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The Enzymological Properties of Immobilized Thiaminase I Prepared by Means of Multi-Immobilization Method

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多重固定化法によって調製した固定化チアミナーゼ I の酵素学的性質

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Thiaminase I (EC 2.5.1.2) was immobilized by the combined use of Sepharose 4B, glutaraldehyde, and κ-carrageenan in order to enhance its utility. The native enzyme was inactivated by the addition of organic solvents into an aqueous solution in order to promote the solubility of apolar substrates. However, the multi-immobilized enzymes were not only tolerant to organic solvents but also stable to heat. In addition, the activities of the immobilized enzymes increased during repeated utilizations. Thus, the utility of thiaminase I as a catalyst may be greatly enhanced by the multi-immobilization.

INTRODUCTION

Thiaminase I (EC 2.5.1.2) catalyzes a base-exchange reaction of the thiazole moiety of thiamine (the lst substrate) with base compounds (the 2nd substrate), forming 2-methyl-4-amino-5-pyrimidinylmethyl compounds (Pm compound).[1] The substrate specificity of the 2nd substrate is so broad that a variety of Pm compunds are formed.

The authors prepared some Pm compounds by the use of native or Sepharose 4B-immobilized thiaminase I.[2] However, the base compounds used were confined to those soluble in water, because of the lability of thiaminase I in aqueous organic solvents. If the disadvantage can be eliminated, the utility of thiaminase I as a catalyst would be greatly enlarged to substrates insoluble in water.

It has been observed that immobilization of some enzymes increases the stability to inhibitory reagents.[3] For instance, aminoacylase immobilized with DEAE-Sephadex is not inactivated by urea and activated by guanidine and *n*-propanol.[4] Although the activation mechanism is not clear, it is presumed to be due to conformational change of the protein.

Methods for enzyme immobilization are classified into three basic categories[5]; the binding of enzymes to water-insoluble carriers (carrier-binding method), intermolecular cross-linking of enzymes by means of bifunctional or multifunctional reagents (cross-

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linking method), and incorporating enzymes into the lattice of a semipermeable gel or enclosing the enzymes in a semipermeable polymer membrane (entrapping method). The immobilization of thiaminase I employed at the present study was the carrier-binding method on Sepharose 4B, the cross-linking method with glutaraldehyde, and the entrapping method with κ-carrageenan. Each single immobilization gave relatively a high yield of the activity. Moreover, it was assumed that the combined use of these immobilization methods would convert the flexible structure of thiaminase I into a more rigid structure suitable for catalytic action in aqueous organic solvents.

This paper deals with the preparations of multi-immobilized thiaminase I and their improved enzymological properties including the tolerance to organic solvents.

MATERIALS AND METHODS

1. Microorganisms and materials

The bacterial strains used were *Bacillus thiaminolyticus* Matsukawa et Misawa and *Bacillus thiaminolyticus* strain B which had been isolated from soil by the authors.[6] The chemicals were all reagent grade and used without further purifications.

2. Cultural conditions and preparation of thiaminase I

Bacillus thiaminolyticus Matsukawa et Misawa or strain B was aerobically grown in a medium containing 1% peptone, 1% meat extract, and 0.5% NaCl at 37°C. After 168 hrs of cultivation, the cells were removed by centrifugation under cooling. To the supernatant, 80% saturated $(NH_4)_2SO_4$ was added. The precipitate was collected by centrifugation under cooling, dissolved in a small amount of 0.02M phosphate buffer (pH 6.8) containing 1 mM 2-mercaptoethanol, and dialyzed against a large volume of the same buffer. The supernatant obtained by centrifugation under cooling was used as native thiaminase I in the experiment described below.

3. Preparations of immobilized thiaminase I

(1) *Sepharose 4B-bound thiaminase I*

The native thiaminase I (5–10 mg protein per 1 ml swelling gel) was dissolved in 25 ml of a coupling solution (0.1 M $NaHCO_3$, pH 8.3) containing 0.5 M NaCl and stirred with CNBr-activated Sepharose 4B for 24 hrs at 25°C. The Sepharose gel was collected by filtration, washed with the same coupling solution, stirred with 100 ml of 0.05 M Tris-HCl buffer (pH 8.0) again, collected by filtration, and washed alternately with 0.1 M acetate buffer (pH 4.0) and 0.1 M borate buffer (pH 8.5), each containing 0.5 M NaCl. Finally, the Sepharose gel was washed with water. The Sepharose 4B-immobilized thiaminases from strain Matsukawa et Misawa and strain B were referred to as Sepharose 4B-TMM and Sepharose 4B-TSB, respectively, in the experiment described below.

