Studies on the Polymorphism of Thiaminase I in Seawater Fish

Takahiro NISHIMUNE, Yoshihiro WATANABE and Hideki OKAZAKI

Nutrition Course, Department of Health Science, Musashigaoka College.
Yoshimi-cho, Hiki-gun, Saitama 355–0154, Japan

(Received February 19, 2008)

Summary Thiaminase I from the seawater fish Fisturalia petimba was characterized, and pl polymorphism of the enzyme was first described, together with the active subfragment of thiaminase I. The liver of E. petimba contained thiaminase I of at least three different pIs and the major fraction exhibited pl 5.7. The most evident difference among pl isozymes was the size of the active subfragments into which they were dissociated. pl 5.7 enzyme dissociated into subfragments of 25 kDa, while pl 7–9 enzymes dissociated into approx. 22 kDa. The reaction rate measured by pyridine as the second substrate was three times higher in pl 7–9 enzymes compared with pl 5.7 enzyme. The degree of cadmium inhibition, when aniline was the co-substrate, also showed obvious differences between pl isozymes. When the major pl fraction was further purified by the difference in hydrophobicity, a smaller active fragment of approx. 22 kDa appeared, indicating the possibility that the difference in the size of active subfragment between isozymes is a result of partial fragmentation. The pl 5.7 enzyme was purified 250 times and the size of the most purified preparation was found to be 106 kDa by gel filtration analysis. The purified preparation gave an active 25 kDa subfragment by SDS-PAGE, together with a 15 kDa non-active subfragment. The enzyme was, thus, inferred to contain active subfragments together with the 15 kDa non-active fragments. Amino acid sequencing of the 25 kDa active subfragment revealed, together with the fully processed N-terminal sequence, two N-terminal peptides with extra Pro-Ser and Gly-Pro-Ser attached to it, and the NCBI non-redundant database did not show significant similarity to other known proteins. On the other hand, the molecular mass of the holoenzyme from the viscera of the seawater fish Engraulis japonica was estimated to be approx. 100 kDa by gel filtration chromatography. An SDS-PAGE analysis revealed that it contained an active subfragment of 22 kDa.

Key Words thiaminase I, polymorphism, subfragment, fish thiaminase

Initially, thiaminase I was found in freshwater fish (1). Now, it is well known that many seawater fishes also contain high levels of thiaminase I activity (2–4), sometimes resulting in severe economic losses in farm fisheries when thiaminase-positive seawater fish are given as a sole feed without supplementing thiamin pellets (5, 6). Studies on the purification of thiaminasises of higher organisms have already been reported on Carp with the result of a single 55 kDa SDS-PAGE band (7) and on a fern, Marsilea drummondii, with a 115 kDa major SDS-PAGE band (8). Little biochemical characterization of the enzyme in marine fish, however, has been accomplished to date, and the idea of its molecular characteristics has been based previously on association with other thiaminases by the similarity of its catalyzing reaction.

Although a putative physiological meaning of thiaminase II, which catalyzes the hydrolyzing breakdown of thiamin, has been proposed recently (9), various thiaminase Is are still not understood enough, not only in their physiological meaning but also in the diversity of their molecular structures. We report here some properties of thiaminase I of the seawater fish Fisturalia petimba with much bigger molecular sizes compared with the reported counterparts of carp (7) and bacteria (10–12). The pl polymorphism and dissociation of the molecule into active subunits were also revealed, which had not been reported in other thiaminase Is yet. The similar molecular size and dissociation into active subunits were also confirmed in another seawater fish, Engraulis japonica.

MATERIALS AND METHODS

Seawater fish. Among various seawater fish tested for the activity of thiaminase, E. petimba is one of the strongest (4), and E. japonica (anchovy) is known to be used as a feed in farm fishing. Fresh frozen E. petimba or E. japonica were obtained from retailers’ shops around suburban Tokyo, and, after they thawed out, the liver or total viscera, respectively, was dissected and chilled immediately. The weight of the liver of E. petimba ranged between 15 and 60 g with the average of approx. 35 g. In an ice-cold mortar, 1.5 times the weight of 20 mM sodium potassium phosphate buffer (NaK-phosphate buffer), pH 6.40, containing 1 mM EDTA, 1 mM 2-mercaptoethanol (βME), and 1/200 volume of Wako Protease Inhibitor Mix were added to the fish organs together with approx. 1/3 weight of purified sea sand.
and homogenized by the pestle. The brei obtained was centrifuged at 15,000 × g for 30 min at 4°C and the supernatant was filtered through medical gauze to eliminate insoluble fat (liver or viscera homogenate).

