

Full Length Research Paper

Biochemical properties of thiaminase from *Anaphe venata* Burtler.

Okonji, Raphael Emuebie^{1*}, Bamitale, Kayode Samuel² and Balogun, Rodiat Olusola³

¹Department of Biochemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

²Department of Medical Pharmacology and Therapeutics, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

³Department of Hematology and Immunology, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

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***Anaphe venata* contain a high level activity of thiaminase enzyme. The enzyme was partially purified through Biogel P-200 gel filtration chromatography. The proximate analysis of *A. venata* showed a high protein content of 28.00%, crude fibre content of 6.37 and crude fat content of 14.26. The native molecular weight of the enzyme was found to be 85,000 daltons. The overall results indicates that thiaminase from *A. venata* prefers thiamine as substrate with a K_m value of 0.35 mM as compared to 24.51 mM of aniline. A pH optima of 6.0 was obtained for the enzyme. The temperature optimum was found to be 60°C with activation energy values of 5.01 and 12.5 Kcal.**

Key words: Thiamine, thiaminase, *Anaphe venata*, kinetics properties.

INTRODUCTION

Thiaminases were first recognized in certain species of fishes (Fujita, 1954). Thiaminase is an enzyme that metabolizes or breaks down thiamine into two molecular parts. The enzyme has been classified into thiaminase type I (EC. 2.5.1.2) which catalyses the decomposition of thiamine by a base exchange reaction involving a nucleophilic displacement on the methylene group of the pyrimidine moiety (Bos and Kozik, 2000). The second thiaminase called thiaminase type II (EC. 3.5.99.2) is involved in the hydrolytic fission of thiamine (Murata, 1982). Thiaminase I occurs in a wide range of fish, shellfish, ferns and bacteria (McCleary and Chicks, 1977; Murata, 1982), while the type II has so far been obtained only from culture fluids of various bacteria and yeast like fungi (Murata, 1982).

Thiaminase physiological function in plant, fish, bacterial cell or insect is not known. It was first described as the cause of highly mortal ataxic neuropathy in fur producing foxes eating raw entrails of river fish like carp.

It is also known as the etiology of cerebrocortical necrosis of cattle and polioencephalomalasia of sheep eating thiaminase containing plants (Evans, 1975; Ramos et al., 2003). It has caused economical losses in raising fisheries, example in yellowtail fed raw anchovy as a sole feed for a certain period, and also in sea bream and rainbow trout. The same problem is being seen in a natural food chain system (Fisher et al., 1998). *Anaphe venata* larvae (African Silkworm) are found commonly in Western part of Nigeria (Adamolekun et al., 1994; Iwalewa et al., 2005). The larvae of the wild silk worm are being consumed as supplemental protein nutrition, and the heat resistant thiaminase in it has been reported to cause an acute seasonal cerebellar ataxia (Adamolekun et al., 1994; Nishimune et al., 2000).

Thiaminase I enzymes present in the culture solutions of *Bacillus thiaminolyticus* (Wittliff and Airth, 1968) and *Clostridium sporogenes* (Princewill, 1980) have been highly purified and detailed kinetic studies performed. McCleary and Chicks (1977) showed that fronds of the fern nardoo (*Marsilea drummondii*) contain a thiaminase I enzyme with molecular mass values in the range of 93,000 to 115,000 dalton. Bos and Kozik (2000) reported a homogeneous preparation of thiaminase I from carp

*Corresponding author. E-mail: okonjire@yahoo.co.uk or reokonji@oauife.edu.ng. Tel: +2348060164991.

liver with molecular mass of 55,000 dalton. The thiaminase of *Anaphe infracta* pupae was reported by Nishimune et al. (2000) with a molecular mass of 200,000 dalton.

The present work describes the partial purification and some biochemical properties of thiaminase from *A. venata* larvae. The nutrient composition was also determined.

MATERIALS AND METHODS

Trizma base, Trizma-HCl, ethylenediamine tetraacetic acid (EDTA) and thiamine were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Other reagents used were of analytical grades.

Collection of samples

The larvae of *A. venata* are eaten in several parts of western Nigeria. They were obtained as commercial sample from a local market in Ile Ife, Nigeria and authenticated by comparison with known samples with similar vernacular names by Professor W. A. Muse of the Department of Zoology, Obafemi Awolowo University, Ile Ife, Nigeria.

