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Comparison of thiaminase activity in fish using the radiometric and 4-nitrothiophenol colorimetric methods

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ABSTRACT

Thiaminase induced thiamine deficiency occurs in fish, humans, livestock and wild animals. A non-radioactive thiaminase assay was described in 2007, but a direct comparison with the radioactive ^{14}C-thiamine method which has been in use for more than 30 years has not been reported. The objective was to measure thiaminase activity in forage fish (alewife *Alosa pseudoharengus*, rainbow smelt *Osmerus mordax*, and slimy sculpin *Cottus cognatus*) consumed by predators that manifest thiamine deficiency using both methods. Modifications were made to the colorimetric assay to improve repeatability. Modification included a change in assay pH, enhanced sample clean-up, constant assay temperature (37 °C), increase in the concentration of 4-nitrothiophenol (4NTP) and use of a spectrophotometer fitted with a 0.2 cm cell. A strong relationship between the two assays was found for 51 alewife ($R^2 = 0.85$), 36 smelt ($R^2 = 0.87$) and 20 sculpin ($R^2 = 0.82$). Thiaminase activity in the colorimetric assay was about 1000 times higher than activity measured by the radioactive method. Application of the assay to fish species from which no thiaminase activity has previously been reported resulted in no 4NTP thiaminase activity being found in bloater *Coregonus hoyi*, lake trout *Salvelinus namaycusch*, steelhead trout *Oncorhynchus mykiss* or Chinook salmon *Oncorhynchus tshawytscha*. In species previously reported to contain thiaminase, 4NTP thiaminase activity was measured in bacteria *Paenibacillus thiaminolyticus*, gizzard shad *Dorosoma cepedianum*, bracken fern *Pteridium aquilinum*, quagga mussel *Dreissena bugensis* and zebra mussels *D. polymorpha*.

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Introduction

Thiamine (vitamin B_1) is an essential dietary ingredient for animals, including humans. Insufficient uptake of dietary thiamine can lead to morbidity and mortality. Despite our understanding of the importance of thiamine in metabolic processes, deficiencies of this vitamin continue to be reported in humans (Ahoua et al., 2007; Nishimune et al., 2000), domestic livestock (Edwin and Jackman, 1974; Ramos et al., 2003, 2005) and aquatic animals (Honeyfield et al., 2005, 2008). Although low dietary intake can result in thiamine deficiency, in many reports thiamine deficiency is not a result of low dietary thiamine but rather the presence of a thiamine degrading enzyme, thiaminase.

Thiaminase is found in nature in two forms (thiaminase I and II) but thiamine deficiency appears to only be linked to thiaminase I (enzyme number 2.5.1.2). No experimental data or anecdotal evidence has been published associating thiaminase II with thiamine deficiency in any species. Thiaminase I catalyzes a base substitution reaction (Fig. 1). During the reaction the thiazole moiety of thiamine is replaced by several possible Lewis bases or nucleophiles. Aniline, pyridine and naturally occurring compounds, such as nicotinic acid are example nucleophiles (Fujita et al., 1952a,b; Fujita, 1954). Thiaminase II only utilizes water as the nucleophile in the reaction. The biological function of thiaminase II has recently been shown to be involved in a salvage pathway leading to the synthesis of thiamine as described by Jenkins et al. (2007) and summarized by Bettendorff (2007). Thiaminase II (referred to as "TenA", "transcriptional enhancer A") salvages pyrimidine from ring-opened thiamine thiazole within the thiamine synthesis pathway found in some bacteria and yeast (Jenkins et al., 2007; Toms et al., 2005). Besides the observations that thiaminase I degrades thiamine, no other biological function has been identified for this enzyme to date.

Thiaminase activity can be detected 1) in bacteria growing on Petri plates with a soft-agar overlay (Abe et al., 1986), 2) by measuring thiamine disappearance (Harris et al., 1951) or 3) by measuring the liberation of ^{14}C-thiazole from radioactive labeled thiamine (Edwin and Jackman, 1974; McCleary and Chick, 1977; Zajicek et al., 2005). For more than 30 years, the radiometric assay has been the preferred method but its use is limited to laboratories licensed to use radioactive

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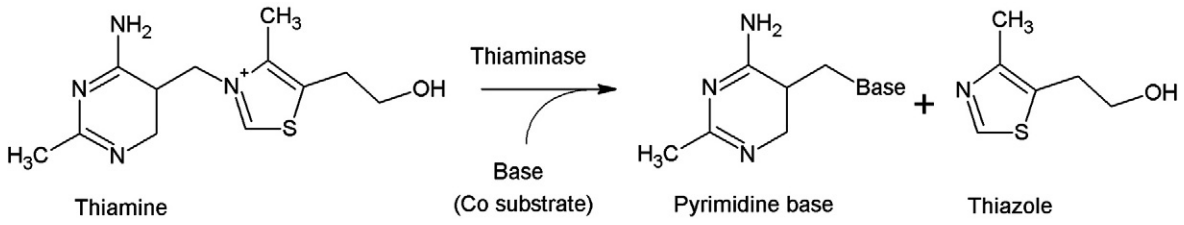

Fig. 1. Chemical base substitution reaction of thiaminase I. For type I thiaminase activity the Lewis base substituted in the reaction can include but is not limited to pyridine, aniline, 4-nitrothiophenol and naturally occurring compounds, such as nicotinic acid. For type II thiaminase (TenA), the only base or nucleophile is water.

material. Furthermore, ^{14}C-thiamine is only commercially available through costly custom synthesis. Recently a novel colorimetric thiaminase assay was reported that has the potential to overcome the limitations stated above (Hanes et al., 2007). This colorimetric assay measures the disappearance of 4-nitrothiophenol (4NTP) absorbance at 411 nm. In the assay, 4NTP serves as the nucleophile rather than aniline (Agee et al., 1973), pyridine (Anglesea and Jackson, 1985) and nicotinic acid (Edwin and Jackman, 1974; Zajicek et al., 2005) used in the radiometric assay. Much of the recently published thiaminase data associated with investigating causes of fish mortality from thiamine deficiency was generated using the radiometric assay with nicotinic acid; therefore it is important to determine the relationship between the 4NTP colorimetric and radiometric assays. The work we report herein measured thiaminase activity in alewife, rainbow smelt, sculpins and round goby using both the radiometric and colorimetric assays. The objective was to provide a repeatable non-radiometric thiaminase assay that is at least as sensitive and reliable as the radiometric assay.