Reagents and apparatus for enzyme purification and characterization. Protease Inhibitor Mixture for Mammalian Cell and Tissue Extracts was the product of Wako Pure Chemical Industries, Ltd., Japan, and phosphatase inhibitor cocktail set II was purchased from Calbiochem. Molecular weight standards for gel filtration chromatography and SDS-PAGE were the products of BIO-RAD. 26/60 Sephacryl S200, HiLoad 16/10 Phenyl Sepharose, and 16/60 Superdex 200 were the products of Amersham Biosciences, and Hydroxy Apa-
tite Ceramic CHT2–1 was a BIO-RAD product. The pre-
cast gel for SDS-PAGE (12.5% gel, 90H×85W×1T mm) was purchased from System Instruments Marysol, Japan, and used with the sample-preparing buffer and the SDS-containing electrode buffer of Wako Pure Chemical Industries. The electric current of 20 mA for 70 min was applied, followed by the silver staining of protein bands by Silver Stain Plus of BIO-RAD or by the activity staining of thiaminase I as described below. The peak areas of the stained bands were estimated by scan-
ing the color picture of the gel with Shimadzu Flying Spot Scanner CS9000 using a 520 nm reflection beam. The Rotofor System of BIO-RAD was used for the iso-
electric focusing (IEF) fractionation with the BioLyte tite Ceramic CHT2–1 was a BIO-RAD product. The pre-
cast gel for SDS-PAGE (12.5% gel, 90H×85W×1T mm) was purchased from System Instruments Marysol, Japan, and used with the sample-preparing buffer and the SDS-containing electrode buffer of Wako Pure Chemical Industries. The electric current of 20 mA for 70 min was applied, followed by the silver staining of protein bands by Silver Stain Plus of BIO-RAD or by the activity staining of thiaminase I as described below. The peak areas of the stained bands were estimated by scan-
ing the color picture of the gel with Shimadzu Flying Spot Scanner CS9000 using a 520 nm reflection beam. The Rotofor System of BIO-RAD was used for the iso-
electric focusing (IEF) fractionation with the BioLyte Ampholyte 5/8 at the final concentration of 2.5%, 0.1% Brij 35, and 1 mM βME in the total sample of 55 mL, and 12 W constant electric current was applied for 6 h. After the focusing, fractions were collected into tubes containing βME to give the final concentration of approx. 1.5 mM. The pH of each tube was measured, and cold 0.2 M NaK-phosphate buffer of pH 6.20 was added immediately to the concentration of approx. 10 mM.

Standard assay protocol. The standard assay mixture for thiaminase I activity contained 0.1 M NaK-phosphate buffer of pH 7.50, 25 mM βME, 30 μM thiamin and enzyme sample in the total volume of 300 μL. After 2 and 7 min incubation at 40°C, the remaining thiamin was oxidized adding 300 μL of BrCN, which was pre-
bred by titrating bromine-saturated water with 10% KCN to the end point of decolorization, and 200 μL of 20% NaOH, and the fluorescence was measured by the excitation and emission wavelength of 375 and 445 nm, respectively.

Activity staining of SDS-PAGE gel (10). The gel was washed, immediately after the run, with 200 mL of 25 mM NaK-phosphate buffer, pH 6.50, containing 5 mM βME for 35 min with vigorous shaking, repeated twice, but the 3rd buffer was without βME. A reducing reagent like βME should be excluded from the last buffer since it interferes with the diazo staining. Then, the gel was immersed in 100 mL of 25 mM NaK-phosphate buffer, pH 6.50, containing 0.89 mM thiamin and 27 mM aniline and shaken gently for 10 min. The solution was discarded completely and the trace amount of remaining solution was absorbed completely by a filter paper from and around the gel. The gel was incubated at 45°C for 20 min in a container to protect it from drying, and stained immediately by 17 mL of the freshly prepared diazo reagent (13) for 5–10 min.

