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***Original article*** ***Biochemistry***

# Biochemical properties of thiaminase, a toxic enzyme in the gut of grasshoppers (*Zonocerus variegatus* Linn)

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**Abstract**
The variegated grasshopper, *Zonocerus variegatus* (Linn) (Orthoptera: Pyrgomorphidae) is eaten mostly in the South western region of Nigeria. Thiaminase is a toxic enzyme present in some foods. The activity of thiaminase in the gut of *Zonocerus variegatus* is described. The enzyme was isolated using DEAE- Cellulose ion exchange chromatography and gel filtration on Biogel P-100. The enzyme had a specific activity of 7.9 unit per milligram of protein. The enzyme exhibited a maximal activity at pH 8.0 and $K_m$ of 5 and 25 µM for thiamine and aniline respectively. The substrate specificity showed that the thiaminase from *Z. variegatus* was specific for thiamine and aniline as substrates. The optimum temperature of *Z. variegatus* thiaminase was 50°C. The native molecular weight of the enzyme as determined by gel filtration was 102,000. The amino acids markedly enhanced the activity of the enzyme. The enzyme was activated by $Mn^{2+}$, $Ni^{2+}$ and $Hg^{2+}$ but inhibited by $Na^+$, $NH_4^+$, $Co^{2+}$ and $Zn^{2+}$. 2-mercaptoethanol and 6-amino hexanoic acid completely inhibited the thiaminase. *Z. variegatus* should be prepared using extensive and prolong cooking to avoid suffering from thiamine deficiency.
**Key Words**: Thiaminase; Zonocerus *variegatus*; Physicochemical Properties; Substrate Inhibition

## INTRODUCTION

Thiaminase I (EC. 2.5.1.2) catalyses the decomposition of thiamine by a base exchange reaction involving a nucleophilic displacement on the methylene group of the pyrimidine moiety [1,2]. Thiaminase I occurs in a wide range of fish, shellfish, ferns and bacteria [3-5]. Different aromatic amines, heterocyclic bases and sulphydryl compounds have been reported to participate in the hydrolysis of thiamine by thiaminase I. Roberts and Boyd [6] also extended the number of possible co-substrate bases to include a range of commonly used anthelmintics, tranquilizers and anti-histamines. The enzyme has been implicated in a number of diseases in different animals [3,7]. Its role in insect metamorphosis has also been established [8].

Hundreds of species of insects have been used as human food [9]. Most people in tropical Africa, Asia and Latin America collect insects for food which has played an important nutritional role [9]. In Africa and the Indians of western North America, the habit is especially well developed among the cultivators of the forest region. It is uncertain whether these insects are eaten because of their nutritional qualities [10, 11]. Insects and meat play the same role in the human body. Most insects are cheap, tasty and a good natural source of protein and minerals. Many species of insects are lower in fat and higher in protein [9, 12]. The variegated grasshopper, *Zonocerus variegatus* (Linn.) (Orthoptera: Pyrgomorphidae), which has a large dry season population in Southwestern Nigeria is reported eaten in the Akoko area of Ondo State [13]. The insect has also been reported to be widely eaten in Borno State, North eastern Nigeria [9]. Proximate analysis of *Z. variegatus* revealed that the insect contains high percentage of crude protein (58.0%) with an array of essential amino acids and numerous mineral elements in right proportions [9, 14]. Ademolu et al. [15] has also shown the nutritive value of various stages of development of *Z. variegatus* with the adult stage having the highest crude protein content. It is possible this insect species is rich in thiaminase that might be harmful to the population eating it. It is therefore interesting to know the biochemical properties of the thiaminase in order to propose way to treat *Z. variegatus* before consumption. Thus, the present study was designed to quantitatively assess the physicochemical properties of thiaminase.

## MATERIALS AND METHODS

### Materials

Trizma base, Trizma-HCl, ethylenediamine tetraacetic acid (EDTA), thiamine dichloride and thiamine were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Sodium dodecyl sulphate (SDS), acrylamide and N, $N^1$-methylene bisacrylamide (MBA), ammonium persulphate, N,N,$N^1$,$N^1$-tetramethylenediamine (TEMED) are from Eastman, Rochester, NY, USA. All other reagents were of analytical grade and were obtained from either Sigma or BDH. *Zonocerus variegatus* were collected from an abandoned farmland at the Ladoke Akintola University of Technology, Ogbomoso, Osun State, Nigeria, between October and November, 2010.

