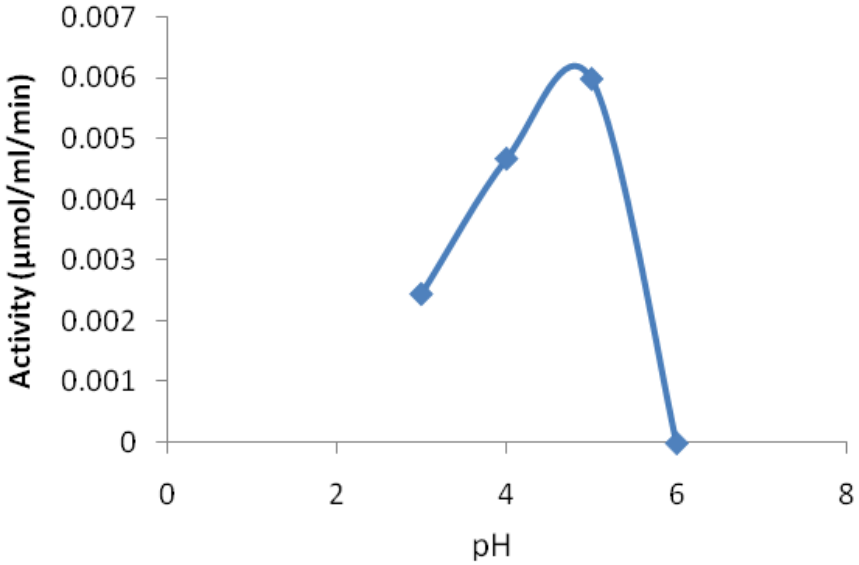

Figure 4: Effect of Ph on *Machrobrachium rosenbergii* Thiaminase

1 ml of the reaction mixture contained 0.4 ml of the appropriate buffer, 0.2 ml thiamine, 0.2 ml aniline and 0.01 ml enzyme preparation. The following buffers of different pH values were used for the pH determination: 5 mM citrate buffer (pH 3.0-5.0), 5 mM phosphate buffer (pH 6.0-8.0).

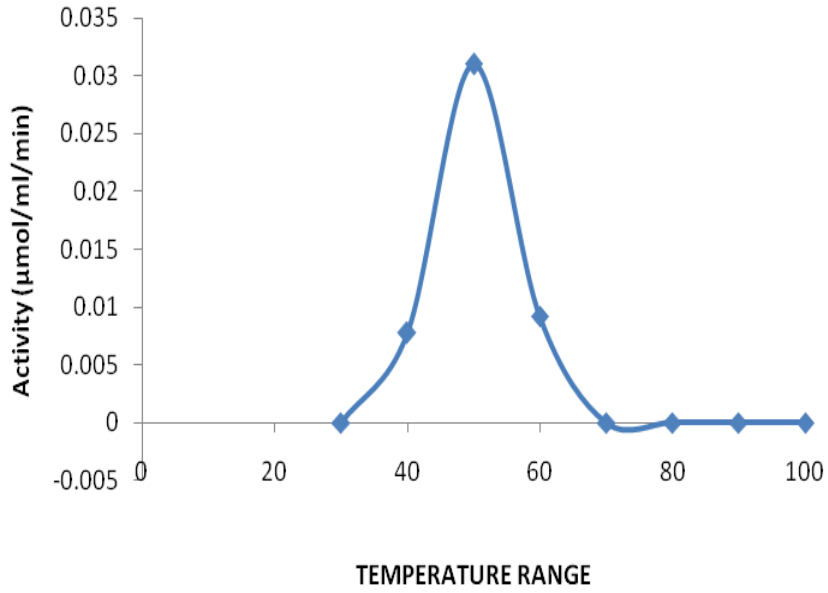

Figure 5: Effect of Temperature on the Activity of Thiaminase

The activity of *Machrobrachium rosenbergii* thiaminase was assayed at temperatures between 30^0 C and 100°C.

Effects of Cations on Thiaminase Activity

Table 2 shows the effects of Cations on Thiaminase Activity. The enzyme activity was markedly enhanced in the presence of Nickel. Mercury showed a slight inhibitory effect, Manganesse, Calcium, Magnessium, and Tin severely inhibited thiaminase activity, while the enzyme had no activity in the presence of Zinc.

Effects of Amino acids on Thiaminase Activity

The result obtained for the effect of various amino acids on thiaminase activity is presented in Table 3. The effects of the amino acids (serine, lysine, valine, cysteine, proline, and aspartate) on *Macrobrachium rosenbergii* thiaminase showed significance inhibition.

