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An Assay for Thiaminase I in Complex Biological Samples

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Abstract

An alternative method for measuring thiaminase I activity in complex samples is described. This assay is based on the selective consumption of the highly chromophoric 4-nitrothiophenolate by thiaminase I resulting in a large decrease in absorbance at 411 nm. This new assay is simple and sensitive and requires only readily available chemicals and a visible region spectrophotometer. In addition, the assay is optimized for high throughput analysis in 96-well format with complex biological samples.

Keywords

Thiaminase I; Thiaminase II; TenA; vitamin B₁; thiamin; early mortality syndrome; salmon; trout; Beriberi; forage fish; Great Lakes; New York Finger Lakes

Introduction

Thiaminases degrade thiamin (vitamin B₁) and were initially observed in foods more than 6 decades ago. This activity has been detected in a wide variety of sources including bacteria, marine organisms, and plants[14;15;23]. Animals that consume thiaminase-containing foods can experience thiamin deficiency related illnesses[8;9;11;16]. Currently problems exist regarding early mortality syndrome (EMS) which occurs in the offspring of large predatory fish (coho salmon, chinook salmon, steelhead trout, brown trout, lake trout and walleye) of the Laurentian Great Lakes and the New York Finger lakes[17]. A similar ailment in Atlantic salmon, called M74, occurs in the Baltic Sea. These maladies have been associated with low egg thiamin concentrations and are manifested clinically as a loss of equilibrium, a spiral swimming pattern, lethargy, hyperexcitability, hemorrhage, and death between hatch and first feeding[21]. This thiamin deficiency occurs as a result of predatory fish consuming forage fish, such as non-native alewife, that often contain high levels of thiaminase[1–4;12;13;17;18;25;27;28;30;31].

Two types of thiaminase activity have been identified. Type I catalyzes the degradation of thiamin by replacing the thiazole moiety with a variety of nucleophiles, while type II is specific for the use of water as the co-substrate (Scheme I).

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Both types operate by a ping-pong kinetic mechanism, but type I is thought to be solely responsible for the cited thiamin deficiencies. Thiaminase I from *Bacillus thiaminolyticus* has been cloned, over-expressed and its mechanism has been studied in detail[7;19;20;24;29]. The enzyme functions as a 42-kDa monomer and has been structurally characterized[5;6]. In contrast to thiaminase II, which is likely to play a role in thiamin salvage, the physiological role of thiaminase I is still unknown after decades of study.

A 30-year old quantitative radiochemical assay for thiaminase I activity in complex samples [10;22] was recently optimized due to the need to further understand the serious environmental issues surrounding thiamin degradation[31]. This assay uses ^{14}C -labeled thiamin and therefore requires a sophisticated laboratory environment for its implementation. In this paper, we describe an alternative method for measuring thiaminase activity in complex samples. This new assay is based on the selective consumption of the highly chromophoric 4-nitrothiophenolate (Scheme II) by thiaminase I, which is able to utilize a variety of nucleophiles as co-substrates. This assay is sensitive and uses readily available chemicals and a visible region spectrophotometer. In addition, the assay can be easily performed in a high throughput fashion, in either 96-well or 384-well plates.

Materials and Methods

Recombinant thiaminase I

B. thiaminolyticus thiaminase I was over-expressed and purified as previously described[7]. After purification, the enzyme was buffer exchanged into 50 mM phosphate pH 7.2 @ RT, 100 mM NaCl and 2 mM DTT in 20% glycerol using a 10 DG column purchased from Bio-Rad (Hercules, CA). The protein was flash frozen in liquid nitrogen and stored at -80°C until use. *B. subtilis* thiaminase II (Ten A) was over-expressed and purified as previously described [26].

Chemicals and reagents

Unless otherwise specified all buffers, salts and chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest purity offered. The 4-nitrothiophenol used in this study was purchased from Sigma-Aldrich and was technical grade (> 80% pure). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Soltec Ventures (Beverly, MA).