### Methods

#### Thiaminase and Protein Assays.

Thiaminase activity was determined according to the modified method of Nishimune *et al*. [8]. The enzyme sample was incubated at 37°C in 0.1 M Tris-HCl buffer, pH 8.0, with $10^{-5}$ M thiamine and $4\times10^{-3}$ M aniline and incubated for 30 min. This was followed with the addition of 1.0 ml of 200 g/l NaOH and assayed spectrophotometrically. Absorbance was read at 411 nm. One unit of enzyme activity is that amount of enzyme which catalyses the formation of one micromole of heteropyrithiamine in 30 min. The protein concentration was routinely determined by the method of Bradford [16] using bovine serum albumin (BSA) as standard.

#### Tissue Extraction and Purification

The gut of *Z. variegatus* was quickly excised and stored in the refrigerator until required. The frozen tissues of *Z. variegatus* were allowed to thaw at room temperature and weighed. A total of 82.8 g of *Z. variegatus* was used for this preparation. The weighed mass was minced and homogenized in a Warring Blender for 1 min in three volumes (v/w) 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 $^{o}$C for 30 min. The supernatant was filtered through a double layer of cheese cloth. The cellular debris was resuspended in one volumes of the homogenization buffer and homogenized in the Warring Blender and centrifuged under the same condition. The supernatant obtained was combined with that from the first centrifugation.

#### DEAE-Cellulose Ion Exchange Chromatography

A column (2.5 x 40 cm) of treated DEAE-Cellulose was packed and equilibrated with 5 mM Tris-HCl buffer, pH 7.2. The dialyzed protein from the preceding step was then layered on the column. The column was first washed with 0.1 M Tris-HCl buffer, pH 7.2 to remove unbound proteins, followed by elution with a 400 ml linear gradient of 0-1.0 M NaCl in 0.1 M Tris-HCl buffer, pH 7.2. Fractions of 3 ml were collected from the column that was maintained at a flow rate of 30 ml per hour. Protein was monitored spectrophotometrically using Bradford method. The fractions were also assayed for thiaminase activity. The active fractions were pooled and stored in the refrigerator.

#### Gel Filtration on Biogel P-100

Biogel P-100 gel filtration resin was washed with several changes of 0.1 M Tris-HCl buffer, pH 7.2. This was then packed unto the column (2.5 × 90) and equilibrated with 0.1 M Tris-HCl buffer, pH 7.2. The enzyme fraction from the preceding step was then applied to the Biogel P-100. Fractions of 5 ml were collected at a flow rate of 10 ml per hour. Protein was monitored spectrophotometrically using Bradford method. The fractions were also assayed for thiaminase activity. The active fractions were pooled.

The native molecular weight was determined on a Biogel P-100 column (2.5 x 90 cm). The standard proteins were α-chymotrypsinigen A ($M_r$ 24,000; 10 mg), ovalbumin ($M_r$ 45,000; 10 mg/ml), bovine serum albumin ($M_r$ 67,000; 10 mg/ml), creatinine phosphokinase ($M_r$ 88,000; 10 mg/ml) and bovine arginase ($M_r$ 120,000: 10 mg/ml).

#### Determination of Kinetic Parameters

The kinetic parameters ($V_{max}$ and $K_m$) 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 [17].

**Substrate Specificity Studies**

The substrate specificity of grasshopper (*Z. variegatus*) thiaminase was investigated by testing the activity of the enzyme with two (aniline and thiamine) compounds. The solutions of above compounds (30 mM) were prepared in 0.1 M Tris-HCl buffer, pH 8.0. Enzyme activity was measured by spectrophotometrically as described by [8]. The reaction mixture contained, in final concentration, 0.05 M Tris-HCl buffer, pH 8.0 containing 30 mM solution of the substrates and 100 µl of the enzyme preparation was added in a final volume of 1.0 ml.