In the case of *E. japonica* thiaminase I, the gel was washed once additionally (4th wash), after the SDS had been taken out as above, in 100 mL of 25 mM sodium acetate buffer, pH 5.50 for 10 min to adjust the pH optimum for the anchovy enzyme. Further, thiamin and aniline were dissolved in the same pH 5.50 buffer in the case of *E. japonica* enzyme. Other procedures were the same as for the *F. petimba* enzyme.

Purification of thiaminase I. Gel filtration through Sephacryl S200 or Superdex 200 was carried out in 25 mM NaK-phosphate buffer, pH 6.20, containing 0.15 M NaCl, 17% glycerol and 2 mM βME (S200G6.2 buffer). Phenyl Sepharose column and the sample were first equilibrated with 50 mM NaK-phosphate buffer, pH 6.20, containing 20% glycerol, 1.35 M ammonium sulfate, and 2 mM βME (Phenyl Sepharose adsorption buffer). After applying the sample at 0.4 mL/min flow rate on the column, it was developed by a linear gradient elution between 105 mL each of 1.2 and 0.4 M ammonium sulfate in 50 mM NaK-phosphate buffer, pH 6.20, containing 20% glycerol and 2 mM βME. A Hydroxyapatite column and the sample were first equil-
bribated with 20 mM NaK-phosphate buffer, pH 6.20, containing 20% glycerol and 2 mM βME (Hydroxyapa-
tite adsorption buffer). After the sample was adsorbed on the column, it was developed by a linear gradient from 200 to 500 mM of NaK phosphate buffer, pH 6.20, containing 20% glycerol and 2 mM βME at a flow rate of 0.15 mL/min. For the concentration of enzyme samples or the exchange of buffers, Amicon Ultra 15, 4, or Microcon of the cut-off molecular weight of 10,000, from Millipore Corporation, was used. All the column chromatographies were carried out at 18°C and the elu-
ate fractions were cooled in ice immediately. Edman sequencing was performed by Aproscience Co., Japan.

RESULTS

### pl polymorphism of seawater fish thiaminase I

The liver homogenate of *F. petimba* was fractionated by 30–75% ammonium sulfate saturation, and the insoluble fat was eliminated. After the ammonium sul-
fate precipitate was dissolved into 10 mM NaK phos-
phate buffer, pH 6.40, containing 1 mM EDTA and 1 mM βME, it was dialyzed against the same buffer over-
night at 4°C and the salt was eliminated. After the dial-
ysis, 1/200 volume of the Protease Inhibitor Mix for a run) was added on the IEF apparatus. The results (a typical result is shown in Fig. 1) gave a major pl fraction of pl 5.7 and 2–3 minor peak fractions of pl 7–9, and this result was reproducible even with a high enough con-
centration of carrier ampholite. The same isoelectric fractionation profile was also obtained when 1/100 vol-
ume of Calbiochem Phosphatase Inhibitor Cocktail was added together with the protease inhibitor mixture to the liver homogenate and dialysate of ammonium sul-
Fish Thiaminase I

341

fate fractionation. Fractions were combined into two groups (named tentatively pI 5.7 enzyme and pI 7–9 enzymes) and concentrated, and the buffer was exchanged into S200G6.2 buffer. The two pI preparations gave activity bands of different sizes when SDS-PAGE was carried out and the activity was stained. The pI 5.7 enzyme exhibited its activity at the size of 25 kDa, while pI 7–9 enzymes gave approx. 22 kDa active band (Fig. 2, lanes 1 and 2).

Purification by gel filtration and phenyl sepharose chromatography

In order to confirm whether those pI isozymes could be separated by their difference in molecular size, pI 5.7 enzyme and pI 7–9 enzymes were applied, first on a Sephacryl S75 (data not shown), and then on a Sephacryl S200 column, and a substantially singular activity peak was obtained for each sample at the same elution position corresponding to the molecular size of 100 kDa (data not shown). The activity-containing fractions obtained from the gel column were combined and concentrated, and the buffer was exchanged into Hydroxyapatite adsorption buffer temporarily. The results of SDS-PAGE and activity staining for these samples proved the sizes of active bands to be kept unchanged (Fig. 2, lanes 4, 5, 7 and 8). Thus, the two holoenzymes of approx. 100 kDa with different pIs were revealed to dissociate into active subunits of different molecular sizes.