Methods

Lake Ontario prey fish (51 alewife *Alosa pseudoharengus*, 36 rainbow smelt *Osmerus mordax*, 20 slimy sculpin *Cottus cognatus* and 24 round goby *Neogobius melanostomus*) were captured using trawl-nets in 2006 and 2007. Trawl-netting was conducted offshore near Toronto, Canada and along the New York coastline from Olcott to Oswego. Only live, fresh-caught fish were selected and immediately frozen and stored at −80 °C, after which these samples were not allowed to thaw until assayed for thiaminase activity. Individual frozen fish were pulverized in dry ice as described in Zajicek et al. (2005). Once pulverized, the dry ice in the samples was allowed to totally sublimate (~24–48 h at -80 °C) before pulverized samples were analyzed for thiaminase.

Thiaminase activity

The radiometric thiaminase assay was conducted according to Zajicek et al. (2005) using nicotinic acid as co-substrate. At least two subsamples of pulverized sample from each fish or biological material were analyzed in duplicate. Duplicate analysis of samples was conducted independently in time. Preliminary analyses using the colorimetric thiaminase method of Hanes et al. (2007) resulted in day-to-day variation of thiaminase activity from the same sample. This variation was considered too large for effectively comparing activity among samples, even though on any given day the relative activity among the samples produced the same relative relationship as activity measures from the radiometric assay. Therefore, subsequent modifications were implemented that substantially reduced this day-to-day variation.

Unless otherwise specified all buffer reagents, salts and chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest purity offered. The 4-nitrothiophenol was purchased from Sigma-Aldrich as technical grade (>80% pure). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was obtained from Soltec Ventures (Beverly, MA). Freshly prepared TCEP buffer was degassed using helium sparge for 10–15 min and chilled on ice. The TCEP buffer consisted of 50 mM phosphate (adjusted to pH 6.9 at room temperature), 100 mM NaCl and 10 mM TCEP. On the day of an experiment approximately 0.25–0.5 g of finely ground biological material was taken out of the freezer and placed into a pre-weighed 15 mL screw cap tube. A volume of 5 mL TCEP buffer per gram of biological material was added to each sample tube and placed on wet ice. Three rounds of vortexing (~10 seconds each at maximum speed) were performed in intervals separated by 1 min incubations on ice. Samples were then centrifuged at 17,200×*g* relative centrifugal force (RCF) at 4 °C for 20 min. The supernatants were transferred into 2 mL Pierce Centrifuge Columns (~30 μm pore size, Thermo Scientific, Rockford, Il) and centrifuged at 1500×*g* RCF for 5–10 min to remove any residual particulate matter. The supernatant was then assayed for thiaminase I activity directly or diluted using the TCEP buffer. Two assay cocktails were prepared for the measurement of thiaminase in complex biological samples. Cocktail A consisted of (final concentrations during reaction): 50 mM phosphate, 100 mM NaCl, 10 mM TCEP, 400 μM thiamine and 200 μM 4-nitrothiophenol (pH 6.9 adjusted at room temperature). Cocktail B contained 50 mM phosphate, 100 mM NaCl, 10 mM TCEP, 200 μM 4-nitrothiophenol and no thiamine. For each sample four glass test tubes (16×100 mm) were placed in water bath preheated to 37 °C. Assay cocktail A (2.9 mL) was pipetted into two tubes and cocktail B (2.9 mL) was pipetted into the remaining two tubes. To start the assay, 0.1 mL sample extract being analyzed for thiaminase was pipetted into each of the four tubes. The two tubes containing cocktail B served as sample blanks. Filling of test tubes with cocktails A and B, and sample extract being analyzed for thiaminase (0.1 mL) were spaced in time so that the absorbance of each sample was read exactly 60 min from the time the reaction was initiated by the addition of thiaminase. Absorbance was read at 411 nm in a cuvette with light path length of 0.2 cm. To convert the absorbance measurement to a 4-nitrothiophenolate concentration, the absorbance value was first divided by the light path length. The resulting value was divided by the extinction coefficient of 4NTP (13,650 M^{-1} cm^{-1}) as determined for TCEP buffer by Hanes et al. (2007). Furthermore, because an observed decrease in absorbance at 411 nm in complex samples lacking thiamine was determined to not be a consequence of thiaminase I activity, tubes containing all constituents except thiamine were run for each dilution of the complex mixture so that the raw data could be corrected by calculating the difference in the absorbance with and without thiamine. It was observed that the background decrease in absorbance at 411 nm varied with dilution of the sample; therefore, it is particularly important to run a control without thiamine under identical conditions to allow for reliable data correction.

Reactivity of co-substrates

In the thiaminase I reaction, a co-substrate (nucleophile) displaces the thiazole portion of the thiamine molecule. The relative reactivity of several co-substrates was measured using a stopped-flow apparatus (SF-2004, KinTek, Austin, TX) equipped with fluorescence detection capabilities. A large quenching of the intrinsic protein fluorescence