**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 routinely at 30 mins interval.

**Effect of pH on Thiaminase Activity**

The effect of pH on thiaminase was studied by assaying the enzyme at 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 8.0-9.0). A reaction mixture of 1 ml contained 0.4ml of buffer, 0.2 ml of thiamine, 0.2 ml of aniline, 0.1ml of enzyme solution.

**Effect of Mono and Divalent Metals on Thiaminase Activity**

The effect of cations on thiaminase activity was studied using the following cations $Zn^{2+}$, $Ni^{2+}$, $NH_4^+$, $Hg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Na^+$. The enzyme was assayed in a typical enzyme assay. A reaction mixture of 1 ml contained 0.1 mM and 0.2 mM metal concentration.

**Effect of Amino Acids on Thiaminase Activity**

The effect of amino acids on thiaminase activity was studied using the following amino acids; proline, cysteine, 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 of enzyme solution.

**Effect of Some Inhibitors on Thiaminase Activity**

The effects of 2-mercaptoethanol and 6-amino hexanoic acid on the activity of *Z. variegatus* thiaminase were investigated. 0.5 mM each of the compounds was used in the typical assay reactions.

**RESULTS**

**Purification of Protein**

The purification of *Z. variegatus* was carried out as described in the materials and methods. The elution profile of the DEAE-Cellulose ion exchange chromatography are shown in Figure 1.

**Molecular Weight**

The calibration curve on Biogel P-100 for native molecular weight determination is shown in Figure 2. Gel filtration on Biogel P-100 resulted in a molecular weight estimate of approximately 102,000 daltons.

**Determination of Kinetic Parameters**

The substrate specificity and kinetic of thiaminase from gut *Z. variegatus* was investigated by testing its activity towards structurally similar compounds. The compounds include aniline, thiamine and thiamine dihydrochloride. The kinetic parameters ($V_{max}$ and $K_m$) of thiaminase from the gut of *Z. variegatus* were determined as described in the text. The kinetic parameter values are shown in Table 1. Figures 3 and 4 shows the Lineweaver - Burk plot of thiamine and aniline respectively.

**Table 1: Kinetic Properties of *Z. variegatus* Thiaminase**

| | Thiamine | Aniline |
|---|---|---|
| $K_m$ (µM) | 5.00 | 25.0 |
| $V_{max}$(µmole/ml/min) | 5.00 | 1.11 |

**Effect of Temperature and pH on Thiaminase Activity**

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 $^oC$ at pH 8.0 (Figure 5). The enzyme activity was markedly enhanced by the amino acids. While the optimum pH of the enzyme was found to be around pH 8.0 (Figure 6).

