Thiamin Is Decomposed Due to *Anaphe* spp. Entomophagy in Seasonal Ataxia Patients in Nigeria[1]

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ABSTRACT 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. The thiaminase in the buffer extract of *Anaphe* pupae was type I (thiamin: base 2-methyl-4-aminopyrimidine methyl transferase EC 2.5.1.2), and the optimal temperature and pH were 70°C and 8.0–8.5, respectively. Based on gel filtration chromatography, the molecules were estimated to be 200 kDa. Second substrates which could be utilized by the thiaminase were pyridoxine, amino acids, glutathione, taurine and 4-aminopyridine. Thiamin phosphate esters were inactive as substrates. This is the first report describing an insect thiaminase. Our results indicate the necessity of thorough heat treatment for the detoxification of the African silkworm, making the worm a safe source of high-quality protein. J. Nutr. 130: 1625–1628, 2000.

KEY WORDS: • thiaminase • thiamin • entomophagy • ataxia • silk worm • Anaphe venata

An acute, seasonal ataxic syndrome has been observed annually for more than 40 y in a rain forest district of Nigeria (Adamolekun and Ibikunde 1994a, Wright and Morley 1958). The syndrome is deduced to be caused by an acute thiamin deficiency based on the fact that the administration of thiamin could overcome the clinical severity in a randomized, double-blind test (Adamolekun et al. 1994). The traditional practice of *Anaphe venata* entomophagy also has been implicated in the etiopathogenesis of the seasonal ataxia (Adamolekun 1993a, Adamolekun et al. 1994). However, the larvae of *Anaphe* are a protein source, with a nutritional value comparable to that of chicken eggs, and mass rearing as an alternative protein source has been advocated (Adamolekun 1993b, Ashiru 1988).

We investigated the possibility of detoxifying the *Anaphe* larvae, since Australian Aborigines detoxified a heat-stable thiaminase in a plant called *Marsilea drummondii*, which was harmful enough to kill three expeditionists (Earl and McCleary 1994).

MATERIALS AND METHODS

African silkmoth. Silk nests containing pupae of *Anaphe* spp. (*A. infracta* or *A. panda*) were collected in a suburb of Kampara, Uganda, through the National Sericulture Development Center. Nearly 40% of the dried cocoons contained pupae while the remainder held moths. Dry pupae (3 g) were extracted with 20 mL of 0.2 mol/L sodium potassium phosphate buffer, pH 6.5, by grinding in a mortar with 2 g of purified sea sand. The ground mixture was centrifuged at 4°C and 15,000 × *g* for 10 min, and the supernatant was used as the crude extract. The total amount of protein extracted was ~100 mg.

Thiaminase assay. The enzyme sample was incubated at 37°C in 0.1 mol/L TrisHCl buffer, pH 8.0, with 10^{-5} mol/L thiamin and 4×10^{-3} mol/L pyridoxine for 30 min. The remaining thiamin was oxidized with the addition of 1.5 mL BrCN and 1.0 mL of 200 g/L NaOH and assayed fluorometrically (Nishimune et al. 1988). Reagents used were obtained from Wako Pure Chemicals (Osaka, Japan), and thiamin analogs were purchased from Sigma Chemical Co. (St. Louis, MO).

Protein assay. The amount of protein was assayed using bicinchoninic acid–Cu^{+1} complex reagent (Pierce, Rockford, IL).

Estimation of molecular size. The crude extract of *Anaphe* pupae was concentrated ~10 times with a Millipore Ultrafree Biomax 10K (Millipore Co., Bedford, MA) and loaded on a Hiprep Sephacryl S300 (16/60) column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.05 mol/L sodium, potassium phosphate buffer, pH 7.2 at 20°C and eluted at a flow rate of 0.8 mL/min. The elution position of thiamin-decomposing activity was detected after fractionation of the eluate into 4-mL fractions. The size was estimated against a gel filtration standard (Bio-Rad Laboratories, Hercules, CA) which contained bovine thyroglobulin; 670 kDa, bovine γ-globulin; 158 kDa, chick ovalbumin; 44 kDa, equine myoglobin; 17 kDa and vitamin B-12; 1350 Da. Absorbance at 280 nm was monitored continuously.