Equipment

A Hitachi (Berkshire, UK) U-2010 UV/visible spectrophotometer was used to characterize the spectral changes during the enzymatic reaction. A model SF-2004 stopped-flow apparatus (KinTek Corporation, Austin, TX) was used to measure the steady state kinetic parameters of the recombinant enzyme. Complex biological samples were pulverized in a SPEX SamplePrep freezer mill model 6850 (SPEX CertiPrep, Metuchen, NJ). Absorbance measurements on complex samples were performed in 96-well plates (plate model #655801 (Greiner)) purchased from Omega Scientific, Inc (Tarzana, CA) in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT).

Steady-state measurement on the recombinant enzyme

On the day of any experiment in which 4-nitrothiophenol was used, a fresh stock solution was made by dissolving it in dimethyl sulfoxide (DMSO) to a final concentration of 20–200 mM. The reaction conditions under which the steady state parameters of the recombinant enzyme were measured were as follow: 20 nM thiaminase I (as judged by Bradford assay), 50 mM phosphate (Na^+) pH 7.2 @ RT, 100 mM NaCl, 2 mM TCEP and variable substrate

concentrations as described in the main text. The decrease in absorbance at 411 nm was recorded during the initial linear phase of the reaction using a stopped flow apparatus. Each rate is the result of an average of at least 5 separate mixing events. The absorbance signal was converted to concentration using an extinction coefficient of $13,650 \text{ M}^{-1}\text{cm}^{-1}$, which was estimated from the absorbance of a known concentration of 4-nitrothiophenolate at 411 nm under the same buffer conditions.

Evaluation of 4-nitrothiophenolate as a co-substrate for thiaminase II

B. subtilis thiaminase II was assayed for activity using 4-nitrothiophenolate as a potential co-substrate under the following conditions: 1 μM thiaminase II (as judged by Bradford assay), 50 mM phosphate (Na^+) pH 7.2 @ RT, 100 mM NaCl, 2 mM TCEP, 400 μM thiamin and 80 μM 4-nitrothiophenolate. The reaction was initiated the addition of enzyme and was monitored for 3 hours at room temperature.

Preparation of complex samples

Whole gizzard shad and alewife from aquatic ecosystems (north-central US) of high (gizzard shad from “pond 6S”), moderate (alewife from Cayuga lake), presumably low (alewife from Cayuta lake) and unknown (alewife from Canadarago lake) thiaminase I levels were a gift from Dr. Clifford E. Kraft (Department of Natural Resources, Cornell University). Upon receipt, the frozen samples were stored at -80°C until pulverization. Prior to and leading up to pulverization, the samples were kept in a cooler filled approximately halfway with finely ground dry ice. Samples (12–18 g fish) were cut into approximately 3 gram segments with diagonal cutting pliers until the entire fish was inside the polycarbonate sample container. The tube was closed and inserted into the freezer mill and submerged in liquid nitrogen. The grinding was preformed in cycles according to the following protocol: 1–10 minute pre-cool followed by 3–2 minute grinding cycles with 2 minute interruptions in between each grinding cycle to allow for re-cooling of the samples. When grinding was completed the samples were quickly taken out of the tube and transferred into 50 mL plastic screw cap tubes and stored at -80°C until further use. The resulting biological material was a fine gray powder of uniform consistency.