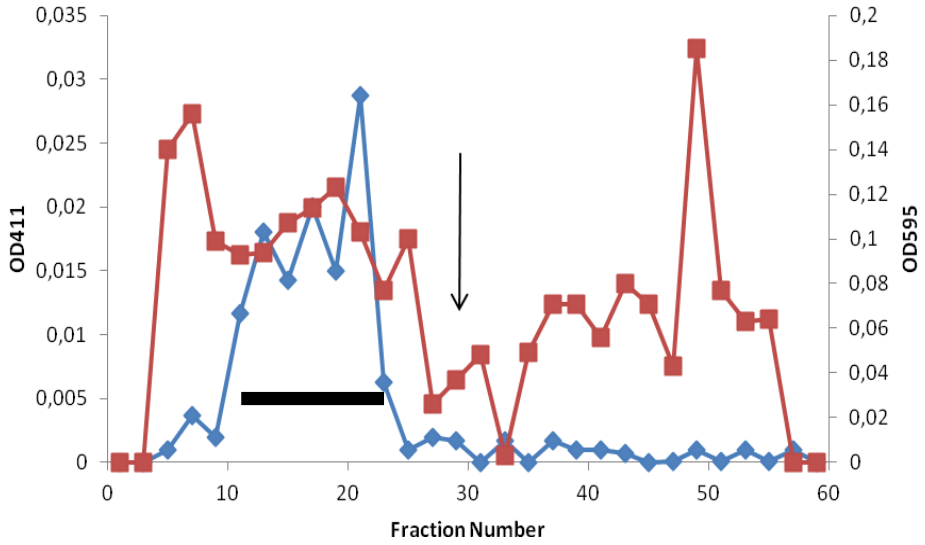


**Figure 1:** DEAE cellulose Ion exchange chromatography of *Z. variegatus* thiaminases. The column was first washed with 100 ml 0.1 M Tris-HCl buffer, pH 7.2. The enzyme solution was layered on the packed column and eluted with a 200 ml linear gradient of 0-1 M NaCl in 0.1 M Tris-HCl buffer, pH 7.2. Fractions of 1 ml were collected from the column. -■-■-■- Activity profile; -■-■-■- Protein profile; ⟶ salt concentration gradient; ▬▬ pooled enzyme fraction.

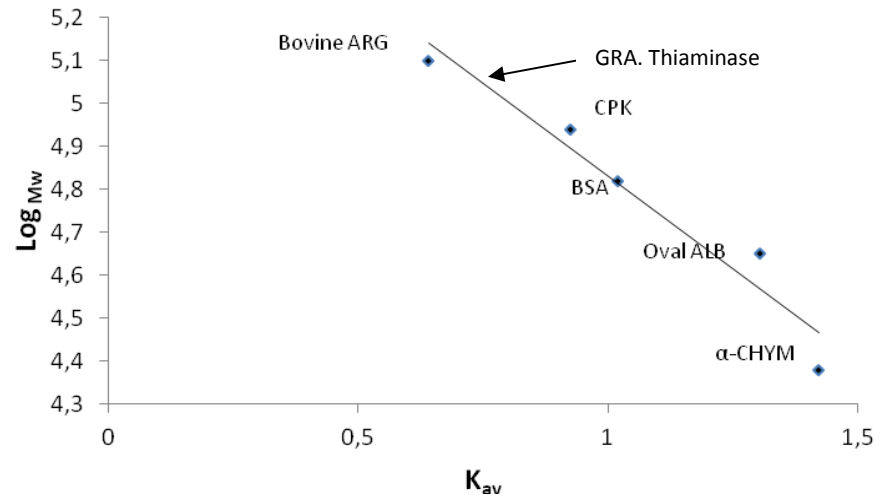


**Figure 2:** Calibration curve on Biogel P-100 for native molecular weight determination of grasshopper (*Z. variegatus* Thiaminase). The native molecular weight was determined on a Biogel P-100 column (2.5 × 90 cm). The standard proteins as indicated on the curve are α-CHYM (α-chymotrypsinogen A), Oval ALB (ovalbumin), BSA (bovine serum albumin), CPK (creatinine phosphokinase) and Bovine ARG (bovine arginase). Grasshopper thiaminase (GRA. Thiaminase) is indicated by the arrow.

**Effects of Mono and Divalent ions on Thiaminase Activity**

The effects of the various cations show that $Mn^{+2}$, $Ni^{+2}$ and $Hg^{+2}$ enhanced the activity of the enzyme while the enzyme was completely inhibited by $Na^{+}$, $NH_4^{+}$, $Zn^{+2}$ and $Hg^{+2}$ (Table 2).

**Effects of Amino Acid and pH**

The results of the data obtained with the effect of various amino acids on the activity of thiaminase are presented in Table 3.

**Effect of Some Inhibitors on Thiaminase Activity**

The effects of 2-mercaptoethanol is shown in Figure 7. The *Z. variegatus* thiaminase was completely inhibited by 6-amino hexanoic acid.

**Table 2: Effect of Mono and Divalent Ions on *Z. variegatus* Thiaminase Activity**

| Metal ion | % of Residual Activity | |
|---|---|---|
| | 100 µM | 500 µM |
| $Zn^{2+}$ | 2.10% | 4.96% |
| $NH_4^{+}$ | 24.49% | 10.05% |
| $Na^{+}$ | 223% | 6.55% |
| $Co^{2+}$ | 34.24% | 14.55% |
| $Ni^{2+}$ | 100% | 80.65% |
| $Hg^{2+}$ | 87.68% | 14.57% |
| $Mn^{2+}$ | 100% | 100% |

Enzyme assay was carried out using the standard assay mixture containing 0.05 ml of each salt at the final concentrations of 100 µM and 500 µM. The values are mean of duplicate determinations.