In order to understand the structure of the whole enzyme of approx. 100 kDa containing 25 kDa active subunit(s) more clearly, we purified the pI 5.7 enzyme. We selected pI 5.7 enzyme from the fact that pI 7–9 enzymes could contain a degradation product of pI 5.7 enzyme as described below. After the buffer was exchanged into Phenyl Sepharose adsorption buffer, the gel eluate was applied on Phenyl Sepharose and the result of linear gradient elution is shown in Fig. 3. The multiplicity of activity peaks was highly reproducible except for the “4” fractions. Moreover, only the fastest eluting activity peak “1” gave the 25 kDa active subunit separated from the 22 kDa protein (Fig. 4, lanes 4 and
and it became clear that the “2” peak contained an active 22 kDa subunit (Fig. 2, lanes 6 and 11 and Fig. 4, lane 3) together with the 25 kDa active subunit. From this result, we deduced that the smaller active subunit of 22 kDa found in pI 7–9 enzymes is a product of partial fragmentation derived from the 25 kDa active subunit.

Hydroxyapatite chromatography and Superdex 200 gel filtration

As shown in Fig. 4, lane 4, even the fastest eluting activity fractions of Phenyl Sepharose “1” still contained contaminating bands other than the 25 kDa active thiaminase I. Thus the “1” fractions were combined and concentrated, and applied onto the Hydroxyapatite column. The eluate of Hydroxyapatite gave a single activity peak (Fig. 5) and combined peak fractions were analyzed (Fig. 4, lane 5). Together with the 25 kDa, an approx. 15 kDa band had been rather concentrated.

In order to eliminate the 15 kDa band, the eluate of Hydroxyapatite was applied onto a Superdex 200 column. The size was estimated to be 106 kDa from the relative position of elution of a single activity peak against the molecular standards (data not shown). The peak fractions again gave the activity band of 25 kDa, together with the 15 kDa band (Fig. 4, lane 6). The elution position of contaminating 15 kDa protein, if any, from the Superdex 200 column was completely apart from the activity peak of 106 kDa. Further, the Superdex 200 gel re-chromatography of the combined peak fractions gave a similar result (Fig. 4, lanes 6 and 7). We tried to estimate the ratio of these two peak areas obtained with silver staining by scanning the photos of SDS-PAGE gel. Relative peak area (%) obtained from 25 and 15 kDa bands were, respectively, 55.5 and 44.5 for Phenyl Sepharose eluate (data not shown), 44.8 and 55.2 for Hydroxyapatite eluate, 38.7 and 61.3 for Superdex 200 eluate, and 51.2 and 48.8 for Superdex 200 re-chromatography eluate (Fig. 6). These values did not reflect precisely the amount of peptide since the color intensity of the gel picture was not necessarily reflected on the peak area by the method used. The amount of 15 kDa peptide is, nevertheless, not decreased at all through those purification steps. From those results, it was assumed that the 15 kDa peptide constitutes a part of the pure F. petimba thiaminase I molecule.

The result of purification is summarized in Table 1. Although the recoveries in the activity and protein were...
not high, more than 250 times purification was accomplished. The recovery in total activity was around 10% in Phenyl Sepharose chromatography, as was expected, and IEF fractionation also compelled us to find a large amount of activity loss.

Amino acid analysis of 25 kDa active subunit
It was ascertained from the result of Edman sequencing that three N-terminals coexisted in the 25 kDa active subunit. In addition to the fully processed RDVYEKLWEDNKDIAEKT, PS- and GPS-attached terminals were found to remain as minor components. The internal N-terminal sequences detected after trypsin digestion were YLVTEDEILK, FHMEGV(S)EIIP, and DRFE(S). The non-redundant protein databases of the NCBI did not show significant similarity to other known proteins.

