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THE PURIFICATION AND PROPERTIES OF A THIAMINASE I ENZYME FROM NARDOO (*MARSILEA DRUMMONDII*)

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Key Word Index—Pteridophyta; nardoo (*Marsilea drummondii*); bracken fern; rock fern; freshwater mussel; thiaminase I; co-substrates.

Abstract—Fronds of the fern nardoo (*Marsilea drummondii*) contain a thiaminase I enzyme at very high levels of activity. Highest levels of enzyme activity were found in vigorously growing plant material. The thiaminase I has been purified to a final sp act value of 2.07 μkat/mg protein at 30° and pH 6.5. It was shown to have similar properties to thiaminase I enzymes purified from bracken fern, rock fern and freshwater mussels. These enzymes have MW values in the range 93 000–115 000, energies of activation of 14 000 cal mol, pH optima of 8–9 and are quite stable in the pH range 3 to 12 and to extended incubation at 55°. The temperature for 50% denaturation is 60–65°. *p*-CMB, mersalyl acid and $HgCl_2$ (10^{-6} M) are potent inhibitors, but monoiodacetic acid (10^{-4} M) has no effect. A wide range of heterocyclic bases, sulphydryl compounds, and amines, including the non-aromatic amines 6-aminohexanoic acid and ethanolamine, act as co-substrates in the thiaminase I reaction; however, their effectiveness is dependent on both their degrees of basicity and to some extent, their stereochemistry. When the co-substrate activity of a range of substituted anilines were compared, no correlation was found between the degree to which the base activates the reaction and the pK_b (or Hammett's sigma constant) of the base.

INTRODUCTION

Two mechanisms for the enzymatic fission of thiamine have been reported. The first involves the enzyme thiaminase 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 [1]. The second mechanism is a hydrolytic fission of thiamine involving the enzyme thiaminase II (EC. 3.5.99.2). Thiaminase I occurs in a wide range of fish, shellfish, ferns and bacteria [2] whereas thiaminase II has so far been obtained only from culture fluids of various bacteria and yeastlike fungi [1].

Thiaminase I enzymes present in the culture solutions of *Bacillus thiaminolyticus* [3–5] and *Clostridium sporogenes* [6–8] have been highly purified and detailed kinetic studies performed. But, despite the importance of thiaminase I enzyme in stock poisoning by bracken fern [9], there is limited information available on the properties of this or other fern thiaminases. The thiaminase I enzyme of bracken has been partially purified by Evans and Jones [10] and more recently by Kenten [11] who obtained a preparation with final sp act of 3150 μmol/hr/mg N (0.14 μkat/mg protein).

The ability of a wide range of aromatic amines, heterocyclic bases and sulphydryl compounds to participate in the hydrolysis of thiamine by thiaminase I has been recognised for many years. Fujita *et al.* [2, 12] showed that some bases were better co-substrates than others. They also found that the rates with a particular base and thiaminase I enzymes from different sources, varied. Recently, Roberts and Boyd [13] extended the number of possible co-substrate bases to include a range of commonly used anthelmintics, tranquilizers and antihistamines. In a study employing a partially purified thiaminase I enzyme from carp viscera, Mazrimas *et al.* [14] found a correlation between the Hammett's sigma constant [15] of *m*- and *p*- substituted anilines and the degree to which they modify the rate of reaction of the thiaminase I enzyme. The inhibition of bacterial thiaminase I enzymes by *p*-chloromercuribenzoate, monoiodoacetic acid, various metal ions and by primary substrates such as thiamine and heteropyrithiamine [16, 17], has been reported.

Recently, Evans *et al.* [9] induced a thiamine deficiency in mature ruminating sheep by feeding dried and milled bracken rhizomes which contained high thiaminase I activity. A similar condition is at present under investigation in north-western New South Wales, Australia, where the plant responsible is the fern nardoo (*Marsilea drummondii*). Nardoo is found in most of the river watercourse areas of Eastern Australia with particularly dense growths near the Gwyder River, N.S.W. We have found that this fern contains extremely high levels of thiaminase I activity under certain seasonal conditions.