Measurement of thiaminase I activity in complex samples

On the day of an experiment approximately 0.25–0.5 grams of finely ground biological material was taken out and placed into a pre-weighed 15 mL screw cap tube. A volume of 5 mLs buffer per gram of biological material was added to each sample tube and placed on wet ice. The buffer was pre-chilled and consisted of 50 mM phosphate (pH 7.2 @ RT), 100 mM NaCl and 10 mM TCEP. Three rounds of vortexing (~10 seconds each at maximum speed) were performed in intervals separated by 1 min incubations on ice. After all samples were vortexed, they were transferred into 1.5 mL Eppendorf tubes and centrifuged at 17,200 RCF @ 4°C for 20 min. At this time the supernatant was assayed for thiaminase I activity directly or diluted using the same buffer and assayed as described in the main text. The assay cocktail for the complex samples consisted of (final concentrations during reaction): 50 mM phosphate, 100 mM NaCl, 10 mM TCEP, 400 μM thiamin and 80 μM 4-nitrothiophenol (pH 7.2 @ RT). To start the reaction 50 μL s of sample supernatant (or dilution of the sample supernatant) was added to 250 μL s of assay cocktail (in a 96 well plate) and the absorbance @ 411 nm was recorded as a function of time using the microplate reader as described above. To convert the absorbance measurement to a 4-nitrothiophenolate concentration, the absorbance value was multiplied by 91.7, which normalized the initial “Y-value” to approximately 80 μM . The number of 91.7 was determined by measuring the absorbance of samples (at 411 nm) containing all of the assay cocktail components minus the 50 μL s of complex biological mixture, but plus 50 μL s of buffer in its place (the complex samples tend to increase the absorbance at 411 nm

slightly). Furthermore, because the decrease in absorbance at 411 nm in complex samples lacking thiamin was determined to not be a consequence of thiaminase I activity, wells containing everything except thiamin were run for each dilution of the complex mixture so that the raw data could be corrected by calculating the difference in the raw data. It was observed that the background decrease in absorbance at 411 nm varies with dilution of the sample, therefore, it is particularly important to run a “– thiamin” sample under identical conditions to allow for reliable data correction.

Data analysis

All data fitting by linear and non-linear regression was performed in the computer program GraFit 5 (Erithacus Software Ltd., Horley, Surrey, UK) by the least squares method. All other data normalization and manipulation was done in Microsoft Excel (Redmond, WA). Steady state parameters were obtained by fitting the observed rate as a function of concentration by nonlinear regression to the following Michaelis-Menten equation.

$$k_{obs} = \frac{k_{cat} \cdot [S]}{K_m + [S]}$$

Results

4-nitrothiophenolate as a substrate for thiaminase I

As thiaminase I is able to utilize a variety of nucleophiles as co-substrates, we tested the enzyme with the highly chromophoric 4-nitrothiophenolate with the expectation that there would be a large change in the extinction coefficient upon formation of product (Scheme II). The λ_{max} of the 4-nitrothiophenolate anion, under the buffer conditions used during this study, was 411 nm with an extinction coefficient of approximately $13,650 \text{ M}^{-1}\text{cm}^{-1}$. Shown in Figure 1a. are UV/visible scans taken as a function of time under the following conditions: 300 pM thiaminase I, 800 μM thiamin, 100 μM 4-nitrothiophenolate, 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 100 mM NaCl buffered in 50 mM phosphate (pH 7.2 @ RT). There is a large change in absorbance that occurs as the reaction proceeds; the absorbance initially present in the visible region of the spectrum drops to less than 15% of the original value (estimated as the sum of absorbance $> 400 \text{ nm}$). Furthermore, the product absorbs predominantly in the ultraviolet region. There is an isosbestic point centered at 362 nm. The absorbance change is sufficiently large that the reaction can easily be followed in real-time, by eye. Figure 1b. shows that the total drop in absorbance is about 1.2 units at 411 nm under the reaction conditions.

Kinetic characterization of product formation

The steady state kinetic parameters were obtained by measuring the initial linear decrease in absorbance at 411 nm as a function of time in a stopped-flow apparatus (see Materials and Methods section). Figure 2a. shows the change in the steady state rate of consumption of 4-nitrothiophenolate as a function of concentration. For this experiment the thiamin concentration was held constant at 400 μM . The data were fit by nonlinear regression as described in the Materials and Methods section. The nitrothiophenolate anion is a surprisingly good substrate for thiaminase I with a k_{cat} of $297 \pm 5 \text{ s}^{-1}$ and a K_m of $36.4 \pm 1.4 \mu\text{M}$ to yield a specificity constant (k_{cat}/K_m) of $8.38 \pm 1.6 \mu\text{M}^{-1}\text{s}^{-1}$. Another experiment was performed to obtain the steady state kinetic parameters for thiamin in the presence of a fixed concentration of 4-nitrothiophenolate (80 μM) and the results are shown in Figure 2b. A k_{cat} of $260 \pm 10 \text{ s}^{-1}$ and a K_m of $21.1 \pm 1.3 \mu\text{M}$ were determined for thiamin to define a specificity constant of $12.4 \pm 1.0 \mu\text{M}^{-1}\text{s}^{-1}$. The k_{cat} for thiamin is lower than the one obtained for the nitrothiophenolate because the experiment in which the thiamin concentration was varied was done with a slightly