Active subunit of anchovy thiaminase I
The whole viscera of *E. japonica* was homogenized and fractionated by ammonium sulfate in the same way as *F. petimba* thiaminase I. The dialyzed crude enzyme preparation was analyzed as in the case of *F. petimba*, and the results of activity staining showed the size of active subunit to be 22 kDa (Fig. 2, lane 14). The enzyme preparation was applied on a Sephacryl S200 column as in the case of *F. petimba* enzyme, and a single activity peak eluted at the position corresponding to the molecular size of 100 kDa (data not shown).

**Table 1. Summary of purification.**

|                | Total volume (mL) | Total protein (mg) | Total activity ([H9004 B1 nmol/min]) | Specific activity ([H9004 B1 nmol/min/mg]) |
|----------------|-------------------|--------------------|--------------------------------------|-------------------------------------------|
| Crude homogenate | 236.00            | 4,320              | 19,600                               | 4.55                                      |
| (NH₄)₂SO₄ 30–75% saturation | 150.00            | 2,470              | 15,500                               | 6.26                                      |
| IEF pI 5.7 fraction | 2.20             | 134                 | 1,770                                | 13.2                                      |
| Sephacryl S200 eluate | 2.82             | 26.8               | 810                                  | 30.2                                      |
| Phenyl Sepharose eluate | 0.40             | 0.065               | 75.3                                 | 960                                       |
| Hydroxyapatite eluate | 0.14             | 0.008               | 8.48                                 | 1,060                                     |
| Superdex 200 eluate | 0.08             | 0.004               | 4.07                                 | 1,160                                     |

Starting from 4 fish, 150 g of *F. petimba* liver was purified as described in the text. The recovery in total activity was around 10% in Phenyl Sepharose chromatography, as was expected, and IEF fractionation also compelled us to find a large amount of activity loss.

**Amino acid analysis of 25 kDa active subunit**

It was ascertained from the result of Edman sequencing that three N-terminals coexisted in the 25 kDa active subunit. In addition to the fully processed RDVYEKLWEDNKDIAEKT, PS- and GPS-attached terminals were found to remain as minor components. The internal N-terminal sequences detected after trypsin digestion were YLVTEDEILK, FHMEGV(S)EIIP, and DRFE(S). The non-redundant protein databases of the NCBI did not show significant similarity to other known proteins.

**Active subunit of anchovy thiaminase I**

The whole viscera of *E. japonica* was homogenized and fractionated by ammonium sulfate in the same way as *F. petimba* thiaminase I. The dialyzed crude enzyme preparation was analyzed as in the case of *F. petimba*, and the results of activity staining showed the size of active subunit to be 22 kDa (Fig. 2, lane 14). The enzyme preparation was applied on a Sephacryl S200 column as in the case of *F. petimba* enzyme, and a single activity peak eluted at the position corresponding to the molecular size of 100 kDa (data not shown).

**Difference in the co-substrate activity for isozymes**

In order to study the effect of partial fragmentation in the active subunit and of the resultant pI shift on enzyme characteristics, co-substrate activity of typical compounds in the two pI isozymes was assayed. As the average with SD of three measurements are shown in Table 2, the two pI isozymes showed a clear difference in pyridine utilization as the co-substrate. Although the measurement was not repeated, amino-pyridines and pyridoxine also showed considerable difference in the co-substrate activity. Cysteine expressed the highest co-substrate activity within the tested compounds. We used, however, βME in the standard assay mixture, since βME had been added in samples of all purification steps as a SH protecting reagent.