The aim of the present investigation was to study some of the properties of this enzyme from nardoo and to compare these to properties of similar enzymes obtained from bracken fern, rock fern and freshwater mussel. The thiaminase assay technique of Edwin and Jackman [18] employing radioactive thiamine has been slightly modified and its usefulness in studying detailed kinetic parameters of the enzymes demonstrated. The properties of co-substrate bases which affect their ability to participate in the thiaminase I reaction will be discussed.

RESULTS AND DISCUSSION

Assay procedure

Improved techniques have recently been developed for the assay of thiaminase I enzymes [4, 18]. The system we used in the present studies is a slight modification of the technique of Edwin and Jackman [18] which employs [thiazole-2-^{14}C] thiamine HCl. Whereas, in their assay mixture the concentration of both thiamine (2.2–2.9 μM) and the co-substrate base, nicotinic acid (1.5 μM), were less than saturating, in the modified assay mixtures for the measurement of 'Potential' thiaminase I activity, the concentration of both thiamine and the co-substrate base (pyridine, 50 mM) were at least 5 times their corresponding K_m values. Pyridine was replaced for nicotonic acid because, in general thiaminase I enzymes have greater affinity for this co-substrate (Ref. Fig. 1 and Table 3). By replacing nicotinic acid (1.5 μM) with pyridine (50 mM) the rate of thiamine hydrolysis and thus the potential sensitivity of the assay were increased × *ca.* 10^4. 'Actual' activity was assayed using mixtures which contained no added co-substrates. Since the thiaminase I enzyme has no activity in the absence of a co-substrate base, the amounts of these compounds in the plant extracts is a major factor controlling the level of this activity. The substrate mixtures in 0.1 M phosphate (pH 6.5), when stored in Quickfit glass containers at −40° between use, showed no detectable thiamine degradation over a 6 month period as judged by the lack of increase of [2-^{14}C] thiazole extractable with ethyl acetate.

Nardoo thiaminase I

Fronds of nardoo contain extremely high levels of thiaminase I enzyme. Activity appears to be related to lushness of growth of the plant. Changes in nardoo thiaminase I activity over a 12 months period (1975–1976) are shown in Fig. 2. High activities, relative to those in bracken fern, were found all year round. However, highest values appear to be associated with the initial surge of growth following heavy rainfall and flooding (Jan.–April). In June, 1976, even though the fronds still appeared quite lush and there was still an ample supply of water,

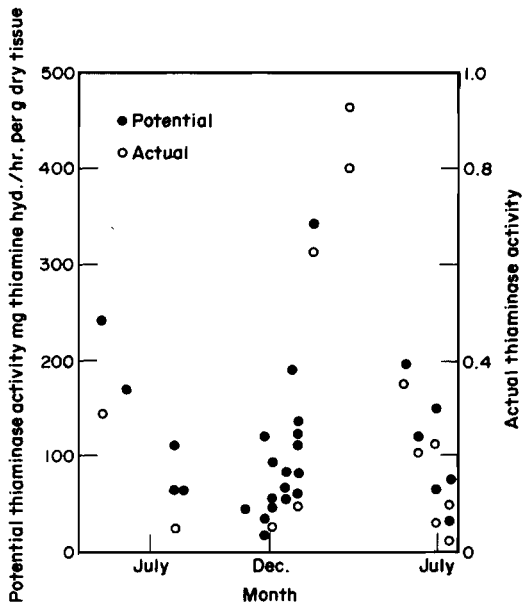

Fig. 2. Seasonal changes in the thiaminase I enzyme activity in nardoo fronds.

