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A New Method for the Determination of Thiaminase Activity by the Use of Quinothiamine and L-Cysteine Ethylester as Substrates

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キノチアミンおよび L-システインエチルエステルを
基質とする新チアミナーゼ活性測定法

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A new method for the determination of transferase activity of thiaminase I and II was proposed. Thiaminase II which has been classified into a hydrolase showed transferase activity by the use of quinothiamine (Pm-quinoline) and L-cysteine ethylester as substrates.

Transferase activity of thiaminase I or II at 37°C was able to be evaluated from the time course of absorbance (315 nm) for the reaction solution prepared by mixing 0.3 ml of 2 mM Pm-quinoline, 0.3 ml of 500 mM L-cysteine ethylester, 2.0 ml of 0.1 M Tris-HCl buffer (pH 8.8), and 0.4 ml of the enzyme solution. The *K*m values for Pm-quinoline and L-cysteine ethylester were determined to be 0.025 and 7.1 mM, respectively, in the case of thiaminase I and 0.094 and 0.9 mM, respectively, in the case of thiaminase II.

INTRODUCTION

Thiaminases are classified into either thiaminase I (EC 2.5.1.2) or thiaminase II (EC 3.5.99.2)[1]. The former catalyzes a base-exchange reaction in which the thiazole moiety of thiamine (the lst substrate) is exchanged with other base compounds (the 2nd substrate), forming 2-methyl-4-amino-5-pyrimidinylmethyl compounds (Pm compound)[2]. The latter catalyzes the hydrolysis of thiamine, forming 2-methyl-4-amino-5-hydroxymethylpyrimidine (OMP)[2]. The transferase activity of thiaminase I and the hydrolase activity of thiaminase II have been assayed by the colorimetric determinations of heteropyrithiamine (HPT) formed in thiamine-pyridine system and substrate remained in thiamine alone system, respectively[3].

The authors have prepared some Pm compounds by using the base-exchange reaction with thiaminase I[4], have examined their bioactive properties to some organisms, and have found several bioactive Pm compounds[5]. However, several enzymes of which substrate specificities are broad or differ from one another were required for the preparation of various kinds of Pm compounds. Suzuki, one of the authors, had found that thiaminase II catalyzes the base-

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exchange reaction of quinothiamine (Pm-quinoline) with L-cysteine ethylester or L-cysteamine, forming the corresponding Pm compounds.[6] This finding suggests that thiaminase II as well as thiaminase I is usable as a catalyst for the preparation of Pm compounds. However, the transferase activity of thiaminase II cannot be measured by the method for thiaminase I described above because of the poor affinity for pyridine being the 2nd substrate. Moreover, a common determination method for either transferase activities was desirable in order to search new strains of microorganisms as the enzyme source.

This paper deals with a new method for the determination of the transferase activities of thiaminase I and II by using Pm-quinoline and L-cysteine ethylester as substrates.

MATERIALS AND METHODS

1. Enzyme preparations and materials

Thiaminase I used was extracted from *Bacillus thiaminolyticus* Matsukawa et Misawa and purified according to the method of Wittliff *et al.*[7] Thiaminase II used was extracted from *Bacillus thiaminolyticus* Kimura et Aoyama and purified according to the method of Ikehata.[8] Each enzyme was homogeneous on polyacrylamide disk gel electrophoresis. Pm-quinoline was prepared according to the enzymatic method described previously.[4] OMP was synthesized according to the method of Takamizawa *et al.*[9] The other chemicals were all reagent grade and used without further purification.

2. Standard assays of transferase and hydrolase activities of thiaminase I and II

The transferase activities of thiaminase I and II were measured by following the change in absorbance at 315 nm with a Hitachi Spectrophotometer, Model 200-20. A typical run is as follows. The mixture (3.0 ml) of 2mM Pm-quinoline (0.3 ml), 500 mM L-cysteine ethylester (0.3 ml), 0.1 M Tris-HCl buffer (pH 8.8, 2.0 ml), and H_2O (0.4 ml) was pipetted into a pair of 1.00-cm quartz cells, one of which was used as a reference cell, and the other, as a sample cell. After thermal equilibrium at 37°C had been reached, 10 μl of an enzyme solution was added to the sample cell and the change in absorbance was followed. As the initial part of absorbance decreased linearly with time, the transferase activity was determined as an initial rate from a slope of the straight line.