**Table 3: Effect of Amino Acids on *Z. variegatus* Thiaminase Activity**

| Amino acid (100 mM) | % Residual Activity |
|---|---|
| Proline | 100% |
| Valine | 76.61% |
| Lysine | 60.31% |
| Cysteine | 100% |

The reaction mixture contained 0.1 M Tris-HCl buffer pH 8.0, 30 mM of the required amino acid and 10 µl of the enzyme.

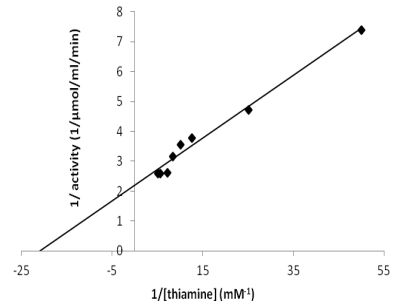


**Figure 3:** The Lineweaver-Burk plot showing the effect of varying concentrations of thiamine at fixed concentration of aniline on the initial reaction velocity at pH 8.0. The reaction mixture is as described in the text.

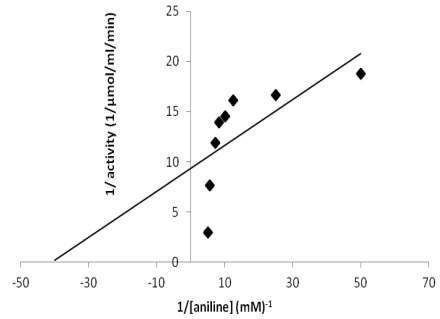


**Figure 4:** The Lineweaver-Burk plot showing the effect of varying concentrations of aniline at fixed concentration of thiamine on the initial reaction velocity at pH 8.0. The reaction mixture is as described in the text.

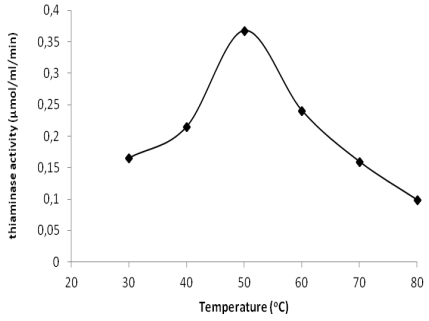


**Figure 5:** The activity-temperature profile showing the optimum temperature. The activity of *Z. variegatus* thiaminase was assayed at temperatures between 30°C and 100°C. The assay mixture containing 50 µl was first incubated at the indicated temperature for 10 min before initiating the reaction by the addition of an aliquot of the enzyme.

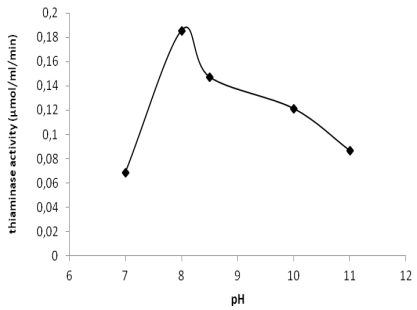


**Figure 6:** Effect of pH on Thiaminase Activity of *Z. variegatus.* One ml of the reaction mixture contained 0.4 ml of the appropriate buffer, 0.2 ml thiamine, 0.2 ml aniline and 0.01 ml enzyme preparation. The following buffers of different pH values were used for the pH determination: 5 mM citrate buffer (pH 6.0-6.5), 5 mM phosphate (pH 6.5-8.0), 5 mM Tris-HCl buffer (pH 8.0-11.0).