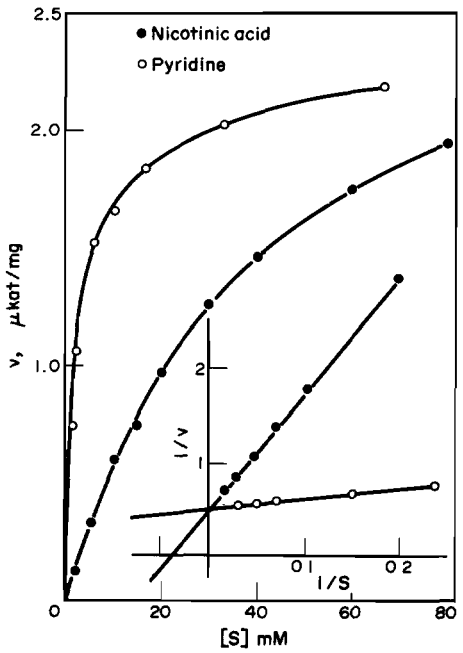

Fig. 1. Kinetic plots for nardoo thiaminase I with the co-substrates pyridine and nicotinic acid.

Table 1. Thiaminase I activity in ferns and mussel

Source of enzyme	Thiaminase activity (mg thiamine hydrolysed per hr per g dry wt
Marsilea drummondii (nardoo)	
Lush fronds	460
Old fronds	50
Roots (lush growth)	100
Sporocarps (mature nuts)	10
Marsilea augustifolia	
Lush fronds	2
Marsilea mutica	
Lush fronds	43
Pteridium esculentum (Bracken fern)	
Young fronds	4
Old fronds	3
Tubers	3
Cheilanthes sieberi (Rock fern)	
Lush fronds	110
Velesunio ambiguus (Freshwater mussel)	
Whole	90

activity values had decreased by about 5 fold. Hot dry weather (August to December, 1975) also resulted in a rapid decrease in activity. The difference between potential and actual activities in nardoo extracts was × *ca.* 500.

Thiaminase I activity in a few fern species and in freeze-dried, freshwater mussel (*Velesunio ambiguus*) is shown in Table 1. Lush nardoo fronds contain much higher levels of activity than any of the other species investigated—5 times that of rock fern and mussel and 100 times that of bracken. Activity in lush bracken fronds did not exceed a value of 4 mg thiamine hydrolysed/hr/g dry wt. Similar values were reported by Kenten [11] and by Evans *et al.* [9]. However, Evans *et al.* used the substrate mixture developed by Edwin and Jackman [18] which contains sub-optimal levels of amine.

In the present study, thiaminases from nardoo, rock fern. bracken fern and fresh-water mussel were extracted and purified and some of their properties compared. Details of the purification of nardoo thiaminase are shown in Table 2. Crude extracts of nardoo were extremely viscous and the protein could not be precipitated even with 100% $(NH_4)_2SO_4$. The enzyme preparation from DEAE-cellulose chromatography was very unstable to dialysis. Dialysis for 18 hr gave *ca.* 100% loss of activity. Further studies showed that soaking pieces of dialysis bag in the enzyme preparation also gave complete loss of activity, suggesting a reaction between the dialysis bag and enzyme protein. More highly purified enzyme preparations were stable to dialysis. The final sp act of the enzyme was 2.07 μkat/mg protein compared to values of 1.08 [3] 0.10 [4] and 1.01 μkat [6]/mg for highly purified bacterial preparations. However, in contrast to the purified bacterial enzymes which were homogeneous by the criterion of gel disc electrophoresis, the nardoo preparation contained some minor protein bands as well as the major band of enzyme activity. Bracken fern, rock fern and mussel enzymes were partially purified to final sp acts of 0.30, 0.25 and 0.10 μkat/m protein, respectively.

Some of the properties of the four thiaminases are compared in Table 3. MW values obtained by gel filtration were very similar and in the range of 93–115 000. Other properties such as K_m for thiamine, pH optima, energy of activation, energy of activation for denaturation and degrees of inhibition by *p*-CMB and monoidoacetic acid were the same. Nardoo thiaminase has a pI of 3.5 which is consistent with its chromatographic properties on DEAE- and phospho-cellulose. This enzyme hydrolyses thiamine pyrophosphate at a rate similar to that for thiamine and the K_m values are the same.