RESULTS

Type of thiaminase and the second substrate. The thiamin-decomposing activity detected was shown to be time-dependent, co-substrate-requiring, temperature-dependent and heat-labile. The active co-substrates at a concentration of 4×10^{-3} mol/L were pyridoxine (**Fig. 1**) and compounds shown in **Table 1.** 4-Amino-pyridine enhanced the activity the most. The enzyme was practically inactive when thiamin phosphate esters were substrates (**Table 2**). Thiamin analogs competed with thiamin to various degrees (Table 2). The thiaminase activity was also nondialyzable through a cellophane dialysis bag that excluded molecular weights above ~10 kDa.

When the crude *Anaphe* extract was assayed without dialysis or dilution, weak thiamin-decomposing activity was present even when no co-substrate was added, indicating the presence of a certain amount of co-substrate, such as vitamin B-6 or nicotinic acid. After dialysis, thiaminase activity was no

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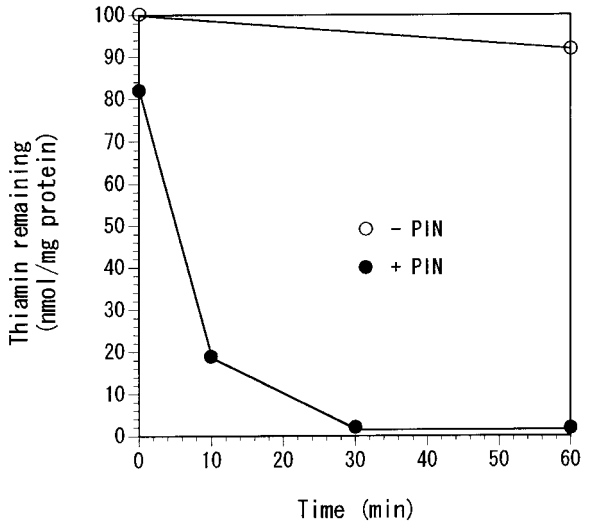

FIGURE 1 Pyridoxine-dependent decomposing reaction of insect thiaminase. Crude *Anaphe* extract (0.097 mg protein) was incubated both with and without 4×10^{-3} mol/L pyridoxine (PIN) for various periods of time at 37°C in 0.2 mol/L potassium, sodium phosphate buffer, pH 6.5 containing 10^{-5} mol/L thiamin, and the remaining thiamin was oxidized by BrCN and NaOH prior to fluorometry. A typical result among similar experiments is shown.

longer present (data not shown). We conclude from these results that the thiaminase is a type I or base-exchanging type of enzyme.

Optimal temperature and heat stability. Thiamin-decomposing activity was assayed at various temperatures for 15 min at pH 6.5 using a 1:20 dilution of crude extract, and the highest activity was detected at ~70°C (**Fig. 2**). Even at 100°C some activity remained. The crude enzyme was thus relatively heat-resistant. Heat treatment at 100°C for 15 min before the standard assay at 37°C inactivated the crude extract of *Anaphe* pupae nearly completely (data not shown).

The pH optimum. The highest thiaminase activity, after subtracting the nonenzymatic alkaline destruction of the substrate, was observed at pH 8.0–8.5 in TrisHCl buffer (data not shown).

Estimation of molecular size. When the concentrated crude extract was analyzed on a column of Sephacryl S300, thiaminase activity was recovered as a single peak in a position different from the major A_{280} nm elution position (**Fig. 3**), where it was expected that fragments of various cell structures and membrane-bound enzymes would elute. As a result, ~10 times higher activity per unit protein [90 nmol/(mg protein • min)] was obtained. The size of the thiaminase was estimated to be 200 kDa.

TABLE 1

Relative activity of aromatic amines and amino acids as co-substrates for insect thiaminase

Co-substrate	Activity (%)[1]	Co-substrate	Activity (%)[1]
Pyridoxine	100	L-Cysteine	94
Aniline	94	Glutathione	95
4-Aminopyridine	273	L-Histidine	39
o-Aminobenzoic acid	78	Hypotaurine	120
m-Aminobenzoic acid	76	Taurine	87
p-Aminobenzoic acid	43	L-Lysine	80
Histamine	80	L-Proline	80

[1] Activity of co-substrates (4×10^{-3} mol/L) was measured using the peak thiaminase fraction shown in Figure 3 by the procedures described in the Materials and Methods section. Activity is expressed as a percentage of thiamin destruction observed with pyridoxine as a co-substrate.

