Studies on Thiaminase of Fungi

I. Thiaminase of *Lentinus edodes* (Berg.) Sing.¹

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1. It was confirmed that thiaminase and thermostable factor existed in "Shiitake", *Lentinus edodes* (Berg.) Sing. by chemical and microbiological methods. 2. The optimum pH of the enzyme was 6.5, and the optimum temperature 20°. It was inactivated by heating at 45° for 10 minutes. Organic bases such as pyridine, aniline, quinoline and *p*-aminobenzoic acid activated the activity, as was the case with thiaminase I. Pyrichrome was produced by ferricyanide oxidation in the presence of pyridine. 3. The thermostable factor was active at pH 6 or above, and at 60° or above. 4. The enzyme activity in the fungus was greatest in the gillus, followed by the stipe and the pileus. 5. The content of thiamine in the fungus was greatest in the gillus, followed by the pileus, and the stipe, and about 70 % of the vitamin was in the esterified form.

The presence of thiaminase in the certain plants (1-7) and molds (8-12) has hitherto been studied extensively. However, there are few investigations dealing with the presence of thiaminase in fungi. It was reported that "Matsutake", *Tricholima Matsutake* Sing. lacked the enzyme (7). The authors have noticed the marked difference in thiamine contents between dried and fresh "Shiitake", *Lentinus edodes* (Berg.) Sing. and suspected the presence of the enzyme. Research on that fungus has led to the recognition of thiaminase. The properties of the enzyme are described in this paper.

MATERIALS AND METHODS

1. Materials

L. edodes Sing. used were mostly harvested in Osaka district in spring, 1966.

2. Determination of Thiaminase

The enzyme was extracted as follows. *L. edodes* was extracted with water by grinding with sea sand, and centrifugation at 3,000 rpm for 10 minutes. The

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supernatant was used as the enzyme preparation. The reaction system contained 2 ml of 0.2 M acetate buffer (pH 4-5) or 0.2 M phosphate buffer (pH 6-8), 3 μ g of thiamine, and 1 ml of the enzyme solution in a total volume of 4 ml. It was incubated at a certain temperature for 1 hour. Then, the enzyme was inactivated by heating at 100° for 5 minutes after adjusting the pH to 4.5, and the amount of residual thiamine was estimated by the thiochrome assay and microbiological assay (using *Lactobacillus fermenti* 36) after Takadiastase hydrolysis (14). Thiaminase potency was expressed according to Fujita (15), as the amount of thiamine degraded in μ g per g dry matter of the sample under the optimal conditions of pH and temperature in 1 hour when more than 50 per cent of the thiamine added is degraded. As a control test, the enzyme solution was heated at 100° for 5 minutes, and the pH of this reaction mixture was adjusted to 4.5 to prevent the action of thermostable factor.

3. Determination of Thermostable Factor

Thermostable factor was determined according to Okamoto (7). The reaction mixture contained 2 ml of 0.2 M phosphate buffer (pH 7.0), 1 ml of thermostable factor solution (heated for 30 minutes at 100°) and 3 μ g of thiamine in a total volume of 4 ml. After incubation at 80° for 1 hour, the amount of the thiamine degraded was estimated, and the thermostable factor potency was calculated.

4. Estimation of Thiamine in *Lentinus edodes*

The tissue of the fungus was heated at 100° for 5 minutes, and homogenized. The extract was adjusted to pH 4.5, Takadiastase solution was added, and the whole was incubated at 45° for 2 hours. Then, the amount of thiamine remaining in the extract was estimated by thiochrome assay.

RESULTS

1. Detection of Thiaminase Activity by Chemical and Microbiological Methods

The thiamine remaining in the reaction system was measured by chemical and microbiological methods, and the results shown in Table 1 were obtained. The degradation rate of thiamine was found to be 65 and 60 % respectively. There was no significant difference between both values.

TABLE 1
Thiaminase Activity Determined by Microbiological and Chemical Assays

Assay method	Thiamine degradation
	<i>per cent</i>
Chemical	65
Microbiological	60

Reaction system consisted of 1 ml of thiamine solution containing 3 μ g, 2 ml phosphate buffer solution (pH 6.5) and 1 ml of the enzyme. It was incubated at 20° for 1 hr.

2. Properties of the Thiaminase

Optimum pH—Acetate buffer (pH 4 to 5) and phosphate buffer (pH 6 to 8) were used as buffer solutions. As shown in Fig. 1, the optimum pH was 6.5.

Optimum Temperature—The effects of temperature were investigated in the range of 15–45°. As shown in Fig. 2, the optimum temperature was 20°. The activity was markedly dropped at 30°, and completely inactivated at 45°.

FIG. 1 Effect of pH on Thiamine Degradation

Reaction system contained 1 ml of thiamine solution (3 μ g), 2 ml of buffer solution and 1 ml of the enzyme. It was incubated at 20° for 1 hr.

FIG. 2 Effect of Temperature on Thiamine Degradation

Reaction system contained 1 ml of thiamine solution (3 μ g), 2 ml of buffer solution and 1 ml of the enzyme. It was incubated at different temperatures for 1 hr.