The similarities in properties of the above four enzymes contrasts with the quite different properties reported for bacterial thiaminases [1, 7]. In comparison to nardoo thiaminase I, the bacterial enzymes have much lower MW values (40–44 000), lower optimal temperatures for activity (30–37°) and are much more sensitive to monoiodoacetic acid, being potently inhibited at concentrations of 10^{-4} M. In general, energies of activation and optimal pH values for activity are quite different.

A partially purified enzyme from bracken [11] with a sp. act. of *ca* 0.13 μkat/mg protein showed maximum activity at a pyridine concentration of 80 mM and a thiamine concentration in excess of 74 μM. The enzyme had an optimal pH for activity of 7.8 in a Pi–borate buffer system. In the same buffer the nardoo thiaminase also had an optimal pH of about 7.8, whereas a value of 8 to 9 was obtained in a Pi–Tris or Pi–pyrophosphate buffer. It would thus appear possible that at higher pH values the borate ion in some way inhibits the enzyme catalysed reaction.

In a study of the effect of pH on the K_m of nardoo thiaminase I for pyridine and nicotinic acid it was found that there was a decrease in K_m as the pH increased above 7. This correlates with the requirement of the enzyme for the base in the uncharged form, i.e. as pH increases the proportion of this form of the base increases. This effect of pH on K_m appears to be a factor when determining pH-activity curves, particularly if sub-optimal levels of the base are employed. For example, as shown in Fig. 3, at pH values less than the pK_b of the base, there is a difference in the pH activity patterns with nicotinic acid at saturating (200 mM) and at less than saturating (20 mM) concentrations. At present no explanation can be given for the differences in the pH activity patterns with different bases, each at saturating concentration.

Each of the four thiaminases presently studied were potently inhibited by concentrations of *p*-CMB, mersalyl acid and mercuric chloride at concentrations as low as 0.1 to 1 μM. The reactivation with various sulphydryl compounds of *p*-CMB inhibited nardoo thiaminase I is shown in Table 4. The stronger reducing and metal chelating compounds, mercaptoethanol and cysteine at 3.3 mM gave complete reactivation of the enzyme as well as about a 40% increase in activity above the control. The weaker reducing agents thiosulphate and thioglycollate gave a greater reactivation at 33 mM than at 3.3 mM. At 33 mM concentration, mercaptoethanol and cysteine inhibited the enzyme *ca.* 20–40%. This inhibition by high concentrations of cysteine and mercaptoethanol is a common occurrence with enzymes containing disulphide links or heavy metal prosthetic groups.

Table 2. Purification of a thiaminase I enzyme from nardoo

Stage of purification	Total protein (mg)	Total activity (μ kat)	Specific activity (μkat/mg)	Recovery per step (%)	Purification fold
Crude extract	9000	64.0	0.007	—	0.1
DEAE-Cellulose (pH 6.5)	555	48.5	0.087	75.5	12.2
Ultrogel AcA 34	118	35.5	0.300	73.3	42.1
Sephadex G100	32	24.2	0.757	68.3	106.0
Phosphocellulose (pH 3)	8	16.6	2.066	68.5	290.0

Table 3. Comparative properties of thiaminase I enzymes

Property	Source of thiaminase I			
	Nardoo	Rock fern	Bracken fern	Mussel
MW (gel filtration)	115 000	107 000	93 000	110 000
K_m for thiamine (μM)	3	3	3	3
p-CMB inhibition (10^{-6} M)	100	100	100	100
Monoiodoacetic acid inhibition (10^{-4} M)	0	0	0	0
pH Optima*	8·0–9·0	8·0–9·0	8·0–9·0	8·0–9·0
pH stability range†	3–12	3–12	3–12	3–12
Energy of activation (cal mol^{-1})	14 400	14 400	14 400	14 400
Energy of activation for denaturation	16 600	16 600	16600	16 600
Temp. for 50% denaturation	65°	60°	63°	65°
K_m for pyridine	2.5	0.7	5.0	0.7
rel V_{max}‡	100	160	167	200
K_m for nicotinic acid	25	29	55	0.9
rel V_{max}	100	100	100	100
K_m for aniline	1.4	1.4	5.1	2.0
rel V_{max}	45	50	35	33

* Pyridine as co-substrate in Pi–PPi buffer. † at 4° for 24 hr. ‡ Relative to a value of 100 for nictonic acid.