(2) *Glutaraldehyde-crosslinked thiaminase I*

After the addition of the native thiaminase I (1 mg protein per 2 mg gluaraldehyde) to 2.5% glutaraldehyde-phosphate buffer solution containing 1 mM 2-mercaptoethanol,

the mixture was allowed to stand for 24 hrs at 25 °C. The resulting gel was homogenized by Potter homogenizer and then collected by centrifugation under cooling. The gel was washed 5 times with 0.02M phosphate buffer containing 2-mercaptoethanol.

The gels of the enzymes obtained from strain Matsukawa et Misawa and strain B were referred to as glutaraldehyde-TMM and glutaraldehyde-TSB, respectively, in the subsequent experiments.

(3) *κ-Carrageenan-entrapped thiaminase I*

The native thiaminase I (20-30 mg protein per 1 g κ-carrageenan) was added to 4.5 % κ-carrageenan-0.02M phosphate buffer solution containing 1mM 2-mercaptoethanol maintained at 37-40 °C. The mixture was cooled below 10 °C for 30 min and then allowed to stand for 1 hr in 0.3M KCl solution to give gels. After collected by filtration, the gels were cut into *ca.* 1 mm^3 and washed with 0.02M phosphate buffer containing 0.1M KCl.

The gels containing the enzymes from strain Matsukawa et Misawa and strain B were referred to as κ-carrageenan-TMM and κ-carrageenan-TSB, respectively, in the subsequent experiments.

(4) *Thiaminase I bi-immobilized with Sepharose 4B and κ-carrageenan*

Ten ml of the suspension of Sepharose 4B-TMM or Sepharose 4B-TSB (10-15 mg protein per 1 g κ-carrageenan) was added to 40 ml 4.0 % κ-carrageenan-0.02 M phosphate buffer solution containing 1 mM 2-mercaptoethanol maintained at 37-40 °C. The mixture was allowed to stand for 30 min at 0 °C. The gels obtained were treated according to the similar procedure described above.

The gels with the enzsmes from strain Matsukawa et Misawa and strain B were referred to as Sepharose 4B+κ-carrageenan-TMM and Sepharose 4B+κ-carrageenan-TSB, respectively, in the subsequent experiments.

(5) *Thiaminase I bi-immobilized with Sepharose 4B and glutaraldehyde*

Sepharose 4B-TMM or Sepharose 4B-TSB (1 mg protein per 10 mg glutaraldehyde) was added to 2.5 % glutaraldehyde-phosphate buffer solution containing 1 mM 2-mercaptoethanol. The mixture was treated according to the similar procedure described above.

The gels containing the enzymes from strain Matsukawa et Misawa and strain B were referred to as Sepharose 4B+glutaraldehyde-TMM and Sepharose 4B+glutaraldehyde-TSB, respectively, in the subsequent experiments.

(6) *Thiaminase I bi-immobilized with glutaraldehyde and κ-carrageenan*

Ten ml of the suspension of glutaraldehyde-TMM or glutaraldehyde-TSB (15-25 mg protein per 1 g κ-carrageenan) was added to 40 ml of 4.0 % κ-carrageenan-0.02M phosphate buffer solution containing 1 mM 2-mercaptoethanol maintained at 37-40 °C. The mixture was treated according to the procedure described above.

The gels immobilizing the enzymes from strain Matsukawa et Misawa and strain B were referred to as glutaraldehyde+κ-carrageenan-TMM and glutaraldehyde+κ-carrageenan-TSB, respectively.

(7) *Thiaminase I tri-immobilized with Sepharose 4B, glutaraldehyde, and κ-carrageenan*

Ten ml of the suspension of Sepharose 4B+glutaraldehyde-TMM or Sepharose 4B+glutaraldehyde-TSB (10–15 mg protein per 1 g κ-carrageenan) was added to 40 ml of 4.0 % κ-carrageenan-0.02M phosphate buffer solution containing 1mM 2-mercaptoethanol maintained at 37–40 °C. The mixture was treated according to the similar procedure described above.