Effect of Inhibitors on Thiaminase Activity

The effects of some inhibitors on Machrobrachium rosenbergii thiaminase is presented in Table 4. Citrate was found to greatly enhance the activity of the enzyme, while significant inhibition was depicted by MCPE, GSH, EDTA, and Ascorbate,

Table 2: Effects of Cations on Thiaminase Activity

Metals	Nickel	Mercury	Manganesse	Calcium	Magnessium	Tin	Zinc
% RESIDUAL ACTIVITY	372.52	58.43	24.02	6.26	2.82	1.16	0

Table 3: Effect of Amino Acids on Thiaminase Activity

Acids	Serine	Lysine	Valine	Cysteine	Proline	Aspartate
%RESIDUAL ACTIVITY	31.6397	21.7090	18.4758	18.0139	14.3187	13.3949

Table 4: Effect of Inhibitors on Thiaminase Activity

Inhibitors	MCPE	Citrate	GSH	EDTA	Ascorbate
%RESIDUAL ACTIVITY	21.0162	96.9977	7.8522	0.1547	36.9515

DISCUSSIONS

The presence of thiaminase in the diet has been associated with the occurrence of thiamine deficiency symptoms among mammals, including humans (Green and Evans 1940; Evans 1975; Chick *et al*. 1989; Earl and McCleary 1994; Nishimune *et al*. 2000), and fish (Saunders and Henderson 1974; Fisher *et al*. 1996, 1998a, 1998b; Fitzsimons and Brown 1998; Honeyfield *et al*. 2005). *Machrobrachium rosenbergii* have been reported to contain significant amount of thiaminase, along with other digestive enzymes (Chuang *et al.*, 1985). The digestive enzymes activities reflect the feeding habits of the aquatic animals and reflect their ability to utilize different feed ingredients.

The results of the Michealis-Menten constant (K_m) for the two substrates (thiamine and aniline) as shown in Figures 2 and 3 indicated that *Machrobrachium rosenbergii* thiaminase preferred thiamine as substrate with a K_m value of 0.5 mM as compared to 1 mM of aniline. Kinetic studies of thiaminase in extracts of ruminant faeces showed that the affinity for one substrate varied with the concentration of the other substrate in the manner of a two-step transfer mechanism. When the alternate substrate (aniline) concentration was optimal, the apparent Michaelis constant (*Km*) for thiamine was 176 µM and the apparent *Km* for aniline was 3·19 mM (Boyd, 1985). Second substrates which could be utilized by the thiaminase were pyridoxine, amino acids (such as cysteine, histidine, lysine, tyrosine and proline), glutathione, taurine and 4-aminopyridine. Thiamine phosphate esters were inactive as substrates (Nishimune *et al*., 2000). Bos and Kozik (2000) also supported the use of numerous nucleophiles such as aniline, pyridine and 2-mercaptoethanol by the enzyme as cosubstrates, and also the best among compounds tested.

The activity of thiaminase was assayed at temperatures between 30°C and 100°C. The optimum temperature of 50^o c was obtained for *Machrobrachium rosenbergii* thiaminase. A much higher temperature of 65°C was obtained for thiaminase enzyme from *Marsilea drummondii* (McCleary and Chick, 1977). The thiaminase in the buffer extract of *Anaphe* pupae was reported to have an optimal temperature of 70°C (Nishimune *et al*., 2000). An optimum temperature of 37°C was also reported for extracellular thiaminase of *Basillus thiaminolyticus* (Wittliff and Airth, 1968).

Thiaminases from different sources exhibits varied responses to change in pH and to differing activators, cosubstrates and inhibitors (Edwin *et al*., 1982). A pH optimum of 5.0 was obtained for *Machrobrachium rosenbergii* thiaminase. McCleary and Chick (1977) reported pH optima of 8 to 9 for the thiaminase of fern nardoo fronds (*M. drummondii*). They also reported the stability of *M. Drummondii* thiaminase in the pH range of 3 to 12. It was also reported that the extracellular thiaminase of *B. thiaminolyticus* showed optimum pH over a broad pH range of 5.8 to 6.8 (Wittliff and Airth, 1968). The study of the effects of different concentrations of cations on the activity of the enzymes

reveals that thiaminase from *Machrobrachium rosenbergii* was greatly inhibited by calcium, (Bos and Kozik, 2000), manganese, magnesium and tin. The enzyme activity was markedly enhanced in the presence of nickel. Mercury showed a slight inhibitory effect while the enzyme had no activity in the presence of zinc.

Table 3 showed the results obtained for the effect of various amino acids (serine, proline, valine, lysine and cysteine, and aspartate) on the activity of *Machrobrachium rosenbergii* in the hepatopancreas tissue thiaminase. The effect of the amino acids on the enzyme activity showed significant inhibition. The study of the effects of different concentrations of Inhibitors (MCPE, Citrate, GSH, EDTA, and ascorbate) on the activity of the enzymes also showed significant inhibition of the enzyme activity except for citrate which showed an activation of approximately 97%.

CONCLUSIONS

Our investigation shows that the hepatopancreas of giant fresh water prawn (*Machrobrachium rosembergii*) is endowed with significant quantity of thiaminase activity. Although the physiological importance of thiaminase is still an enigma, however, the enzyme has been shown to decrease cellular thiamine concentration thereby impairing carbohydrate metabolism and energy production. The results from this work and previous studies (Fujita *et al*., 1954; Abe *et al*., 1987 ; Adamolekun *et al*., 1994; Nishimune, 2000) suggest that in looking for the so-far-unidentified factor(s) influencing thiaminase activity in aquatic organisms, we should focus more on the state of health of these organisms and that thorough heat treatment for inactivation of thiaminase enzyme is needed in order to make aquatic organisms safe for human consumption.

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