Metal ions other than Hg^{2+} inhibited the enzymes, thus at 1 mM concentration, Ag^+ gave 100% inhibition, Fe^{2+} and Fe^{3+} gave 70% and Cu^{2+} gave 50%. Other cations including Ca^{2+}, Mo^+ Mn^{2+}, Zn^{2+}, Mg^{2+}, K^+ and Na^+ gave no significant increase or decrease in activity. In contrast to the enzyme purified from *Cl. sporogenes* by Kobayashi, [6, 7] the nardoo thiaminase I was neither inhibited by EDTA (up to 100 mM) nor activated by Ca^{2+} (1 mM) at 30°.

As mentioned, a number of aromatic amines, heterocyclic bases and sulphydryl reagents can act as co-substrates in the thiaminase I reaction [3, 4, 7]. In the present investigation a highly purified nardoo thiaminase I enzyme was employed to study the relationship between the relative V_{max} and K_m of the enzyme for a particular base and the pK_b of that base. Table 5 compares the relative effectiveness of a number of substituted anilines as co-substrates in the nardoo thiaminase I scission of thiamine. Where a base has more than one ionizable *N*,

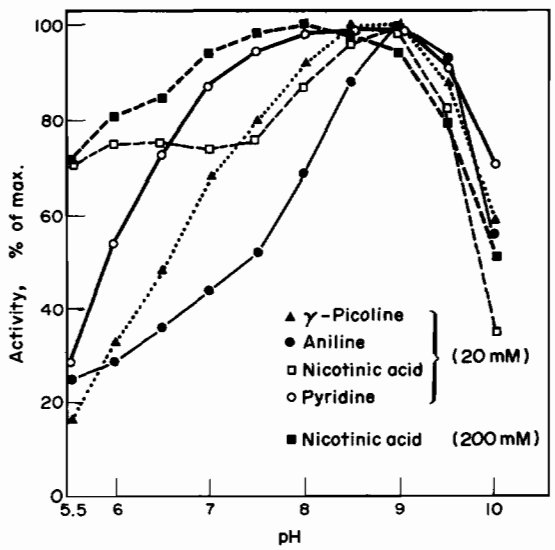

Fig. 3. pH activity patterns of nardoo thiaminase I with a number of co-substrate bases.

Table 4. Reactivation with sulphydryl compounds of *p*-CMB (10^{-4} M) inactivated nardoo thiaminase I

Reducing or metal chelating agent added	Thiaminase activity % of control	
Control (No *p*-CMB or reducing agent)	100	
	3.3 mM*	33 mM*
Nil	0	0
EDTA	0	0
Thioglycollate	18	103
Thiosulphate	15	85
Cysteine	140	55
Mercaptoethanol	142	78

* Concentration of reducing or metal chelating agent in the incubation solution.

Table 5. Effectiveness of a range of aromatic amines as co-substrates for nardoo thiaminase I at pH 6.5

Substrate	Structure	pK_b	K_m (mM)	Relative* V_{max}
Aniline	 (=R)	9.39	1.4	45
o-Phenylene diamine	R—NH_2	9.26	1.7	25
m-Phenylene diamine		9.0	1.7	39
p-Phenylene diamine		7.8	3.3	23
o-Toluidine	R—Me	9.55	6.3	14
m-Toluidine		9.28	2.4	25
p-Toluidine		8.90	9.1	20
2-Hydroxyaniline	R—OH	9.26	4.2	28
3-Hydroxyaniline		9.70	2.8	41
4-Hydroxyaniline		9.35	20.0	32
o-Aminobenzoic Acid	R—COOH	9.05	37.0	17
m-Aminobenzoic Acid		9.27	37.0	24
p-Aminobenzoic Acid		9.61	200.0	6
o-Nitroaniline	R—NO_2	14.26	—	0
m-Nitroaniline		11.53	50	12
p-Nitroaniline		13.00	—	0
Sulphanilamide	R—SO_2—NH_2		77	2.6
Sulphanilic Acid	R—SO_3	10.78	50	1.8