Extraction of thiaminase

Three grams of *A. venata* larvae was used in the extraction using 20 ml of 0.2 mol/L sodium phosphate buffer, pH 6.5, by stirring overnight at 37°C. The ground mixture was centrifuged at 4°C and 4000 g for 10 min, and the supernatant was used as the crude extract. pH measurement was made at 25°C using Mettler MP 200 Digital pH meter.

Thiaminase and protein assays

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 taken 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 determined by the method of Bradford (1976). Absorbance was read at 595 nm. Protein concentration was extrapolated from standard curve using bovine serum albumin (BSA) as standard.

Proximate analysis

Proximate analysis was carried out using the procedure of the Association of Official Analytical Chemistry (A.O.A.C. 2006) in the Department of Animal Science, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

Gel filtration on Biogel P-200

Biogel P-200 gel filtration resin was washed with several changes of 5 mM Tris-HCl buffer, pH 7.5. This was then packed into column (2.5 × 90) and equilibrated with 5 mM Tris-HCl buffer, pH 7.5. The enzyme fraction from the preceding step was then applied to the

Biogel P-200 column. Fractions of 4 ml were collected at a flow rate of 10 ml per hour. Protein was monitored spectrophotometrically at 280 nm. The fractions were also assayed for thiaminase activity. The active fractions were pooled and dialysed against 50% glycerol in 5 mM Tris-HCl buffer, pH 7.5.

Native molecular weight determination

The native molecular weight of the partially purified thiaminase enzyme was determined by Sephadex G-150 gel filtration column chromatography. The standard proteins were ovalbumin (M_r 45,000; 3 mg/ml), bovine serum albumin (M_r 67,000; 5 mg/ml), creatinine phosphokinase (M_r 88,000; 5 mg/ml), γ -globulin (M_r 150,000; 5 mg/ml) and pyruvate kinase (M_r 230,000; 5 mg/ml).

Kinetic study

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 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).

Optimum temperature

The enzyme was assayed at different temperatures between 0 and 70°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 min interval. The heat stability of the enzyme was also determined by incubating 1 ml of the enzyme for 1 h at 30, 40, 50 and 60°C, respectively. 0.1 ml was withdrawn at 30 min interval and assayed for residual activity.

Optimum pH

The effect of pH on thiaminase was studied by assaying the enzyme at different pH values: 5 mM citrate buffer (pH 5.0 to 6.5), 5 mM phosphate (pH 6.5 to 8.0), 5 mM Tris-HCl buffer (pH 8.0 to 9.0). A reaction mixture of 1 ml contained 0.4 ml of buffer, 0.2 ml of thiamine, 0.2 ml of aniline and 0.1 ml of enzyme solution.

RESULTS

Table 1 shows the proximate composition of *A. venata*. Moisture content and crude fibre were found to be very low. The result reveals high protein and fat contents. The thiaminase from *A. venata* was partially purified by Sephadex G-200 gel filtration chromatography. The elution profiles of the gel filtration chromatography are shown in Figure 1.

Molecular weight

The calibration curve on Sephadex G-150 for native molecular weight determination is shown in Figure 2 and

Table 1. Proximate analysis of *A. venata* (percentage dry weight).

Nutrient composition	Dry weight (%)
Moisture content	11.56
Ash content	5.44
Crude fibre	6.37
Crude fat	14.26
Crude protein	28.00

Dry weight of the *A. venata* was used for the analysis.