**pH dependency of isozymes**

The difference in optimal pH between pI isozymes was not as clear (Fig. 7A). When the co-substrate was βME, optimal pHs were 8.2 for pI 5.7 enzyme and 7.8 for pI 7–9 enzymes. When the co-substrate was aniline, on the other hand, the pH dependency was clearly dif-

**Fig. 7.** pH dependency of isozymes. A: 25 mM βME was used as the co-substrate. The activity was assayed by 11.6 μg (pI 5.7 enzyme) or 21.5 μg (pI 7–9 enzymes) protein at 40˚C for 10 min in 0.2 M Na acetate buffer (pH 4.0–5.5), 0.2 M NaK-phosphate buffer (pH 6.0–8.0), or 0.1 M Tris HCl buffer (pH 8.5–9.0). The amount of non-enzymatic degradation had been subtracted from the plotted data. B: 10 mM aniline was used as the co-substrate. The activity was assayed by 4.0 μg (pI 5.7 enzyme) or 26.5 μg (pI 7–9 enzymes) protein at 37˚C for 15 min in the same buffers as in Fig. 7A.
different from that of βME supplied reaction (Fig. 7B). We tried to add aniline and βME simultaneously, and the result was less steep pH-activity curves with nearly the same peak pH as in Fig. 7A (data not shown).

**Optimal temperature of seawater fish thiaminase I**

When βME was used as the co-substrate, the optimal temperature was 45°C in both isozymes (Fig. 8). When aniline was added to the reaction mixture, the optimal activity level extended from 45°C up to 55°C (data not shown). In the standard assay protocol, however, the reaction temperature of 40°C was used for the smaller non-enzymatic degradation of thiamin.

**Effect of mineral ions and thiamin analogues on isozymes**

In order to see whether or not the low recovery of total activity in Hydroxyapatite chromatography resulted from a loss of any indispensable metal ion, the effect of additional minerals on the activity was measured. Only the Cd ion showed a weak activation in the βME-supplied assay (Fig. 9). The Cd ion, however, strongly inhibited the activity when aniline was the cosubstrate, and it could not be an essential component of the enzyme. Two pI isozymes showed a clear difference in the degree of cadmium inhibition when aniline was the co-substrate.

Within the three thiamin analogues tested, Neopyrithiamin showed strong inhibition in twice the concentration of thiamin (Fig. 10). This inhibition totally disappeared when aniline was the co-substrate for pI 7–9 enzyme.

**DISCUSSION**

*F. petimba* is an edible fish and, although it is not very popular, its white muscle is one of the favorite foods for a hot-pot cooked at the table in winter in Japan. The white muscle of *F. petimba* is reported to contain, however, rather high concentrations of thiaminase and the specific activity is approx. one third of the viscera (4). We have reported a strong and heat-resistant thiaminase activity in a wild silkworm (14), and wanted to characterize the silkworm thiaminase. The material was, however, not easily available for us, and instead, we studied the seawater fish first. As shown in Fig. 8, it became evident that the heat resistancy of *F. petimba* thiaminase is not as high, and the fish muscle seemed safe so long as it was cooked and thiaminase was inactivated.

The existence of isozymes in fish and shellfish thiaminases has long been proposed, mainly from the observation of more than one optimal pH for their activity (15–
Initially, we expected the possibility that the pI isozymes could explain the multiplicity of optimal pH in those early reports. However, the pH-activity curves did not exhibit significant differences among pI isozymes (Fig. 7A, B). Instead, we could show a clear difference in pH-dependency between reactions using βME and aniline as the co-substrate. Thus, there is a possibility that some other co-substrate might show an activity peak in an acidic area. When such co-substrates existed together, multiple optimal activity peaks could be possible in crude enzyme preparations.

In this report, we have presented evidence indicating the presence of isozymes in seawater fish thiaminase I, and deduced the mechanism for the presence of pI isozymes to be partial fragmentation of active subunits from 25 to 22 kDa. In the case when the whole molecule is composed of 2 to 3 active subunits, together with some 15 kDa peptides, the result of partial fragmentation will be the reduction of 6 to 9 kDa in the pI 7–9 enzymes compared to the pI 5.7 enzyme. As described in “Results”, the reduction of molecular size of this amount was within the limit of our experimental error of 100 kDa estimation using a preparative gel filtration column. Any additional fragmentation in the non-active 15 kDa peptides, however, seemed unlikely because further reduction in the size of the whole molecule would be detectable even in the 26/60 Sephacryl S200 gel filtration.