* Relative to a value of 100 for nicotinic acid

the only pK_b value reported is that in the range 7–10. For a particular ligand the enzyme has a greater affinity and relative V_{max} when the group is *meta*-substituted. However, the high pK_b value of the base is the overriding factor when compounds such as *o*-, *m*- and *p*-nitroanilines and sulphanilic acid are considered. Bases with pK_b values above 10 are poor co-substrates. No obvious relationship was found between minor changes in pK_b values (or the corresponding Hammetts' sigma constants [15]) of substituted anilines and their effectiveness as co-substrates in the nardoo thiaminase I reaction. Thus it is proposed that the effectiveness of an amine as a co-substrate in this reaction is not solely dependent on its chemical and stereochemical properties but that the nature of the enzyme active site is also relevant (cf. ref [14]).

The relative effectiveness of a number of heterocyclic compounds based on pyridine as co-substrates in the nardoo thiaminase I reaction is shown in Table 6. Here again the *m*-substituted derivatives are more effective than either the *o*- or *p*-compounds. When the substituent is relatively small e.g. $-NH_2$ or $-Me$ groups, the *o*-substituted base is almost as effective as the corresponding *m*-substituted derivative as a co-substrate. However, substitution at the *o*-position with larger groups, such as the carboxyl group of α-picolinic acid, greatly affects the V_{max} values.

The activity of thiaminase I with a miscellaneous group of compounds, some of which are naturally occurring, is shown in Table 7. The most active co-substrates found in bracken fern extracts by Watkin *et al.* [19] were proline and hydroxyproline. These two and adenine are good co-

Table 6. Effectiveness of a range of heterocyclic bases as co-substrates for nardoo thiaminase I at pH 6.5

Substrate	Structure	pK_b	K_m (mM)	Relative* V_{max}
Pyridine	 (=R)	8.82	2.5	100
2-Aminopyridine	R—NH_2	7.18	12.5	62
3-Aminopyridine		8.02	2.9	100
4-Aminopyridine		4.89	15.4	83
α-Picoline	R—Me	7.80	3.3	45
β-Picoline		8.48	0.5	65
γ-Picoline		7.92	0.9	64
α-Picolinic acid	R—COOH	8.75	21.2	2.7
β-Picolinic acid		9.23	25	100
γ-Picolinic acid		9.16	3.4	54

* Relative to a value of 100 for nicotonic acid.

Table 7. Effectiveness of a miscellaneous group of bases as co-substrates for nardoo thiaminase I at pH 6.5

Substrate	pK_b	K_m (mM)	Relative† V_{max}
Proline	10.6	111	28
Hydroxyproline	12.15	400	28
Adenine	9.9	9.5	40
Creatine	11.4	—	0
Quinoline	9.5	3.3	22
Imidazole	6.9	125	100
Methylamine	3.4	32	0.3
6-Aminohexanoic acid	9.6	38	16
Ethanolamine	8.15	*	22
Tris	9.80	200	1
Cysteine	5.79(NH_2)	33	6
2,3 Dimercapto propanol	—	0.8	3
Sodium thiosulphate	—	33	6
Mercaptoethanol	—	33	9
Dithiothreitol	—	22	9
Sodium metabisulphite	—	50	7
o-Mercapto-benzoic aicd	—	5	24
Sodium thioglycolate	—	1250	0.2
Sodium dithionite‡	—	—	—

* Ethanolamine did not give a linear Lineweaver–Burk plot. Maximum activity was obtained at 40 mM concentration. Above this concentration the enzyme was inhibited and below this value the plot was not linear. † Relative to a value of 100 for nicotinic acid. ‡ Refer to text.

substrates for the thiaminase I from nardoo. In previous reports it has been suggested that the thiaminase I hydrolysis of thiamine requires either an aromatic amine, heterocyclic base or a sulphydryl containing compound, [2] however the present results show that the reason why aliphatic amines are generally not successful co-substrates is because they are too basic (e.g. methylamine). Aliphatic amines with pK_b values in the range 8 to 10 are good co-substrates (e.g. 6-aminohexanoic acid and ethanolamine).