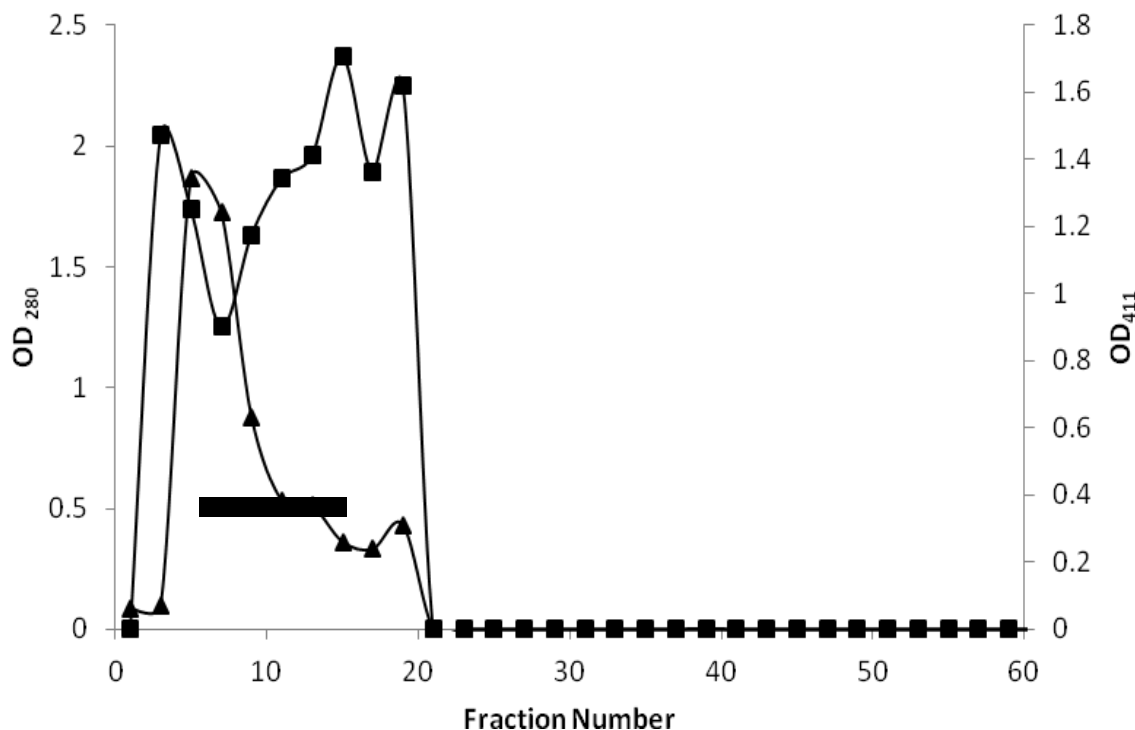


Figure 1. Biogel P-200 gel filtration chromatography (column 2.5 × 90) of *A. venata* thiaminase. The eluent was 5 mM Tris-HCl buffer, pH 7.5. Fractions of 4 ml were collected at a flow rate of 10 ml per hour. -▲-▲-▲- Protein profile; -■-■-■- activity profile; — pooled enzyme fraction.

a molecular weight of approximately 85,000 dalton was obtained for thiaminase.

Kinetic parameters

The Lineweaver-Burk plot of thiaminase using thiamine as substrate is shown in Figure 3 while Figure 4 shows that of aniline. The overall results indicate that thiaminase from *A. venata* shows more affinity towards thiamine as substrate with a K_m value of 0.35 mM as compared to 24.51 mM of aniline.

Effect of temperature and pH on enzyme activity

The optimum temperature of the enzyme was found to be

60°C at pH 6.5 (Figure 5). Arrhenius plot of log of activity against the reciprocal of temperature (1/Kelvin) is shown in Figure 5b. The plot is biphasic with a break at 60°C. The activation energy values obtained from the two phases are 5.01 and 12.5 Kcal, respectively. The optimum pH of *A. venata* was 6.0 (Figure 6).

DISCUSSION

In South-western Nigeria, edible insects are conceived as food and as source of nutrient (Banjo et al., 2006). Insects have played an important part in the history of human nutrition in Africa, Asia and Latin America (Banjo et al., 2006). The proximate analyses of the *A. venata* shown in Table 1 compares very well with other reported