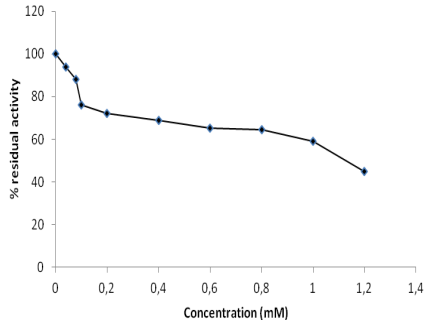


**Figure 7:** Effect of 2-mercaptoethanol on the activity of thiaminase from gut of *Z. variegatus*

## DISCUSSION

Edible insects constitute an important part of the daily diet of a large proportion of the population in Southwestern Nigeria. These insects provide high quality proteins and supplements (minerals and vitamins) even when dried [9]. The consumption of *Z. variegatus* is becoming popular in Africa [9]. Thiaminase, an antinutritional enzyme was isolated from the gut of *Zonocerus variegatus* with DEAE-Cellulose ion exchange chromatography and Biogel P-100 gel filtration chromatography. The enzyme had a specific activity of 7.2 micromole/mg of protein. A specific activity of 2.07 micromole/mg of protein was obtained for Nadoo fern thiaminase [1]. [8] obtained a relatively heat-resistant thiaminase from the pupae of an African silkworm *Anaphe* spp.

Molecular weight of the enzyme was 102,000 dalton from gel filtration chromatography on Biogel P-100. This value is close to the molecular weight values of 93,000 - 115,000 dalton for fern nardoo fronds *(Marsilea drummondii)* and shellfish [1, 18, 19, 20]. A very high molecular weight value of 200,000 dalton was reported for *Anaphe species* [8].

The kinetic parameters obtained are similar to the $K_m$ reported for the enzyme in other sources. The $K_m$ presented in Table 1 shows that the enzyme has affinity for thiamine as substrate as compared to the other substrates. [21] reported an apparent Michaelis constant ($K_m$) of 176 mM and 3·19 mM for thiamine and aniline respectively. *Zonocerus variegatus* thiaminase is specific for catalyzing thiamine and aniline as the co-substrates. The use of numerous nucleophiles such as aniline, pyridine, and 2-mercaptoethanol by thiaminase enzyme as cosubstrates was reported [22]. [23] used 4-nitrothiophenolate as an alternative substrate for thiaminase I.

Temperature is the most important determinant of metabolic rate in ectothermic animals [24-26] and controls nearly all physiological and biochemical processes [27].

Optimum temperature of 50 $^{o}$C was obtained for *Z. variegatus* thiaminase enzyme. The optimum temperature is in the range of optimum temperature reported for the enzyme from other sources. [8] obtained an optimum temperature of 70$^{o}$C with extract of *Anaphe* pupae thiaminase, a relatively heat-resistant and stable enzyme. Similarly, the optimum pH of 8.0 of *Z. variegatus* thiaminase compares very well with pH values reported for thiaminases from other organisms [1, 8]. Increases in temperature generally result in increases in physiological processes including metabolic rate. In arthropods, the metabolic rate varies with gender, climate, body weight and parasitism [28-31].

Effect of metal on the enzyme showed the inhibition of the enzyme by $Co^{2+}$, $Na^{+}$, $NH_4^{+}$ and $Zn^{2+}$ while $Mn^{2+}$, $Ni^{2+}$ and $Hg^{2+}$ activated the enzyme. [32] had earlier reported the activating effect of $Mn^{2+}$ and $K^{+}$ on *Macrotermes* fungus *termitomyces* and its sensitivity to $Zn^{2+}$, $Fe^{2+}$, and $Cu^{2+}$. Some amino acids (lysine and valine) were found to slightly inhibit the enzyme while proline and cysteine activated the enzyme. Mercaptoethanol also inhibited the enzyme considerably. [22] had earlier reported the use of 2-mercaptoethanol as a nucleophile substrate in thiamine catalysis.

## CONCLUSION

The present study has shown the thiaminase from *Z. variegatus* to be thermostable and the physicochemical properties are similar to results obtained from other sources. Though the biological importance of thiaminases in living organism is still unknown, they are involved in impairment of energy producing reactions. In Southwestern Nigeria, edible insects are conceived as food and source of nutrient and the practice of eating *Z. variegatus* is on the increase [9]. This practice should be followed with extensive and prolong cooking of *Z. variegatus* to avoid suffering from thiamine deficiency.

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