The gels entrapping the enzymes from strain Matsukawa et Misawa and strain B were referred to as Sepharose 4B+glutaraldehyde+κ-carrageenan-TMM and Sepharose 4B+glutaraldehyde+κ-carrageenan-TSB, respectively, in the subsequent experiments.

4. Standard enzyme assay

(1) *Native enzyme*

The activity of the native thiaminase I was assayed by the colorimetric determination of heteropyrithiamine (HPT) according to the method of Murata *et al.*[7]

After the mixture of 6 mM thiamine (0.2 ml), 100 mM pyridine (0.2 ml), and 150 mM citrate-phosphate buffer (pH 5.5, 0.2 ml) was preincubated at 50 °C, the enzyme reaction was started by the addition of an enzyme solution (0.2 ml). The mixture was incubated at 50 °C for 20 min. The reaction was stopped by the addition of 20 % metaphosphoric acid (0.2 ml). The mixture was incubated with 2 ml of 10 % NaOH at 37 °C for 60 min. After adding 1 % ferricyanide-20 % NaOH (2/3, v/v), the mixture was allowed to stand at room temperature for 15 min and then 0.5 ml of 1 % H_2O_2 was added to the mixture. The absorbance of HPT formed was measured at 386 nm.

One unit of the activity was defined as the amount of enzyme producing 1 μmol HPT per min.

(2) *Immobilized enzyme*

The enzyme activity of the immobilized thiaminase I was assayed by a modifying HPT method.

The mixture of 6 mM thiamine (2 ml), 100 mM pyridine (2 ml), 150 mM citrate-phosphate buffer (pH 5.5, 4 ml), and an adequate amount of immobilized enzyme was shaked at 50 °C for 20 min. For the determination of the enzyme activity of thiaminase I immobilized by use of κ-carrageenan, 0.1 M KCl was added to the mixture. The enzyme reaction was stopped by adding 20 % metaphosphoric acid (0.2 ml) to an aliquot (0.8 ml) of the mixture. The mixture was treated according to the similar procedure described above.

5. Protein assay

Protein concentrations were measured by the method of Lowry *et al.*[8] with bovine serum albumin as the standard. The amount of immobilized proteins were determined by subtracting the amount of unimmobilized proteins from that of total proteins used.

6. Stability of enzyme in water-organic solvent systems

(1) *Influence of organic solvents on the colorimetric determination of HPT*

After incubating the mixture of 6 mM thiamine (2 ml), 100 mM pyridine (2 ml), 150

Table. 1.　Yields of immobilization of thiaminase I in each step

Step	Method	Used			Immobilized			Yield	
		Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Protein (%)	Activity* (%)
	(TSB)								
1st	Native→Sepharose 4B	140	212	1.5	99	192	1.9	71	91
	Native→Glutaraldehyde	53	80	1.5	44	24	0.6	83	30
	Native→κ-Carrageenan	49	108	2.2	5	16	3.2	10	15
2nd	Sepharose 4B→κ-Carrageenan	23	50	2.2	21	30	1.4	91	60
	Sepharose 4B→Glutaraldehyde	47	92	2.0	44	53	1.2	94	58
	Glutaraldehyde→ κ-Carrageenan	41	30	0.6	39	22	0.6	95	73
3rd	Sepharose 4B+glutaraldehyde→ κ-Carrageenan	25	30	1.2	23	19	0.8	92	63
	(TMM)								
1st	Native→Sepharose 4B	175	283	1.6	124	359	2.9	71	127
	Native→Glutaraldehyde	42	110	2.6	27	32	1.2	64	29
	Native→κ-Carrageenan	40	105	2.6	16	21	1.3	40	20
2nd	Sepharose 4B→κ-Carrageenan	20	58	2.9	18	30	1.7	90	52
	Sepharose4B→Glutaraldehyde	52	149	2.9	51	114	2.2	98	77
	Glutaraldehyde→κ-Carrageenan	18	20	1.1	15	23	1.5	88	115
3rd	Sepharose 4B+glutaraldehyde→ κ-Carrageenan	15	34	2.3	13	23	1.8	87	68

* 100×(Immobilized total activity)/(Used total activity)

mM citrate-phosphate buffer (pH 5.5, 2 ml), and the native enzyme solution (2 ml) at 50 °C for 20 min, the enzyme reaction was stopped by the addition of 20 % metaphosphoric acid (2 ml). Various concentrations of organic solvents (0.4 ml), such as MeOH, EtOH, DMF, and DMSO, and 20 % metaphosphoric acid (0.1 ml) were added to an aliquot (0.5 ml) of the reaction solution. The mixture was treated according to the procedure described above.