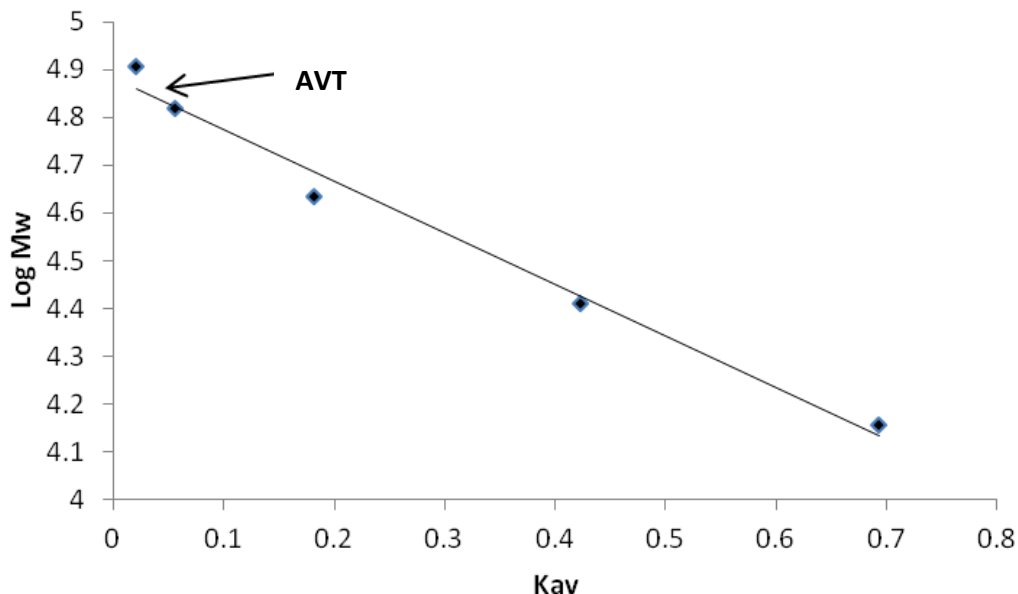


Figure 2. Native molecular weight determination of *A. venata* thiaminase. The native molecular weight was determined on a Biogel P-200 column (2.5 x 90 cm). The standard proteins as indicated on the curve are ovalbumin, bovine serum albumin (BSA), creatinine phosphokinase (CPK), γ -globulin and pyruvate kinase (PK). *A. venata* thiaminase (AVT) is indicated by the arrow.

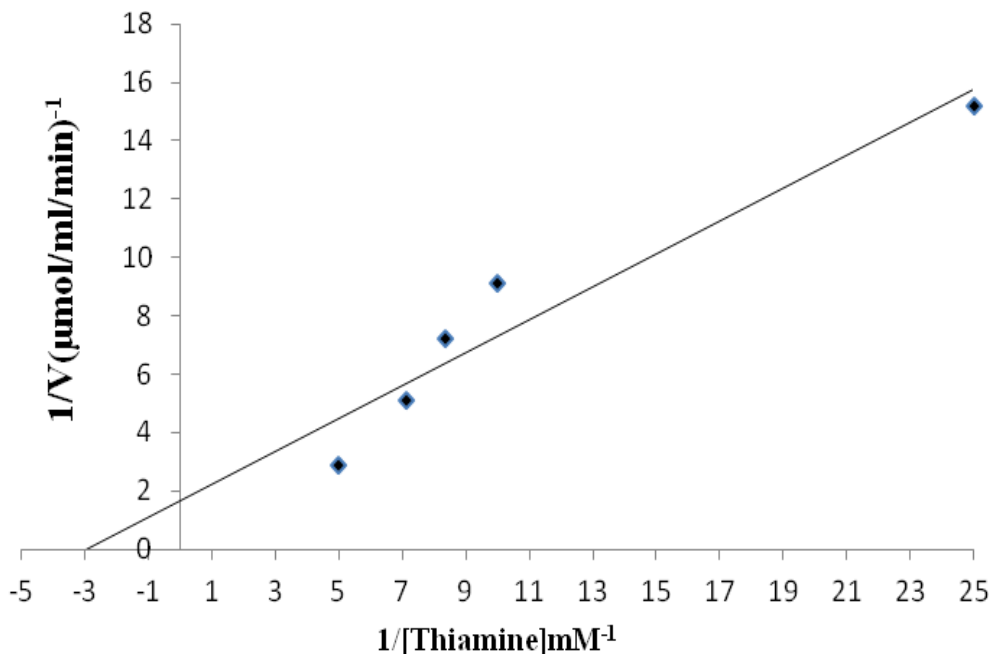


Figure 3. The Lineweaver-Burk plot showing the effect of varying concentrations of thiamine on the initial reaction velocity at pH 8.0. The reaction mixture of 1 ml contained 0.1 mM Tris-HCL buffer, pH 8.0, and varying concentrations of thiamine at fixed concentration of 50 mM aniline.

works. Banjo et al. (2006) reported the nutrient composition of four species of Lepidoptera (*A. infracta*, *Anaphe recticulata*, *Anaphe* spp. and *A. venata*). They obtained 20.0, 23.0, 18 and 25.7% for *A. infracta*, *A.*

recticulata, *Anaphe* spp. and *A. venata* crude protein content, respectively. A higher value of 44.12% was reported for *Macrotermes* spp. (Oyarzum et al., 1996).