Similarly, the hydrolase activity of thiaminase I or II was determined as an initial rate by following the change in absorbance at 315 nm for the same reaction mixture except that H_2O (0.3 ml) was added in place of 500 mM L-cysteine ethylester (0.3 ml).

RESULTS AND DISCUSSION

1. Differential spectrum of reaction mixture

The differential spectrum between initial and final reaction mixtures was measured in order to find the appropriate wavelength for the assay of the transferase activity of thiaminase I or thiaminase II (Fig. 1). Two peaks were observed at 318 and 282 nm. The former was twice as high as the latter and was not subject to interference from various contaminants such as phenols owing to being longer wavelength. The activity was assayed at 315 nm in the present work.

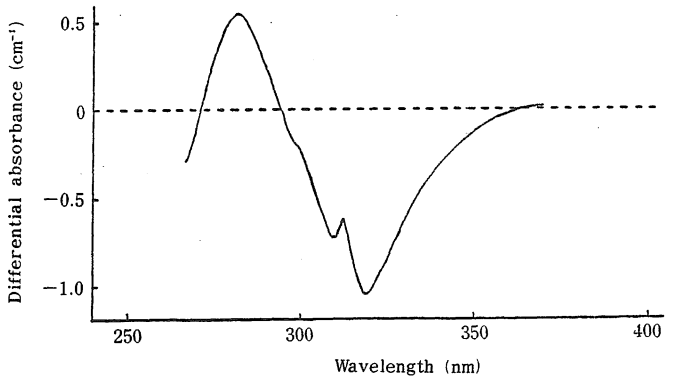

Fig. 1. Differential spectrum of reaction mixture in Tris-HCl buffer solution (pH 8.8) at 37°C

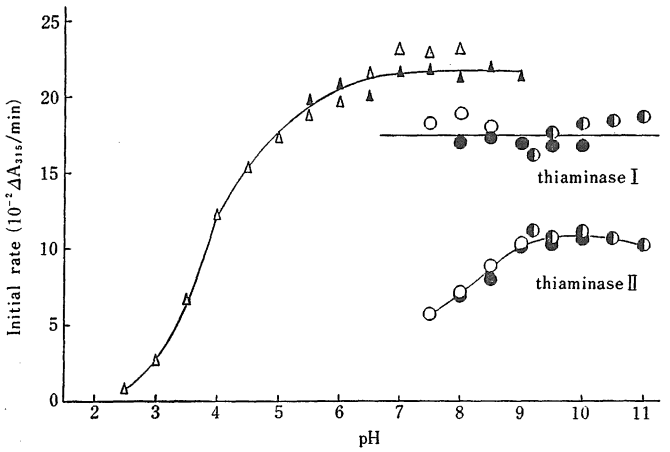

Fig. 2. Effect of pH on the transferase activity of thiaminase I and II

△ : Phosphate-citrate buffer　● : H_3BO_3-KCl-NaOH buffer
▲ : Phosphate buffer　◐ : Na_2CO_3-$Na_2B_4O_7$ buffer
○ : Tris-HCl buffer

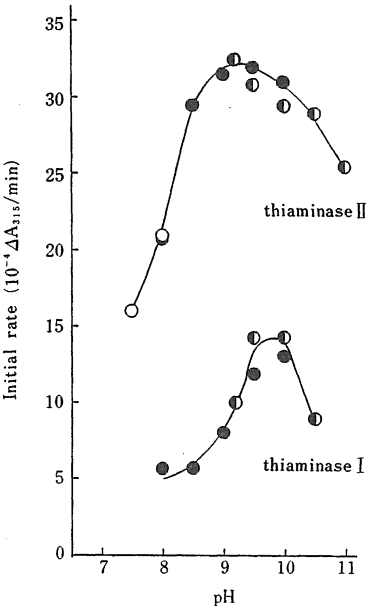

Fig. 3. Effect of pH on the hydrolase activity of thiaminase I and II

2. Optimal pH for transferase and hydrolase activities of thiaminase I and II

Optimal pH for the transferase activity of thiaminase I and II was examined (Fig. 2). Although the maximum rate was alterable with a change in buffer components, the optimal pH for the transferase activity of thiaminase I was 6.5 or above. On the other hand, the optimal pH for the transferase activity of thiaminase II was *ca.* 10. The pH for assay of the transferase activity of thiaminase I and II was decided 8.8 which is in the vicinity of each optimal pH.