less than saturating concentration of 4-nitrothiophenolate. These data show that thiaminase I can efficiently and rapidly use 4-nitrothiophenolate as a substrate.

Examination of 4-nitrothiophenolate as a co-substrate for thiaminase II

An experiment was performed with *Bacillus subtilis* thiaminase II in order to inspect the possibility that 4-nitrothiophenol can function as a co-substrate. Thiaminase II (1 μ M) was mixed with thiamin (400 μ M) and 4-nitrothiophenolate (80 μ M) and allowed to incubate at room temperature for three hours. Under these conditions no significant depletion of 4-nitrothiophenolate was observed.

4-nitrothiophenolate as a substrate for native thiaminase I in a complex biological sample

In order to test whether the 4-nitrothiophenolate would function as a substrate for thiaminase I in a complex biological mixture derived from native sources, we obtained freshwater fish samples which were known to contain measurable amounts of thiaminase I (measurable by the radiochemical assay). The samples were prepared in a manner similar to that of previous methods[31]. The final assay cocktail contained nearly saturating concentrations of both substrates (80 μ M 4-nitrothiophenolate and 400 μ M thiamin). Critical to success of the assay was the inclusion of a non-nucleophilic reducing agent (10 mM TCEP), which prevents the non-enzymatic oxidation of the 4-nitrothiophenolate. Thiaminase I is also sensitive to oxidation, presumably because of its essential active site cysteine residue[7], and TCEP extends its catalytic lifetime (unpublished results).

A time course for 4-nitrothiophenolate consumption in a 72-fold dilution (of the original solid sample W:V) of the complex biological mixtures was run and is shown in Figure 3a (labeled “+ thiamin”). The activity was measured in a 96-well plate by monitoring the changes in absorbance as a function of time using a microplate reader. As evidenced in the “– thiamin” control, this experiment clearly shows that it is important to include such a measurement performed under identical conditions to ensure that there is a quantifiable difference in the rate of decrease in absorbance between the two samples. A slower rate of consumption of the 4-nitrothiophenolate was always detected relative to the sample containing thiamin, but is due to unknown causes. Further experiments were performed that clearly exclude the possibility that the background decrease is due to endogenous thiamin or other potential thiaminase I substrates present in the tissue samples. This was accomplished by adding purified recombinant thiaminase I to the “– thiamin” sample (1.6 μ M final concentration). No change in the rate of absorbance decrease at 411 nm was observed (data not shown). It seemed logical that another possible route to the decrease in absorbance could be due to various possible reactions catalyzed by transition metals present in the complex samples. Therefore, the assay was performed again, but instead, a final concentration of 10 mM EDTA was included and the results are also shown in Figure 3a (see “+ EDTA”). There was a small, but significant, change upon addition of EDTA to the assay cocktail. However, because the relative change was nearly identical when one compares the “+ thiamin” and “– thiamin” data, the assay was essentially unaffected overall. Unexpectedly EDTA increased the rate of 4-nitrothiophenolate consumption in the “blank” sample where buffer was used in place of the tissue supernatant. It seems likely that the addition of EDTA to the assay cocktail may have a greater consequence if the samples are likely to contain higher concentrations of transition metals than the ones used here. Therefore, in those cases it may be advisable to characterize the effect of metal chelation.