Thiaminase activity have been reported in crude

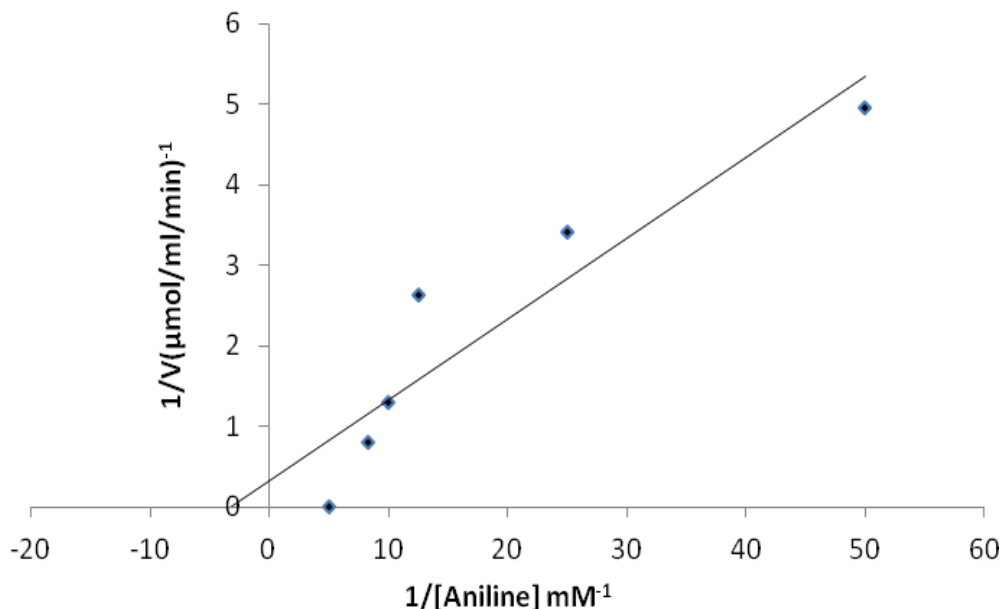


Figure 4. The Lineweaver-Burk plot showing the effect of varying concentrations of aniline on the initial reaction velocity at pH 8.0. The reaction mixture of 1 ml contained 0.1 mM Tris-HCL buffer, pH 8.0, and varying concentrations of aniline at fixed concentration of 20 mM thiamine.

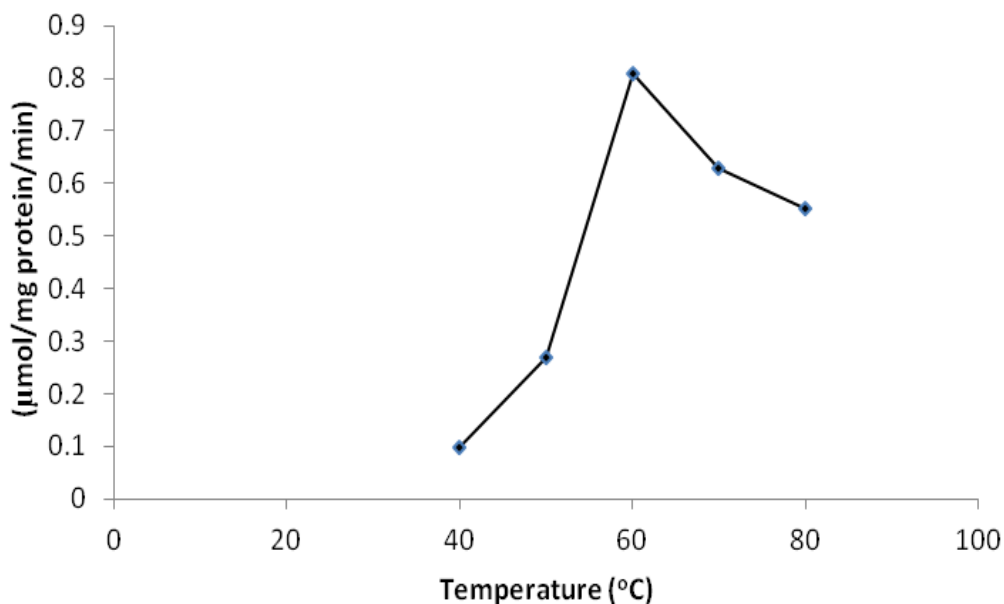


Figure 5a. Effect of temperature on the activity of *A. venata* thiaminase. The activity of *A. venata* thiaminase was assayed at temperatures between 30 and 100°C.

preparation of *A. infracta* and *Anaphe panda* collected in a suburb of Kampala, Uganda (Nishimune et al., 2000). In this work, extract from gel filtration on Biogel P-200 was used to determine the properties of thiaminase from *A. venata* larva. Bos and Kozik (2000) have also reported a homogeneous preparation of thiaminase from carp liver. A fairly high activity of a relatively heat-resistant

thiaminase was detected and characterized from the pupae of an African silkworm *Anaphe* spp. which had been the putative cause of a seasonal ataxia and impaired consciousness in Nigerians (Nishimune et al., 2000).