(2) *Stability of enzyme in organic solvents*

The mixture of the immobilized enzyme (30-40 mg), 150 mM citrate-phosphate buffer (1 ml), and various concentrations of organic solvent (1 ml) was preincubated at 37 °C for 24 hrs. The immobilized enzyme was collected by filtration and washed with 0.02M phosphate buffer (pH 5.5) containing 1 mM 2-mercaptoethanol. An aliquot of the immobilized enzyme was used for the determination of the enzyme activity.

7. Repeated utility of immobilized enzymes

After incubating the mixture of 6 mM thiamine (5 ml), 100 mM pyridine (5 ml),

Table 2. Yields of protein and activity for immobilized preparations based on native thiaminase I

Preparation	Yield(%)	
	Protein	Activity*
Sepharose 4B-TSB	71	91
Glutaraldehyde-TSB	83	30
κ-Carrageenan-TSB	10	15
Sepharose 4B+κ-carrageenan-TSB	65	55
Sepharose 4B+glutaraldehyde-TSB	67	53
Glutaraldehyde+κ-carrageenan-TSB	79	22
Sepharose 4B+glutaraldehyde+κ-carrageenan-TSB	61	33
Sepharose 4B-TMM	71	127
Glutaraldehyde-TMM	64	29
κ-Carrageenan-TMM	40	20
Sepharose 4B+κ-carrageenan-TMM	65	66
Sepharose 4B+glutaraldehyde-TMM	70	98
Glutaraldehyde+κ-carrageenan-TMM	53	33
Sepharose 4B+glutaraldehyde+κ-carrageenan-TMM	61	66

* 100× (Immobilized total activity)/(Used total activity)

150 mM citrate-phosphate buffer (10 ml), and glutaraldehyde+κ-carrageenan-TMM (1.8 g) or glutaraldehyde+κ-carrageenan-TSB (3.1 g) at 37 °C for 20 min, the immobilized enzyme was collected by filtration. The filtrate was used for the determination of the enzyme activity. The enzyme collected was used for repetition of the procedure.

Table 3. Influence of some organic solvents on the colorimetric determination of HPT

Organic solvent	Relative absorbance*			
	Concentration (%)			
	5	10	15	20
EtOH	105	105	107	108
MeOH	103	103	106	105
DMF	101	103	106	106
DMSO	104	107	108	109

* Percentage based on 0% organic solvent

RESULTS AND DISCUSSION

1. Yield of immobilization

The amount, activity, and specific activity of thiaminase I used as well as the yields in each step of immobilization were summarized in Table 1. The immobilization of native thiaminase I on Sepharose 4B gave high yield of activity of 91% or above. In particular, the yield of activity for TMM preparation exceeds 100%. The single immobilization on Sepharose 4B, in contrast to those with glutaraldehyde and κ-carrageenan, is higher in yield of activity than in that of protein. This fact suggests that thiaminase I was immobilized selectively on Sepharose 4B. In the case of combined use of single immobilization, the yields of protein were 83% or above but the yields of activity were somewhat low. However, the enhancement of activity was observed also in the immobilization of glutaraldehyde-TMM with κ-carrageenan. This may be due to removal of inhibitors and/or to activation of enzyme reaction accompanying change of structure in the whole enzyme or in the vicinity of active site of enzyme molecule by immobilization. The yields of protein and activity for immobilized preparations based on native thiaminase I were shown in Table 2. It was

Tab e 4. Stabilities of TSB and TMM in water-organic solvent systems

Solvent	Concentration (%)	Residual activity* TSB	TMM
EtOH	10	92	99
	20	2	18
	30	0	0
	40	0	0
	50	0	0
MeOH	10	98	101
	20	44	79
	30	1	0
	40	1	0
	50	0	0
DMF	10	82	105
	20	0	11
	30	0	0
	40	0	0
	50	0	0
DMSO	10	105	99
	20	89	88
	30	65	75
	40	2	42
	50	0	0