Sensitivity of thiaminase I assay

The thiaminase I activity in four fish samples, with different amounts of native thiaminase I, are shown in Figure 3b. The plot is presented in double log format to better illustrate the relative magnitudes of the rates of 4-nitrothiophenolate consumption as a function of sample dilution. The assays were run in duplicate for 60 minutes and the decrease in absorbance for all samples

was linear in this time window after correcting for the decrease seen in the “– thiamin” control wells. The linear decrease in absorbance was converted into concentration as described in the Materials and Methods section and fit by linear regression to obtain the activity of thiaminase I in units of $\mu\text{M}/\text{min}$. The predicted relative amounts of thiaminase I present in the samples using this assay agreed well with the expected levels and the relationship of the activities remained intact up to a dilution of approximately 1000-fold, easily sensitive enough for a reasonable dynamic range.

Discussion

In order to understand and control thiaminase I catalyzed degradation of thiamin in nature, it will be necessary to know the distribution, seasonal variation and persistence of this enzyme in a variety of environments. In particular, the elucidation of the factors influencing the amount of thiaminase in forage fish will benefit from a robust and facile assay that can be carried out with simple equipment. The assay described in this paper is a step in this direction, requiring only readily available reagents and a simple UV-visible spectrometer. In addition, this assay is specific for thiaminase I. The radioactive assay reports on an undefined combination of thiaminase I and II activity. This difference is well worth noting because only thiaminase I has been documented to be toxic, while thiaminase II likely plays a role in thiamin salvage in bacteria. Eventually, this simplified thiaminase I assay may also aid in the elucidation of the true biological function of this toxic enzyme.

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Reference List

1. Brown SB, Arts MT, Brown LR, Brown M, Moore K, Villella M, Fitzsimons JD, Honeyfield DC, Tillitt DE, Zajicek JL, Wolgamood M, Hnath JG. Can diet-dependent factors help explain fish-to-fish variation in thiamine-dependent early mortality syndrome? *Journal of Aquatic Animal Health* 2005;17:36–47.
2. Brown SB, Brown LR, Brown M, Moore K, Villella M, Fitzsimons JD, Williston B, Honeyfield DC, Hinterkopf JP, Tillitt DE, Zajicek JL, Wolgamood M. Effectiveness of egg immersion in aqueous solutions of thiamine and thiamine analogs for reducing early mortality syndrome. *Journal of Aquatic Animal Health* 2005;17:106–112.
3. Brown SB, Fitzsimons JD, Honeyfield DC, Tillitt DE. Implications of thiamine deficiency in Great Lakes Salmonines. *Journal of Aquatic Animal Health* 2005;17:113–124.
4. Brown SB, Honeyfield DC, Hnath JG, Wolgamood M, Marcquenski SV, Fitzsimons JD, Tillitt DE. Thiamine status in adult salmonines in the great lakes. *Journal of Aquatic Animal Health* 2005;17:59–64.
5. Campobasso N, Begun J, Costello CA, Begley TP, Ealick SE. Crystallization and preliminary X-ray analysis of thiaminase I from *Bacillus thiaminolyticus*: space group change upon freezing of crystals. *Acta Crystallographica Section D-Biological Crystallography* 1998;54:448–450.
6. Campobasso N, Costello CA, Kinsland C, Begley TP, Ealick SE. Crystal structure of thiaminase-I from *Bacillus thiaminolyticus* at 2.0 angstrom resolution. *Biochemistry* 1998;37:15981–15989. [PubMed: 9843405]
7. Costello CA, Kelleher NL, Abe M, McLafferty FW, Begley TP. Mechanistic studies on thiaminase I - Overexpression and identification of the active site nucleophile. *Journal of Biological Chemistry* 1996;271:3445–3452. [PubMed: 8631946]
8. Duffy P, Morris H, Neilson G. Thiamin status of a Melanesian population. *Am J Clin Nutr* 1981;34:1584–1592. [PubMed: 7270482]