FIG. 3 Thermostability of Thiaminase

Reaction system, containing 1 ml of the enzyme and 2 ml of phosphate buffer (pH 6.5), was kept at varying temperatures for 10 min.

Thermostability—The thermostability of the enzyme was tested as follows. The mixture containing 2 ml of phosphate buffer (pH 6.5) and 1 ml of the enzyme solution was heated at various temperatures for 10 minutes, and the activity of the enzyme was determined. As a control test, the mixture was kept at 2° for 10 minutes. As shown in Fig. 3, the enzyme was not affected between 2° and 35°, but was inactivated at 45° completely.

Acceleration by Organic Bases—The effects of organic bases on the activity of the enzyme were investigated at 10^{-3} M of the compound in the reaction mixture. As shown in Table 2, acceleration of the enzyme activity by organic bases such as *p*-aminobenzoic acid, quinoline, aniline and pyridine were 28, 418, 698 and 739 per cent

TABLE 2
Effect of Several Organic Bases

Substance ($10^{-3} M$)	Acceleration rate
	<i>per cent</i>
Pyridine	739
Aniline	698
Quinoline	418
<i>p</i> -Aminobenzoic acid	28

respectively. When the ferricyanide oxidation method was used in the case of pyridine, pyrichrome was produced (16-17), whereas it failed to be produced when the cyanogen bromide oxidation method was used.

3. Determination of Thermostable Factor

Not only thiaminase but also thermostable factor was proved to exist in *L. edodes*. The factor remained active after heating at 100° for 30 minutes. The results of the experiments of the factor on the effects of pH and temperature are shown in Figs. 4 and 5. The activity was undetected at pH 6.0, but it was distinct at pH 7.0. On the other hand, at the temperature under 40° no activity was observed, but it rose markedly above 60°.

FIG. 4 *Effect of pH on Thermostable Factor Activity*

Reaction system, containing 1 ml of thiamine solution (3 μ g), 2 ml of different buffer solution and 1 ml of the thermostable factor solution, was incubated at 80° for 1 hr.

FIG. 5 *Effect of Temperature on Thermostable Factor Activity*

Reaction system, containing 1 ml of thiamine solution (3 μ g), 2 ml of buffer solution and 1 ml of the thermostable factor solution, was incubated at 80° for 1 hr.

4. Test for Thiamine Disulfide

According to Tamaki (18), 2 ml of phosphate buffer (pH 7.0), 1 ml of thiamine solution (3 μ g) and 1 ml of cysteine (100 mg/100 ml) were added to 1 ml of the enzyme solution, the mixture was incubated at 37° for 1 hour, and the amount of remaining thiamine was determined. The result failed to show the conversion into thiamine disulfide.

5. Change of Thiaminase Potency and Thiamine Content in Various Seasons

The thiaminase potencies of the commercial fresh fungi in various seasons were estimated as described above (pH 6.5, 20°, 1 hr). As shown in Table 3, the potency and thiamine content of each fungus showed remarkable fluctuation and no definite dependency upon seasons could be detected.

TABLE 3
Thiaminase Activity of the Commercial Fungus in Various Seasons

Month	Thiaminase potency	Thiamine content
		$\mu\text{g}/100\text{ g fresh weight}$
January		86.4; 148.0; 62.9; 115.7
February		50.0; 88.1; 77.4; 93.5; 54.2; 38.4
March		94.9; 75.8; 116.3; 66.7; 39.3
April	19.6; 12.0	
May	12.8; 13.2; 20.7	
June	24.7; 22.5; 11.9; 9.9	56.0; 87.4; 74.5
July	6.0	
September	2.8	
October	10.3 ^a ; 14.5 ^b	
November	25.1	81.7; 69.3
December	20.5; 11.9	115.0; 158.6

^a Produced in Okayama.

^b Produced in Nara.

6. Thiaminase Potencies and Thiamine Contents in Various Parts of the Fungus

The various parts, pileus, gillus and stipe (Fig. 6), were used for testing the thiaminase potency and thiamine content and the results shown in Table 4 were obtained. Thiaminase was found strongest in the gillus, followed by the stipe and the pileus, and about 70 per cent of the vitamin was in the esterified form (Table

FIG. 6 Fruit Body of *Lentinus edodes* Sing.

TABLE 4
Thiaminase Potency in Various Parts of L. edodes

Part	Thiaminase potency	Thiamine content
		$\mu\text{g}/100\text{ g fresh weight}$
Pileus	7.4; 2.0	
Gillus	22.5; 6.0	
Stipe	13.7; 3.7	
Pileus	Large ^a	23.8
	Middle ^b	24.7
	Small ^c	26.1
Edible Parts	Large	78.0
	Middle	74.5
	Small	113.7

^a Diameter of pileus 8.0 cm

^b Diameter of pileus 5.5 cm

^c Diameter of pileus 4.0 cm

TABLE 5
Thiamine Content in Various Parts of L. edodes

Thiamine	Thiamine content			Total fungus
	Pileus	Gillus	Stipe	
		$\mu\text{g}/100\text{ g}$		$\mu\text{g}/100\text{ g}$
Free	21.8	34.2	12.5	22.8
Ester	40.4	104.5	40.3	61.8
Total	62.2	138.7	52.8	84.6
Ester/total (%)	65	75	76	72