TABLE 2

Activity of insect thiaminase using thiamin phosphate esters as substrates and thiamin analogs as inhibitors

Substrate	Substrate activity (%)[1]	Inhibitor	Control thiaminase activity (%)[1]
Thiamin	100	Pyrithiamin(neo-)	59
Thiamin monophosphate	13.2	Oxythiamin	70
Thiamin diphosphate	6.4	Amprolium	95
Thiamin triphosphate	0		

[1] Assays were conducted as described for Table 1. Inhibitors were added at a concentration of 2×10^{-5} mol/L, and their inhibitory action is expressed as a percentage of the uninhibited activity.

DISCUSSION

Thiaminases have been reported in bacteria (Shida et al. 1994), plants, fish and shellfish (Evans 1975). However, this is the first report of the enzyme in insects, and the hypothesis presented by Adamolekun (1992) concerning the etiopathogenesis of the seasonal ataxia of Nigerians has been confirmed. The characteristics of *Anaphe* thiaminase include its high heat resistance, which could explain why the symptoms of seasonal ataxia occur within hours of consuming a carbohydrate meal with a stew containing roasted larvae of *A. venata*. The Nardoo thiaminase (obtained from *M. drummondii*) has a high optimal temperature of 65°C (McCleary and Chick 1977). *Anaphe* thiaminase in a crude extract preparation showed an

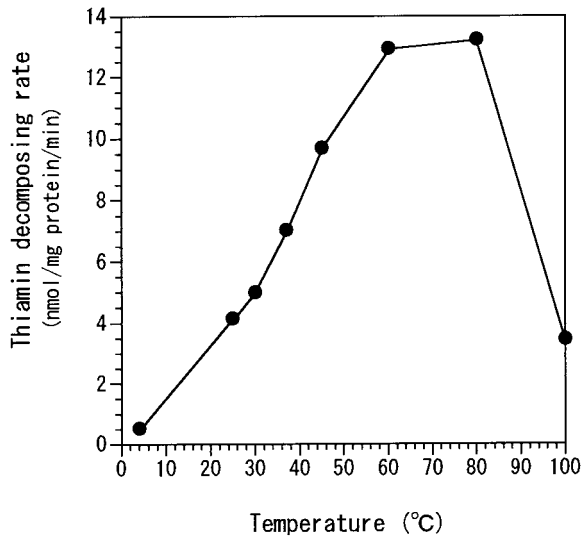

FIGURE 2 Temperature dependence of insect thiaminase. Crude *Anaphe* extract (0.049 mg protein) was incubated at various temperatures, and pH 6.5 with 4×10^{-3} mol/L pyridoxine and 10^{-5} mol/L thiamin for 15 min. The remaining thiamin was measured fluorometrically after oxidization with BrCN and NaOH. A typical result among similar experiments is shown.

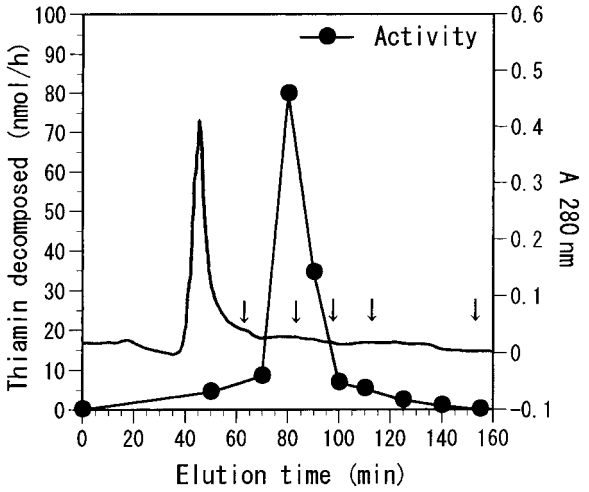

FIGURE 3 Molecular weight estimation of insect thiaminase by gel filtration chromatography. Concentrated crude extract of *Anaphe* pupae was developed through Sephacryl S300 (16/60) column, and the eluate was fractionated into 4 mL fractions after continuous monitoring of A_{280} nm. Thiaminase activity in each fraction was assayed against 10^{-5} mol/L thiamin adding 4×10^{-3} mol/L pyridoxine at pH 8.0 and 37°C for 30 min, and the remaining thiamin was measured fluorometrically after oxidizing with BrCN and NaOH. Elution positions for the standard proteins are shown by arrows.

even higher optimal temperature of 70°C. Although our measurement was accomplished using fairly diluted enzyme, possibly the optimum would shift to one slightly lower when the measurement is made in a purified sample. The heat stability of animal enzymes is, however, higher in many cases when they are located in organs or tissue matrices compared to when they are extracted into buffer solutions. Many bacterial food poisonings have arisen because the temperature of the inner part of foodstuffs is much lower throughout the cooking process than the temperature of the cooking oil or cooking pan. Thus, it is entirely possible that a roasted or stewed larvae of *A. venata* still retains its thiaminase activity.