9. Earl JW, McCleary BV. Mystery of the poisoned expedition. *Nature* 1994;368:683–684. [PubMed: 8152477]
10. Edwin EE, Jackman R. A rapid radioactive method for determination of thiaminase activity and its use in the diagnosis of cerebrocortical necrosis in sheep and cattle. *JSci Food Agric* 1974;25:357–368. [PubMed: 4857780]
11. Evans WC. Thiaminases and their effects on animals. *Vitam Horm* 1975;33:467–504. [PubMed: 779253]
12. Fitzsimons JD, Williston B, Amcoff P, Balk L, Pecor C, Ketola HG, Hinterkopf JP, Honeyfield DC. The effect of thiamine injection on upstream migration, survival, and thiamine status of putative thiamine-deficient Coho salmon. *Journal of Aquatic Animal Health* 2005;17:48–58.
13. Fitzsimons JD, Williston B, Zajicek JL, Tillitt DE, Brown SB, Brown LR, Honeyfield DC, Warner DM, Rudstam LG, Pearsall W. Thiamine content and thiaminase activity of ten freshwater stocks and one marine stock of alewives. *Journal of Aquatic Animal Health* 2005;17:26–35.
14. FUJITA A. Thiaminase. *Adv Enzymol Relat Subj Biochem* 1954;15:389–421. [PubMed: 13158184]
15. FUJITA A. Recollections of the background of the study on thiaminase and thermostable thiamine-decomposing factor in Japan. *JVitaminol (Kyoto)* 1972;18:67–72. [PubMed: 4556266]
16. HAYASHI R. Bacterial synthesis and destruction of thiamine. *Nutr Rev* 1957;15:65–67. [PubMed: 13419184]
17. Honeyfield DC, Brown SB, Fitzsimons JD, Tillitt DE. Early mortality syndrome in great lakes salmonines. *Journal of Aquatic Animal Health* 2005;17:1–3.
18. Honeyfield DC, Hinterkopf JP, Fitzsimons JD, Tillitt DE, Zajicek JL, Brown SB. Development of thiamine deficiencies and early mortality syndrome in lake trout by feeding experimental and feral fish diets containing thiaminase. *Journal of Aquatic Animal Health* 2005;17:4–12.
19. Kelleher NL, Costello CA, Begley TP, McLafferty FW. Thiaminase-I (42-Kda) Heterogeneity, Sequence Refinement, and Active-Site Location from High-Resolution Tandem Mass-Spectrometry. *Journal of the American Society for Mass Spectrometry* 1995;6:981–984.
20. Kelleher NL, Nicewonger RB, Begley TP, McLafferty FW. Identification of modification sites in large biomolecules by stable isotope labeling and tandem high resolution mass spectrometry - The active site nucleophile of thiaminase I. *Journal of Biological Chemistry* 1997;272:32215–32220. [PubMed: 9405424]
21. Marquenski, SV.; Brown, SB. Early mortality syndrome in the Great Lakes. In: Rolland, RM.; Gilbertson, M.; Peterson, RE., editors. *Chemically Induced Alterations in Functional Development and Reproduction in Fishes*. SETAC; Pensacola, FL: 1997. p. 135-152.
22. McCleary BV, Chick BF. Purification and Properties of A Thiaminase I Enzyme from Nardoo (*Marsilea-Drummondii*). *Phytochemistry* 1977;16:207–213.
23. Murata K. Actions of two types of thiaminase on thiamin and its analogues. *Ann NYAcad Sci* 1982;378:146–156.
24. Nicewonger R, Rammelsberg A, Costello CA, Begley TP. Mechanistic studies on thiaminase I.1. The stereochemical course of the reaction. *Bioorganic Chemistry* 1995;23:512–518.
25. Tillitt DE, Zajicek JL, Brown SB, Brown LR, Fitzsimons JD, Honeyfield DC, Holey ME, Wright GM. Thiamine and thiaminase status in forage fish of salmonines from Lake Michigan. *Journal of Aquatic Animal Health* 2005;17:13–25.
26. Toms AV, Haas AL, Park JH, Begley TP, Ealick SE. Structural characterization of the regulatory proteins TenA and TenI from *Bacillus subtilis* and identification of TenA as a thiaminase II. *Biochemistry* 2005;44:2319–2329. [PubMed: 15709744]
27. Wolgamood M, Hnath JG, Brown SB, Moore K, Marcquenski SV, Honeyfield DC, Hinterkopf JP, Fitzsimons JD, Tillitt DE. Temporal and spatial variation of early mortality syndrome in Salmonids from lakes Michigan and Huron. *Journal of Aquatic Animal Health* 2005;17:65–76.
28. Wright GM, Brown SB, Brown LR, Moore K, Villella M, Zajicek JL, Tillitt DE, Fitzsimons JD, Honeyfield DC. Effect of sample handling on thiamine and thiaminolytic activity in alewife. *Journal of Aquatic Animal Health* 2005;17:77–81.
29. Wu M, Papish ET, Begley TP. Mechanistic studies on thiaminase I: Identification of the product of thiamin degradation in the absence of the nucleophilic cosubstrate. *Bioorganic Chemistry* 2000;28:45–48.