The optimal pH for the hydrolase activity of thiaminase I and II was also examined (Fig. 3). The optimal pH for the hydrolase activity was *ca.* 9.7 for thiaminase I and 9.3 for thiaminase II. It is noteworthy that the maximum rate of the transferase activity of thiaminase II was greater than that of its hydrolase activity. As the rate is represented as a differential absorbance (ΔA_{315}) at 315 nm per min, it suggests that the addition of L-cysteine

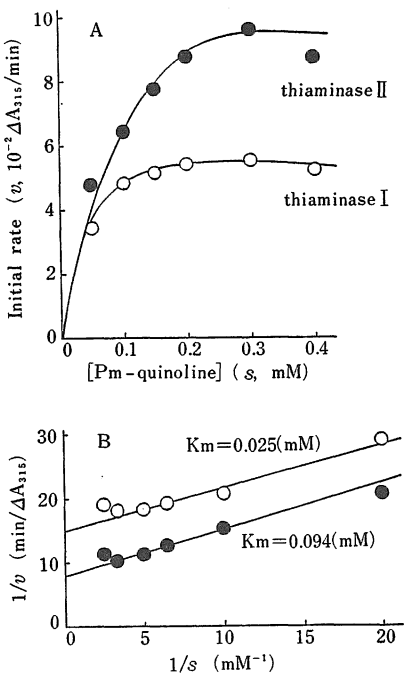

Fig. 4. Effect of concentration of Pm-quinoline on initial rate of transferase activity (A) and Lineweaver-Burk plot (B)

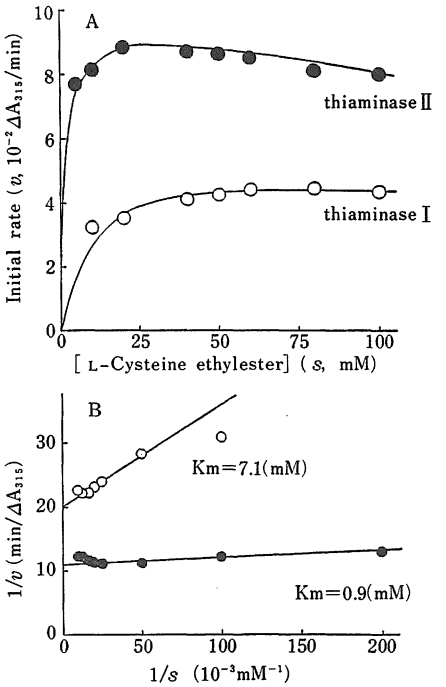

Fig. 5. Effect of concentration of L-cysteine ethylester on initial rate of transferase activity (A) and Lineweaver-Burk Plot (B)

ethylester caused a transfer reaction in preference to a hydrolysis, because the rates in both systems had to agree with each other if a transfer reaction did not proceed by the addition of L-cysteine ethylester.

3. Concentration of Pm-quinoline for assay of transferase activity of thiaminase I and II

The effect of the concentration of Pm-quinoline on the transferase activity of thiaminase I and II was examined in order to decide suitable concentration for the assay (Fig. 4). The rate increased with increasing concentration of Pm-quinoline in each enzyme system and approached nearly a maximum value. However, excess Pm-quinoline caused substrate inhibition. Lineweaver-Burk plot of these data revealed that *K*m's for thiaminase I and II are 0.025 and 0.094 mM, respectively. From these findings, the concentration of Pm-quinoline for assay of transferase activity of thiaminase I and II was decided to be 0.2 mM.