A number of sulphydryl compounds act as co-substrates for the nardoo thiaminase I enzyme (ref. [2]). The compound *o*-mercaptobenzoic acid was the most effective co-substrate but the enzyme had highest affinity for 2,3-dimercaptopropanol. The co-substrate activity of sodium dithionite could not be determined accurately because under the assay conditions employed this compound rapidly hydrolysed thiamine and released free [2-^{14}C] thiazole- in the absence of thiaminase I enzyme.

EXPERIMENTAL

Sources and extraction of thiaminase I enzymes. Thiaminase I was extracted from *Marsilea drummondii* (nardoo fern), *Marsilea augustifolia, Marsileamutica, Pteridium esculentum* (bracken fern). *Cheilanthes siebri* (rock fern) and *Velesunio ambiguus* (freshwater mussel). Comparison of activity among species was based on extracts of lush growth of ferns and of whole mussels (collected in Autumn months). Activity was also measured in stems, roots and older fronds of nardoo and bracken. On harvesting, samples were stored frozen until lyophilised. Lyophilised material was milled to pass 0.8 mm mesh and stored in sealed glass containers at −40°. Milled material (1 g) was suspended in 0.1 M Pi buffer (20 ml, pH 6.5), stirred vigorously for 1 min., allowed to stand for 20 min and again stirred. The soln was centrifuged at 1500 *g* for 10 min, centrifuged at 1500 *g* for 10 min and duplicate aliquots of supernatant removed for assay.

Assay of thiaminase I activity, [18] All substrated contained 0.1 μCi/ml [thiazole-2-^{14}C]thiamine HCl and were prepared in Pi buffer (0.1 M, pH 6.5). Thiamine concentrations employed were 0.02, 0.2, 2 and 20 mM and the substrates contained either 100 mM pyridine or no added base. For a substrate containing a certain concentration of thiamine e.g. 0.02 M and pyridine e.g. 100 mM, the shorthand notation, substrate [0.02, 100] is employed. Unless otherwise stated, all thiaminase I activities were determined in the presence of 50 mM pyridine. For assay of thiaminase I, 0.2 ml of the enzyme preparation was added to an aliquot (0.2 ml) of the appropriate substrate (0.02–20 mM thiamine plus zero or 100 mM pyridine) and the mixture incubated at 30° for 5 to 20 min. The reaction was stopped and released thiazole [2-^{14}C] extracted, by adding EtOAc (2 ml) and blending at max speed on a test tube stirrer for 10 sec. On centrifugation (1500 *g*, 2 min) an aliquot (0.5 ml) of the EtOAc layer was removed and added to 15 ml scintillation cocktail (0.4% w/v PPO, 0.05% w/v POPOP in toluene). After 2 min storage in the dark, radioactivity was measured in a scintillation counter.