The results of the Michealis-Menten constant (K_m) for the two substrates (thiamine and aniline) as shown in

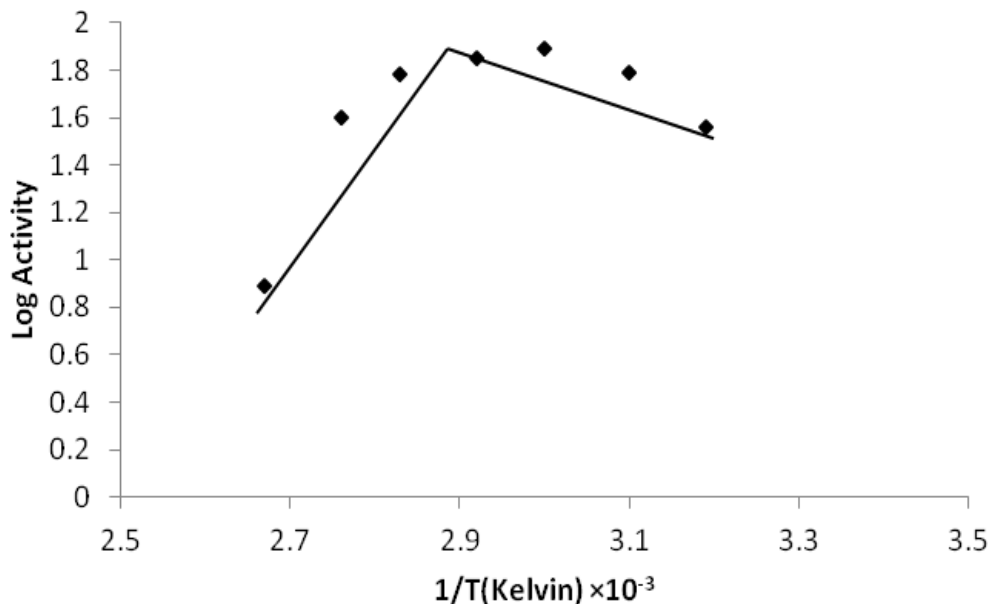


Figure 5b. Effect of temperature on the activity of thiaminase. Arrhenius plot (logarithm of activity against the reciprocal of the incubation temperature in Kelvin).