In the partial fragmentation of active subunits, we infer that main structure of the catalytic center of thiaminase I is conserved, because we could detect roughly similar specific activity in both pI isozymes. Thus, the partial fragmentation of 25 kDa active subunits into 22 kDa seemed to accompany some losses of acidic amino acids residing outside of the catalytic center, resulting in the shift of pI to a more alkaline pH. In the activity assay in neutral pHs such as 6.5 or 7.5, the ionic character of the whole thiaminase molecule is deduced to be opposite between those pI isozymes. The detected difference in co-substrate activity (Table 2) of pyridine and amino-pyridines was favorable to pI 5.7 enzymes whereas in pyridoxine the pI 5.7 enzyme gave a higher activity. This difference might be explained at large by the degree of accessibility of those co-substrates to the two pI isozymes of opposite ionic characteristics.

The ionic character of the whole thiaminase I seemed to exert influence only on the binding of the cosubstrate, and practically no effect was observed on the accessibility of thiamin or its analogues. The effect of thiamin analogues on the pI isozymes was very similar when βME was the cosubstrate (Fig. 10). Once the cosubstrate aniline was on the surface of the thiaminase I molecule, the result of the activity assay led us to deduce that the cosubstrate aniline might repel the access of neopryridoxime, but not of thiamin.

### Table 2. Co-substrate activity for isozymes.

| Co-substrate         | Thiamin degradation (%) |
|----------------------|-------------------------|
|                      | pI 5.7 enzyme | pI 7–9 enzymes |
| Cysteine             | 41.2 ± 4.0      | 40.9 ± 1.7     |
| βME                  | 21.7 ± 9.5      | 18.3 ± 2.8     |
| Aniline              | 10.2 ± 6.4      | 20.6 ± 7.2     |
| Pyridine             | 9.7 ± 9.4       | 31.6 ± 3.0     |
| 4-Amino-pyridine     | 13.7            | 25.4           |
| 3-Amino-pyridine     | 10.7            | 26.1           |
| 2-Amino-pyridine     | 2.6             | 13.2           |
| Lysine               | 17.3            | 18             |
| Pyridoxine           | 6.9             | 2.5            |
| Nicotinic acid       | 0               | 7.9            |
| Hypotaurine          | 0               | 6.8            |
| Taurine              | 0               | 6.6            |
| Histidine            | 0               | 5.7            |
| Anthranilic acid     | 0               | 0              |
| No co-substrate      | 2.8 ± 1.8       | 11.8 ± 7.8     |

Enzyme preparations obtained by the IEF fractionation were used after concentration and buffer exchange into S200G6.2 buffer. All co-substrates were added at 25 mM and the reaction was carried out by 7.9–8.2 µg (pI 5.7 enzyme) or 16.4–17.6 µg (pI 7–9 enzymes) protein at 40°C for 10 min at pH 7.50, and the decrease in thiamin was determined. For compounds with SD, the average of three measurements is shown.

In the course of purification of seawater fish thiaminase I, we could expect a low recovery of total activity in isoelectric focusing and phenyl sepharose chromatography steps. We could not understand, however, the low recovery in Hydroxyapatite chromatography. It is to be elucidated how any indispensable factor(s) for the stability of thiaminase I might be lost in this step. As the result of purification steps, we obtained two SDS-PAGE bands, 25 and 15 kDa in size, and both of them were much smaller compared to the un-denatured whole molecule. The elution position of the 15 kDa peptide and 106 kDa native enzyme in Superdex 200 gel chromatography render untenable the argument that the 15 kDa peptide is a contamination. Thus, we deduce that thiaminase I of *E. petimba* is made up by the combination of either 2 or 3 molecules of 25 kDa active subunits and 4 or 2 of 15 kDa peptides, respectively. We hope this study could provide a clue to cloning and analyzing the gene structure of seawater fish thiaminase I.
In the near future.

In the case of carp thiaminase I, it is reported that the kidney and spleen cells contain the enzyme in lysosome (20), and the pH of the inside of lysosome is usually kept at around pH 5. Thus, optimal pH for lysosomal enzymes is known to be acidic. In the case of *F. petimba* thiaminase I, despite the origin of the enzyme being whole viscera, the optimal pH is reported to be pH 6–6.5 when pyridine is used as the co-substrate (4). If the liver thiaminase I is also localized in lysosome, a co-substrate which can give the maximum activity at acidic pH might be the a priori co-substrate of this enzyme.