* Percentage based on 0% organic solvent

Table 5. Stabilities of various immobilized TSB preparations in DMSO solution

Preparation	Residual activity* DMSO concentration (%) 10	20	30	40	50
Native TSB	105	89	65	2	0
Sepharose 4B-TSB	—	—	112	—	6
Glutaraldehyde-TSB	—	—	95	—	31
Sepharose 4B+κ-carrageenan-TSB	—	—	79	—	10
Sepharose 4B+glutaraldehyde-TSB	—	—	77	—	10
Glutaraldehyde+κ-carrageenan-TSB	—	—	87	—	52
Sepharose 4B+glutaraldehyde+κ-carrageenan-TSB	—	—	88	—	55

* Percentage based on 0% organic solvent

Table 6. Stabilities of various immobilized TMM preparations in DMSO solution

Preparation	Residual activity*				
	DMSO concentration (%)				
	10	20	30	40	50
Native TMM	99	88	75	42	0
Sepharose 4B-TMM	—	—	93	—	7
Glutaraldehyde-TMM	—	—	103	—	59
Sepharose 4B+κ-carrageenan-TMM	—	—	83	—	33
Sepharose 4B+glutaraldehyde-TMM	—	—	91	—	17
Glutaraldehyde+κ-carrageenan-TMM	—	—	92	—	48
Sepharose 4B+glutaraldehyde+ κ-carrageenan-TMM	—	—	93	—	66

* Percentage based on 0% organic solvent

suggested that immobilization on Sepharose 4B was preferred as the first step in combined use of single immobilization.

2. Influence of organic solvents on the colorimetric determination of heteropyrithiamine (HPT)

Whether the presence of organic solvent influences the colorimetric determination of HPT or not was examined prior to the evaluation of influences of organic solvents on the activity and stability of the enzyme (Table 3). The coexistance of organic solvents such as MeOH, EtOH, DMF, and DMSO influenced slightly the colorimetric determination of HPT. The actual concentration of organic solvents in an assay solution for the colorimetric determination was at most 10%, where an error of determination due to an organic solvent is regarded as negligible.

3. Stability of thiaminase I in water-organic solvent systems

The residual activities after incubating native thiaminase I for 24 hrs at 37°C in various organic solvent-water systems are shown in Table 4. The tolerance of the two native enzymes to organic solvents, except for DMSO, was extremely low. In aqueous DMSO solution, the activities of TSB and TMM were relatively high even at DMSO concentration of 30%. On the whole, the tolerance of TMM to the organic solvents was superior to that of TSB.

Table 7. Stabilities of TMM and glutaraldehyde+κ-carrageenan-TMM in various organic solvent systems

Solvent	Residual activity*	
	Native TMM	Glutaraldehyde+κ-carrageenan-TMM
30% *i*-Propanol	—	68
30% Acetone	—	104
30% EtOH	0	100
30% MeOH	0	112
30% DMF	0	115
30% DMSO	75	92

* Percentage based on 0% organic solvent

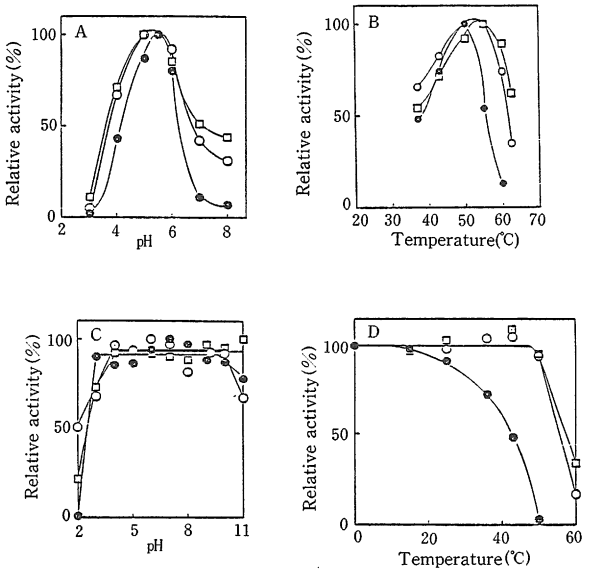

Fig. 1. Optimal reaction pH (A), optimal reaction temperature (B), pH-stability (C), and thermal stability (D) of glutaraldehyde +κ-carrageenan-TSB (□), Sepharose 4B+glutaraldehyde+κ-carrageenan-TSB(○), and TSB (●).