5). In the estimation of thiamine in the fungus the presence of thiaminase has hitherto not been taken into consideration. The authors have measured the thiamine content after inactivation of the enzyme. The thiamine contents seemed to be higher in smaller fungi (Table 4). The thiamine contents in commercial dried fungi obtained in May were as follows ($\mu\text{g}/100\text{ g}$): 56.0 (Osaka); 133.3 (Kyushu); 247.4* (Kiryu, W No. 121); 203.5* (Kiryu, W No. 4). (*dried by an infrared lamp). The individual variation was so marked

7. Loss of Thiamine in the Fungus by Dehydration

To test the loss of thiamine by heat-dehydration of the fungi, one part of the fresh fungus was dried by heat and the other part of the same fresh fungus was used without drying for estimating the thiamine content. As given in Table 6, the contents of the vitamin were almost the same, when calculated on the fresh weight basis, showing that the vitamin was scarcely lost by drying by heat.

TABLE 6
Thiamine Contents of Heat-dried Preparation Prepared from Fresh Fungi

	Thiamine contents (Calculated as $\mu\text{g}/100\text{ g}$ fresh weight)		
Fresh	88.1	77.4	93.5
Dried	85.4	72.1	88.1

8. Thiamine Degradation by Tissue Destruction

A part of the raw fungus was sliced along the gillus, 3 or 0.1 cm thick, and another part was ground with water together with sea sand in a glass mortar. They were allowed to stand separately at 20° for 1 hour. After inactivating the enzyme, the vitamin was estimated. The thiamine contents given in Table 7 show that the thiamine in the slice, 3 cm thick, was not affected, that in the slice, 0.1 cm thick, was somewhat degraded and that in the ground tissue was degraded by more than half.

TABLE 7
Destructive Effect of Fruit Body Treatment on Thiamine Content

	Thiamine content
	$\mu\text{g}/100\text{ g}$
Control	148
Slice, 3 cm thick	150
Slice, 0.1 cm thick	141
Homogenate	55

DISCUSSION

Thiamine contents of fresh and dried *L. edodes* given in the Standard Table of Food Composition of Japan (13) are 640 and 320 μg per 100 g respectively. Comparing the calculated values of thiamine on the dry matter basis, the dried fungus contained the vitamin about 1/20 of the fresh one (Table 8). It suggested the existence of thiaminase in the fungus, and the experiment has proved the assumption. The optimum temperature of the enzyme was 20°, corresponding to the optimum growth temperature of the fungus and the optimum pH 6.5, corresponding to the pH of the fungus tissue. Furthermore, the enzyme is concentrated in the gillus, where the spores of the fungus are localized, and in this part, most thiamine was found to exist. These findings seem to be of biological interest.

The thiaminases, which have been found thus far, are active at 30° to 60°, and are inactivated by heating at 90–100° for 10–20 minutes. However, the thiaminase of *L. edodes* have an optimum temperature of 20° and inactivated by heating at 45° for 10 minutes, a finding characteristic of this enzyme. The molds producing thiaminase were investigated by Jacobson (8), Ozawa *et al.* (10) and Ikehata (12). They were all reported to belong to thiaminase II, whereas the enzyme in *L. edodes* was found to be thiaminase I. The enzyme activity in *L. edodes* hypha and fruit body is being investigated.

Comparison of the thiamine contents of the fungus between sliced and ground one after incubation at the optimum temperature of the enzyme for 1 hour, showed that the values of the sliced ones did not change significantly, whereas the value of the ground one was decreased markedly to about one half. It is therefore considered that the thiamine in the fungus is not significantly degraded by cutting in ordinary cooking.

In the reports related to thiamine contents of *L. edodes*, Chin (19) and Takahashi (20) showed that a trace amount of thiamine is contained in the fresh fungus. In the dried fungus, Sumi (21) concluded from animal experiment that it contained scarcely any thiamine, Miyayoshi *et al.* (22) also reported the same results. However, Haraki (23) reported that the thiamine content in the dried *L. edodes* pileus was 129 μg per 100 g dry matter. The Standard Table of Food Composition of Japan (13) gives the values of thiamine in fresh and dried *L. edodes*, 640 and 320 μg per 100 g respectively, and "Vitaminology" (24) gives the value of 500 μg

TABLE 8
Thiamine Contents in Fresh and Dried Fungus

	Content of thiamine	
	In fresh weight	In dry weight
	$\mu\text{g}/100\text{ g}$	
Fresh fungus	100	1,145 ^b
Fresh fungus	640 ^a	7,800 ^c
Dried fungus	320 ^a	380 ^c

^a Values in the Standard Table of Food Composition of Japan (1963).

^b The value found by the authors.

^c Calculated from water content.

per 100 g dry matter. Thus, the thiamine contents fluctuate markedly. It is partly due to the determination of the vitamin, without the existence of thiaminase being taken into consideration.

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