Acknowledgments

We are grateful to Prof. N. Myoga of this school for his helpful advice in the taxonomy of fish and dissection of fish organs. This work is dedicated to the memory of the late T. N.

REFERENCES

1) Deutsch HF, Hasler AD. 1943. Distribution of a vitamin B1 destructive enzyme in fish. *Proc Soc Exp Biol Med* 53: 63–65.
2) Yudkin WH. 1949. Thiaminase, the Chastec-Paralysis factor. *Physiol Rev* 29: 389–402.
3) Hiller DM, Peter OE. 1966. Anti-thiamin activity in Hawaii fish. *J Nutr* 89: 419–421.
4) Ishihara T, Kinari H, Yasuda M. 1973. Studies on thiaminase I in marine fish II. Distribution of thiaminase in marine fish. *Bull Jap Soc Scientific Fisheries* 39: 55–59.
5) Alexander L, Green RG, Evans CA, Wolf LE. 1941. Alcoholic encephalopathy in man and fish-diet-disease in foxes and fishes. A study in comparative neuropathology. *Trans Am Neurol Assn* 67: 119–122.
6) Ishihara T, Yasuda M, Kashiwagi T, Yagi M. 1974. Studies on thiaminase I in marine fish II. Distribution of thiaminase in marine fish. *Bull Jap Soc Scientific Fisheries* 40: 675–682.
7) Bos M, Koizik A. 2000. Some molecular and enzymatic properties of a homogeneous preparation of thiaminase I purified from carp liver. *J Prot Chem* 19: 75–84.
8) McCleary BV, Chick BF. 1977. The purification and properties of a thiaminase I enzyme from nardoo (*Nardia drummondii*). *Phytochemistry* 16: 207–213.
9) Jenkins AH, Schyns G, Potot S, Sun G, Begley TP. 2007. A new thiamin salvage pathway. *Nature Chem Biol* 3: 492–497.
10) Abe M, Ito S, Kimoto M, Hayashi R, Nishimune T. 1987. Molecular studies on thiaminase I. *Biochim Biophys Acta* 909: 213–221.
11) Costello CA, Kelleher NL, Abe M, McLaugherty FW, Begley TP. 1996. Mechanistic studies on thiaminase I. Over expression and identification of the active site nucleophile. *J Biol Chem* 271: 3445–3452.
12) Campobasso N, Costello CA, Kinsland C, Begley TP, Ealick SE. 1998. Crystal structure of thiaminase I from *Bacillus thiaminolyticus* at 2.0 Å resolution. *Biochemistry* 37: 15981–15989.
13) Abe M, Nishimune T, Ito S, Kimoto M, Hayashi R. 1986. A simple method for the detection of thiaminase producing colonies. *FEMS Microbiol Lett* 34: 129–133.
14) Nishimune T, Watanabe Y, Okazaki H, Akai H. 2000. Thiamin is decomposed due to *Anaphe* spp. Entomophagy in seasonal ataxia patients in Nigeria. *J Nutr* 130: 1625–1628.
15) Reddy KK, Giri KV, Das R. 1948. Thiaminase system in fresh water mussel. *Enzymologia* 12: 238–245.
16) Deolalkar ST, Sohonie K. 1954. Thiaminase from fresh-water, brackish-water and salt-water fish. *Nature* 4402: 489–490.
17) Zizza F. 1954. Research on thiaminase. 1) pH optimum of thiaminase extracted from *Venerupis decussata*. *Bull Soc Ital Biol Sper* 30: 246–247.
18) Fujita A. 1954. Thiaminase. *Adv Enzymol Related Subjects Biochem* 15: 389–421.
19) Nakatsuka T, Suzuki K, Nakano Y, Kitaoka S. 1988. Physicochemical properties of intracellular thiaminase II of *Bacillus aneurinolyticus*. *Vitamins* 62: 15–22.
20) Sato M, Hayashi S, Nishino K. 1994. Subcellular localization of thiaminase I in the kidney and spleen of carp, *Cyprinus carpio*. *Comp Biochem Physiol* 108A: 31–38.