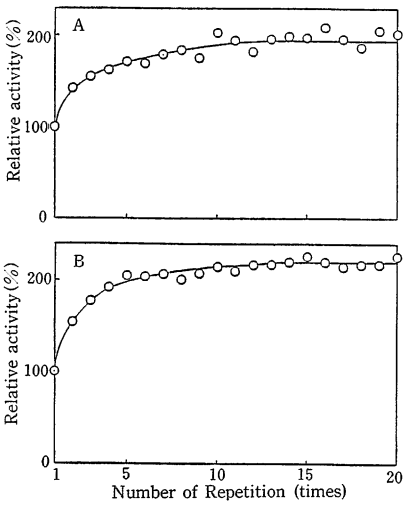

Fig. 2. Stabilities of glutaraldehyde+κ-carrageenan-TMM (A) and glutaraldehyde +κ-carrageenan-TSB (B) in repeated utilization.

The stabilities of various immobilized TSB preparations in a DMSO solution are shown in Table 5. Among the multi-immobilized enzymes examined, the glutaraldehyde+κ-carrageenan preparation and the Sepharose 4B+glutaraldehyde+κ-carrageenan preparation were excellent in tolerance to DMSO. The same tendancy was also observed for the immobilized TMM preparations (Table 6). The glutaraldehyde+κ-carrageenan preparation and Sepharose 4B+glutaraldehyde+κ-carrageenan preparation maintained as high as 48% and 66% of the initial activity, respectively, in 50%

DMSO solution. The stabilities of enzymes in organic solvents tended to be increased by the multi-immobilization to a greater extent than by the single immobilization. This effect of multi-immobilization may arise from the fixation of the conformation of enzyme within the polymer matrix.

The stabilities of glutaraldehyde+κ-carrageenan-TMM preparation in the other organic solvents are shown in Table 7. The preparation maintained satisfactory activities in all organic solvents used.

4. Enzymological properties of the multi-immobilized preparations

Optimal reaction pH, optimal reaction temperature, pH-stability, and thermal stability of the glutaraldehyde+κ-carrageenan-TSB and Sepharose 4B+glutaraldehyde+κ-carrageenan-TSB preparations, which tolerated significantly to organic solvents, were examined and compared with those of the native enzyme (Fig. 1). Thermal stability of the enzyme was increased about 10 °C by the immobilization. The other properties were hardly affected by multi-immobilization. The same tendancy was also observed for the multi-immobilized TMM preparations.

5. Stabilities of the multi-immobilized preparations in repeated utilization

The greatest merit of immobilization is the ability of repeated utilization of enzyme. Changes in the activities of the glutaraldehyde+κ-carrageenan-TSB and-TMM preparations during repeated utilization were examined (Fig. 2). The activities of both preparations increased gradually with repeated utilization and reached approximately twice the initial activity. This phenomenon was not observed for single immobilized preparations. As explanation for the phenomenon, the removal of inhibitors or the change in diffusion state of substrate into κ-carrageenan gel matrix is presumed. However, further investigation is required to make clear the cause.

When thiaminase I was prepared by means of multi-immobilization, not only stability of enzyme in organic solvents but also activity in repeated utilization increased. These improved properties may enhance greatly the utility of thiaminase I as a catalyst.

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摘　　要

チアミナーゼ I (EC 2.5.1.2) の利用性を増大させるために，Sepharose 4B, glutaraldehyde および κ-carrageenan を固定化基材としたチアミナーゼIの多重固定化法を検討した．非固定化チアミナーゼIは，非極性基質の溶解度を増すために水溶液に添加される有機溶媒に不安定であるが，得られた多重固定化チアミナーゼIは，有機溶媒に対して耐性であるだけでなく，熱に対しても安定であった．加えて，多重固定化標品の活性は，繰り返し使用の間増加した．これらのことから，この多重固定化チアミナーゼIは触媒としての利用性を大きく増大するものと期待された．