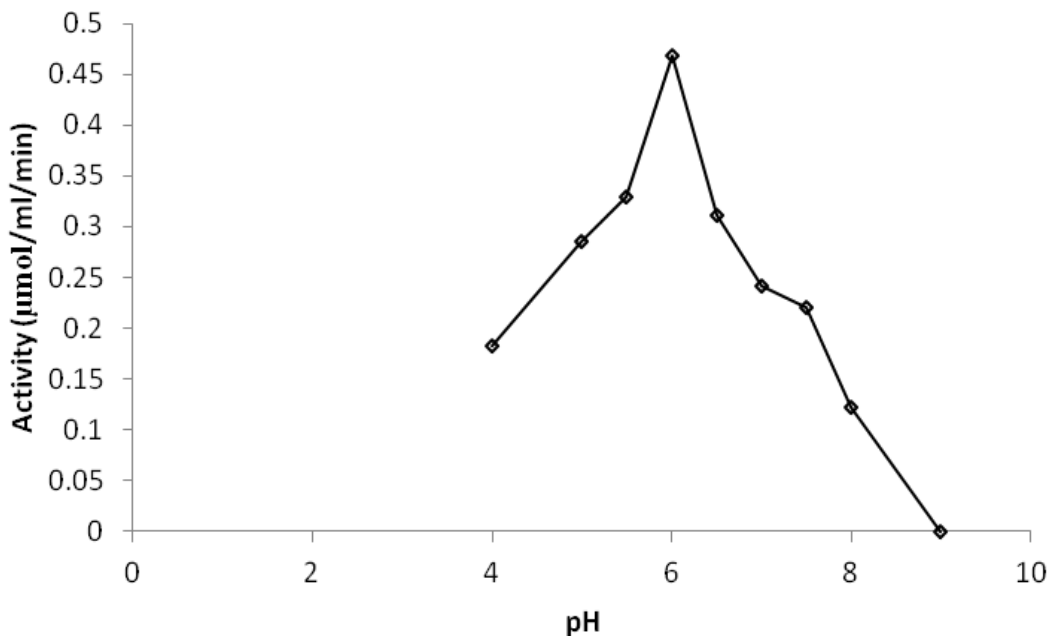


Figure 6. Effect of pH on the activity of *A. venata* thiaminase. One milliliter of the reaction mixture contained 2.5 mM of the appropriate buffer, 0.1 M arginine and 0.02 ml enzyme preparation. The following buffers of different pH values were used for the pH determination: 5 mM citrate buffer (pH 6.0-6.5), 5 mM phosphate (pH 6.5 to 8.0), 5 mM Tris-HCl buffer (pH 8.0 to 11.0).

Figures 3 and 4 indicated that *A. venata* thiaminase preferred thiamine as substrate. Kinetic studies of thiaminase I 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 (K_m) for thiamine was 176 μ M and the apparent K_m 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.

Though the reported molecular sizes of thiaminases ranges from 42,000 to 44,000 dalton (bacterial thiaminase I) (Wittliff and Airth, 1968; Agee et al., 1973; Abe et al., 1987) and 100,000 to 115,000 dalton (bacterial thiaminase II) (Ikehata, 1960) and thiaminase I of plant and shellfish (McCleary and Chick, 1977), the native molecular weight of *A. venata* was found to be 85,000. A similar result was obtained for the crude extract of *A. venata* with a molecular weight range of 23,000 to 80,000 dalton (Bamitale et al., 2011). An estimated molecular weight range of 93,000 to 115,000 dalton was reported for fern nardoo fronds (*Marsilea drummondii*) (McCleary and Chick, 1977). Nishimune et al. (2000) estimated the molecular size of the thiaminase from *A. panda* collected in Uganda to be 200,000 dalton using gel filtration chromatography. A molecular weight value of 55,000 dalton by gel filtration and by SDS-PAGE was reported for homogenous preparation of Carp liver (Bos and Kaz, 2000). Nishimune et al. (2008) recently reported a molecular weight of 106,000 dalton by gel filtration analysis in seawater fish *Fisturalia petimba*. The purified preparation gave an active 25,000 dalton subfragment by SDS-PAGE, together with a 15,000 dalton non-active subfragment.

The optimum temperature of 60°C for *A. venata* thiaminase compares well with values reported for other thiaminases. An optimum 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). A much lower optimum temperature of 37°C was reported for extracellular thiaminase I of *Bacillus thiaminolyticus* (Wittliff and Airth, 1968). Arrhenius plot of the effect of temperature on reaction rate constant was plotted and the apparent activation energy values from the two slopes were 5.01 and 12.5 Kcal. McCleary and Chick (1977) reported an activation energy value of 14.0 kcal for *M. drummondii* thiaminase. Much earlier, activation energy values of 9.8 and 2.7 Kcal were obtained for extracellular thiaminase I of *B. thiaminolyticus* (Wittliff and Airth, 1968). On the basis of its heat stability, the enzyme was found to be stable at 60°C. Similar results have shown the stability of the enzyme at high temperature. The temperature for 50% denaturation was found to be 60 to 65°C for *M. drummondii* thiaminase but was stable at 55°C (McCleary and Chick, 1977).

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 value of 6.0 obtained for *A. venata* is similar to the value reported for *Anaphe* pupae. 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. Also, the extracellular thiaminase I of *B. thiaminolyticus* showed optimum pH over a broad pH range of 5.8 to 6.8 (Wittliff and Airth, 1968).

In conclusion, this present work supports the observation of Nishimune et al. (2000), which reported the effect of consumption of *A. venata* in some part of South-western Nigeria. The biochemical properties of the enzyme compares very well with the reports of the enzyme from other sources. Also, the result of the proximate analysis, which clearly shows the high protein content of the insect infer that the nutritional status of the insect cannot be undermined, however, caution should be taken when the insect is used as a source of protein.

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