Purification of thiaminase I enzymes. Milled nardoo tissue (300 g) was mixed with washed insol PVP (300 g). Pi buffer (6 l., 20 mM, pH 6.5) was added to the mixture and stirred. After 20 min the soln was homogenised and centrifuged (3500 *g*, 30 min). Wet—cake DEAE—cellulose (*ca* 200 g) was added to the very viscous, amber coloured supernatant, and stirred. The DEAE-cellulose was collected by filtration of the soln through Miracloth ® and washed exhaustively with 50 mM Pi buffer (4 l., pH 6.5). It was then transferred to a large sintered-glass funnel and further washed under a slight vacuum. Thiaminase I was almost quantitatively recovered by washing the DEAE-cellulose with 400 mM KCl (800 ml). The enzyme soln was then concentrated by rotary evaporation below 40° to *ca* 60 ml, applied to a preparative Ultrogel AcA 34 column (7 × 70 cm) and eluted with Pi buffer (0.1 M, pH 6.5). The active fraction was concentrated by Diaflo ultrafiltration using a UM 10 membrane, applied to a Sephadex G-100 column (2.5 cm × 80 cm) and eluted with citrate buffer (20 mM, pH 3). It was then directly applied to a pre-equilibrated phosphocellulose column (1.5 × 10 cm, pH 3) and eluted with a linear combined salt–pH gradient (citrate, 20 mM, pH 3—phosphate 50 mM, pH 6.5 plus KCl, 0.2 M). The enzyme was concentrated in a Diaflo Ultrafiltration cell, washed with KCl (1 mM), removed and lyophilised and then redissolved in KCl (5 mM) to a protein concn of 10 μg/μl. The conc protein soln was used directly for Gradient-gel electrophoresis using a Gradipore Electrophoresis unit (100–200 μg protein per sample) and for isoelectric focusing. Isoelectric points were determined by slicing gels into 5 mm sections, extracting with 2 ml H_2O and assaying for both pH and enzyme activity. Thiaminase I enzymes from bracken and rock ferns were purified using a similar technique. The enzyme from mussel was obtained by a similar sequence of purification steps, except that on elution from DEAE-cellulose the enzyme was concentrated by pptn with $(NH_4)_2SO_4$(0–90%).

Properties of thiaminase I enzymes. Molecular sizes of thiaminase I enzymes were determined by chromatography on Sephadex G 100 as described by Andrews [20]. Bovine serum albumin, trypsin, papain, lactate dehydrogenase, alkaline phosphatase, myoglobin, haemoglobin and ovalbumin were used as standards in 0.2 M NaPi (pH 6.5). For the determination of pH-activity curves, solns containing 40 mM pyridine, aniline, γ-picoline or nicotinic acid or 400 mM nicotinic acid were prepared in the pH range 5.5–10 using a Pi–PPi (0.2 M), Pi–Tris (0.2 M) or Pi–borate (0.2 M) buffer system. The assay mixture contained amine/buffer soln (0.2 ml), thiamine substrate (0.1 ml; 0.4 mM thiamine, 0.2 μCi/ml of radioactive thiamine) and enzyme prepn (0.1 ml). pH stability values were estimated by incubating enzyme soln (0.2 ml) with buffer soln (0.1 M, 0.2 ml, pH 3–12) for 24 hr

at 4°. The soln was then diluted 20 fold with 0.1 M Pi buffer (pH 6.5) and assayed using substrate [0.2, 100]. To determine the effect of pH on K_m and V_{max} of nardoo thiaminase I for pyridine and nicotinic acid, the amine solns prepared for the pH activity curves were employed at concns of 1–40 mM (pyridine) and 1–400 mM (nicotinic acid). The assay mixture contained amine–buffer soln (0.2 ml), substrate [0.2, 0] (0.2 ml) and nardoo thiaminase I enzyme (0.2 ml). The energy of activation and energy of activation for denaturation were determined by incubating the enzyme (0.2 ml) in Pi buffer (0.1 M, pH 6.5) in the temp range 20 to 80° with substrate [0.2, 100] (0.2 ml) for 5 min. Temp stability was determined by incubation of enzyme solns (1 ml) at 20–80° for 20 min. Aliquots (0.2 ml) were assayed for residual activity using substrate [0.2, 100] (0.2 ml) at 30°. To determine the effect of metal ions and other possible inhibitors of thiaminase I, enzyme (0.2 ml) in 100 mM Pi (pH 6.5) was incubated with an aliquot (0.2 ml) of 2 μM to 2 mM metal ion or sulphydryl reagent or 0.1 to 10 mM iodoacetic acid at 30° for 30 min. Residual activity was assayed by the addition of substrate [0.2, 100] (0.2 ml). The degree of reactivation of enzymes inhibited with *p*-CMB (0.1 mM) was determined by the addition of a reducing and/or metal chelating agent (0.2 ml), to a final concn of 3.3 or 33 mM, to the inhibited enzyme soln and incubating at 30° for 30 min. Final activity was assayed with substrate [0.2, 100].

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