30. Zajicek JL, Tillitt DE, Brown SB, Brown LR, Honeyfield DC, Fitzsimons JD. A rapid solid-phase extraction fluorometric method for thiamine and riboflavin in salmonid eggs. *Journal of Aquatic Animal Health* 2005;17:95–105.
31. Zajicek JL, Tillitt DE, Honeyfield DC, Brown SB, Fitzsimons JD. Method for measuring total thiaminase activity in fish tissues. *Journal of Aquatic Animal Health* 2005;17:82–94.

Figure 1. Time dependent changes in the UV/visible spectrum of the thiaminase reaction mixture
(a) An assay reaction was performed under steady-state conditions and the changes in the UV/visible spectrum were monitored as a function of time over a period of 30 minutes. The arrows indicate the directionality of the absorbance changes as the reaction proceeded. The reaction was performed with 300 pM thiaminase I, 800 μM thiamin and 100 μM 4-nitrothiophenolate.
(b) Absorbance value at 411 nm plotted as a function of time showing a total change of approximately 1.2 absorbance units.

Figure 2. Steady state kinetic parameters

(a) The initial rate of 4-nitrothiophenolate consumption is plotted as a function of 4-nitrothiophenolate concentration. Error bars are shown, but are obstructed by the data points. The experiment was performed with a constant concentration of thiamin equal to 400 μM. A fit of the data by nonlinear regression to the Michaelis-Menten equation provided a k_{cat} of $297 \pm 5 \text{ s}^{-1}$, a K_m of $36.4 \pm 1.4 \text{ μM}$ and a specificity constant (k_{cat}/K_m) of $8.38 \pm 1.6 \text{ μM}^{-1}\text{s}^{-1}$ for 4-nitrothiophenolate. (b) The initial rate of 4-nitrothiophenolate consumption is plotted as a function of thiamin concentration. A k_{cat} of $260 \pm 10 \text{ s}^{-1}$, a K_m of $21.1 \pm 1.3 \text{ μM}$ and a specificity constant of $12.4 \pm 1.0 \text{ μM}^{-1}\text{s}^{-1}$ were defined for thiamin. The experiment was run in the presence of a constant concentration of 4-nitrothiophenolate equal to 80 μM.

Figure 3. Thiaminase I activity in complex biological samples

(a) Shown is a complete time course for 4-nitrothiophenolate consumption by a 72-fold diluted (of original solid sample W:V) complex biological sample known to contain measurable thiaminase I activity. A total of six samples were run in triplicate as depicted directly on the plot to show that the decrease in the concentration of 4-nitrothiophenolate is largely due to thiaminase I activity and that there is a small change upon the addition of a final concentration of 10 mM EDTA. (b) Four fish samples were tested which originated from sources of differing levels of thiaminase I activity: high ($\bullet$), moderate ($\circ$), low ($\blacksquare$), and unknown ($\triangle$). The linearity of activity dilution was also tested and depicted above as a double log scale of activity in $\mu\text{M}/$

min consumption of 4-nitrothiophenolate versus fold-dilution of the original sample material. The relative activities of the four samples remained intact to nearly a 1000-fold dilution.

Scheme I.

Scheme II.