4. Concentration of L-cysteine ethylester for assay of transferase activity of thiaminase I and II

The effect of the concentration of L-cysteine ethylester on the transferase activity of thiaminase I and II was also examined (Fig. 5). The rate increased with increasing concentration of L-cysteine ethylester in each enzyme system and approached nearly a maximum value. However, L-cysteine ethylester as well as Pm-quinoline caused the inhibition by excess concentration of the substrate. Lineweaver-Burk plot of these data revealed that *K*m's for thiaminase I and II are 7.1 and 0.9 mM, respectively. Taking account of the inhibition by excess substrate, 50 mM of L-cysteine ethylester was decided to be appropriate concentra-

0.1 M Tris-HCl buffer (pH 8.8)	2.0 ml
2 mM Pm-quinoline	0.3 ml
500 mM L-Cysteine ethylester	0.3 ml
Enzyme solution	0.4 ml

—Reaction at 37°C

Determination of ΔA_{315}/min

Fig. 6. The procedure for the determination of transferase activity of thiaminase I or II

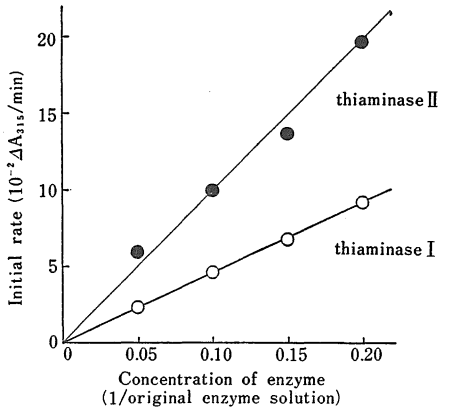

Fig. 7. Efect of concentration of enzyme on initial rate of transferase activity

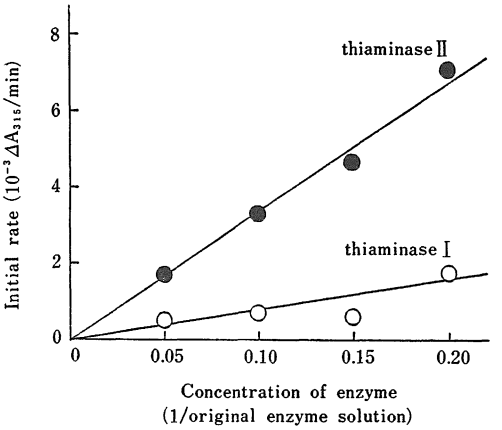

Fig. 8. Effect of concentration of enzyme on initial rate of hydrolase activity

tion of substrate for assay of the transferase activity of thiaminase I and II.

5. Effect of concentration of enzyme on transferase activity and hydrolase activity

On the basis of above-mentioned results, the procedure shown in Fig. 6 was proposed as a method for the determination of transferase activity of thiaminase I or thiaminase II. The hydrolase activity of thiaminase I or thiaminase II can be determined by replacing L-cysteine ethylester with H_2O in the components shown in Fig. 6.

The effect of the concentration of enzyme on transferase activity or hydrolase activity of thiaminase I and II was examined (Fig. 7 and 8). As shown in Fig. 7 and 8, each activity increased linearly with increasing concentration of each enzyme. This shows that the procedure is usable as a method for the determination of transferase activity and hydrolase activity of thiaminase I and II.

Table 1. Molar absorption coefficients of some compounds in 0.1 M Tris-HCl buffer (pH 8.8) at 37°C

Compound	Molar absorption coefficient at 315 nm ($M^{-1}cm^{-1}$)
Pm-quinoline	7950
L-Cysteine ethylester	0.64
Pm-L-cysteine ethylester	2288
Quinoline	1113
OMP	70

If necessary, the rate is able to be represented on the basis of an amount of formed Pm-L-cysteine ethylester for the transferase activity or formed OMP for the hydrolase activity by making use of each molar absorption coefficient shown in Table 1.

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摘　　要

チアミナーゼⅠおよびⅡの転移酵素活性の新測定法を提出した．加水分解酵素に分類されているチアミナーゼⅡは，基質としてキノチアミン（Pm-キノリン）および L-システインエチルエステルを用いると転移酵素活性を示した．

チアミナーゼⅠあるいはⅡの37℃における転移酵素活性は，2mM の Pm-キノリン0.3 ml, 500 mM の L-システインエチルエステル 0.3 ml, 0.1 M のトリス-塩酸緩衝液 (pH 8.8) 2.0 ml および酵素溶液 0.4 ml からなる反応溶液の吸光度 (315 mm) の経時変化から評価することができた．Pm-キノリンおよび L-システインエチルエステルの *Km* 値は，チアミナーゼⅠに対してそれぞれ 0.025 および 7.1 mM，チアミナーゼⅡに対してそれぞれ 0.094 および 0.9 mM であった．