As a rough estimate, we calculated the amount of thiamin decomposed by 100 g of the pupae in 1 h to be 0.9 g, using the optimal reaction rate in Figure 2 [14 nmol thiamin/(mg protein · min), 100 mg of the extracted protein from 3 g of the pupae]. At the human body temperature of 37°C, the reaction rate was nearly one-half of the optimum (Fig. 2). The assay method we used, on the other hand, could not give the maximal rate when the percentage of thiamin decomposed exceeded ~30%. Furthermore, the pH in the small intestine is more alkaline than pH 6.5, and nearer to the optimal pH of this enzyme. Although the amount of *Anaphe* larvae consumed by seasonal ataxia patients in Nigeria has not been reported, the above-estimated value suggests that the entomophagy is a possible reason for thiamin deficiency. On the other hand, thiaminase activities in fresh fish and fish products on the market have been reported (Hilker and Peter 1966). The amount of thiamin destroyed varied from 0 to 1.0 mg by 100 g of the tissue in 1 h. Raw carp entrails, with which a typical thiamin avitaminosis was produced in foxes, destroyed ~10.0 mg of thiamin/100 g wet entrails in 1 h. (Spitzer et al. 1941). Thiaminase activity in bracken ferns has been reviewed (Fujita 1954), and the amounts of thiamin destroyed are 12.0–13.4 mg/h by 100 g of the tissue. In the latter two cases, numerous victims of thiaminase have been reported in foxes and ruminants such as sheep and cattle (Evans 1975, Green et al. 1941). A similar level of thiaminase activity seems to be present in a *Nardoo* fern (M. *drummondii*) (McCleary and Chick 1977). We are interested in the fluctuations of thiaminase activity in the larvae of *Anaphe* during their growth stages. However, we have not yet measured this due to the difficulty of obtaining samples. As Adamolekun (1993b) has indicated, a monotonous diet of carbohydrates and the resulting marginal thiamin deficiency may play a role in the etiology of seasonal ataxia.

From the heat-resistant nature of *Anaphe* thiaminase, we expected a smaller molecular weight than was observed, and a similar molecular size to those reported for thiaminases of other origins. The reported molecular sizes of thiaminases are 42–44 kDa (bacterial thiaminase I) (Abe et al. 1987, Agee et al. 1973), and 100–115 kDa (bacterial thiaminase II (Ikehata 1960) and thiaminase I of plant and shellfish (McCleary and Chick 1977)). The size of *Anaphe* thiaminase was ca. double that of any previously studied enzyme. The importance of the larger molecular weight of *Anaphe* thiaminase remains to be determined.

In M. *drummondii*, which contains plant thiaminase I, detoxification was accomplished by Australian Aborigines through washing the *Nardoo* powder with a large amount of water. Baking the *Nardoo* powder without washing it did not reduce the thiaminase activity because of its stability to heat (Earl and McCleary 1994). A similar procedure seems to have been ineffective with *Anaphe* larvae, since this enzyme can utilize some amino acids as the second substrate, like the *Nardoo* thiaminase, and the larvae are expected to be a source of protein.

Although thiaminases were discovered many decades ago, the physiological importance of these enzymes has been an enigma. Since the enzyme reaction can decrease cellular free thiamin concentration, carbohydrate metabolism or energy production can be impaired. The enzyme could play a role in the metamorphosis of insects. We are interested in its gene expression in the various steps of silkworm metamorphosis, which has many apoptosis-like steps. A thiaminase I gene has been sequenced (Costello et al. 1996) using a bacterial gene that we have cloned (Abe et al. 1987). We have preliminarily measured thiaminase activity in Japanese silkworms (*Bombyx mori*) and found thiaminase I activity to be less than one-third that of *Anaphe* spp. The practice of eating silk worm pupae is declining in Japan, and the eating of larvae was last documented in 1919. In the event of a severe food shortage in the future, we have to be careful to heat-cook the pupae to avoid suffering from thiamin deficiency.

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