REACTION MECHANISMS OF THIAMINE WITH THERMOSTABLE FACTORS

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Distributions of thermostable thiamine-inactivating factors in about 10 kinds of ferns and brackens and 42 kinds of other plants were investigated. It was observed that besides ferns and brackens, some plants (most of them greenish or yellowish in color) had thermostable thiamine-inactivating activities, and that shiitake, okra, fukinoto (butterbur flower stalk), diluted water extract of green tea, and diluted coffee had activities which oxidized thiamine into thiamine disulfide.

The present study showed that one of the factors isolated from fern 3, 4-dihydroxycinnamic acid (caffeic acid) and catechol distributed in plants accelerated decomposition of thiamine at pH 7-7.5 into 2-methyl-4-amino-5-aminomethyl-pyrimidine, decomposing the thiazole moiety, probably into γ-aceto-γ-mercapto-propylalcohol and formic acid. However, some flavonoids, especially 6, 7, 4'-trihydroxyisoflavone (Factor 2) were proved to have the ability to accelerate oxidation of thiamine into thiamine disulfide at pH 7.5. Estimation was made of the decomposition products of thiamine with caffeic acid, catechol and Factor 2 under certain conditions.

Since Weswig et al. (1) reported the existence of thermostable thiamine-inactivating factors in fern, the fact has been confirmed by many workers. From the experimental results of these workers it has been considered that ferns contain not only thermostable factors but also the thermolabile non-dialyzable thiaminase. Nakabayashi (2) isolated two flavonoid pigments, astragalin and isoquercitrin, from bracken and proved the presence of rutin as the so-called “thermostable antithiamine factor.” Hasegawa et al. (3) also suggested that the thermostable thiamine-decomposing factors of fern are flavonoids. They studied the activities of various flavonoids as well as phenol derivatives. Flavonoids possessing o-diphenol in the side chain were found to be most active. Sakamoto and Fujita (4) isolated isoquercitrin as a thermostable thiamine-decomposing sub-

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stance from sweet potato leaves. Hosoda (5) obtained a crystalline product from the reaction mixture of thiamine with catechol or hydroquinone at pH 7, and proved that the crystals were not only identical with the crystals isolated from thiamine reacted with rutin by Hasegawa et al. (6), but also identical with thiamine disulfide.

Later, Berutter and Somogyi (7) isolated 3,4-dihydroxycinnamic acid (caffeic acid) from fern as one of the thermostable antithiamine factors in plants. Somogyi and Bonicke (8) and Davis and Somogyi (9) attempted to clarify the reaction mechanism of the inactivation of thiamine by caffeic acid and other orthodiphenols and reported that the course of the reaction was biphasic, an observation which we could not confirm under our experimental conditions. The reaction was made partially reversible by the addition of cysteine (or other reducing substances) to the assay mixture, indicating that some thiamine disulfide was formed. The latter reaction has been observed by Davis and Somogyi (9) and the present authors. However, the structures of other forms of thiamine made inactive by the thermostable factor have not yet been disclosed.

We have investigated the mechanism of thiamine inactivation by the thermostable factors; caffeic acid, catechol, and flavonoids present in plants. Experiments and results are presented here.

**EXPERIMENTAL**

1. *Test for the thermostable thiamine-inactivating factor in plants*

1) *Extraction of factors.* Samples of fresh plants or dried powdered ferns were homogenized with 10–20 volumes of distilled water and boiled for 30 min to extract thermostable factors and inactivate thiaminase. The water extract was centrifuged to obtain a clear supernatant.

2) *Reaction of thiamine with the extract.* Conditions for the reaction of thiamine with the extract and determination of the remaining thiamine and thiamine disulfide (SSB1) are shown in Table 1.

| Table 1. Reaction condition |
|----------------------------|
| Plant extract | 1 ml<sup>a</sup> |
| Phosphate buffer (pH 7.0) | 2 ml |
| Thiamine hydrochloride (10 µg/ml) | 0.1 ml (A) |
| 60°C, 1 hr | |
| 10% Metaphosphoric acid 2.5 ml | |
| to stop the reaction | |
| Cysteine 2 mg/ml | |
| 60°C 30' | |
| SSB<sub>1</sub> % = \( \frac{C-B}{A-B} \times 100 \) | |
| Determination of thiamine | |
| (B) | (C) |

<sup>a</sup> Dist. water 1 ml for blank test.
2. Reaction condition of thiamine with the thermostable factors

Thiamine hydrochloride (5 µg–5 mg) in 1 ml of water solution was mixed with 0.2–1.0 mole factors per mole of thiamine in 1/15 M phosphate buffer (pH 6–7.7). The solutions of total volume 3–4 ml were placed in a centrifugal tube with a diameter of 3.1 ± 0.1 cm, sealed with a glass stopper, and incubated at 37°C or 45°C from 1–264 hr. Samples were drawn at suitable intervals for the determination of remaining thiamine by the thiochrome method or the colorimetric determination with Prebulda’s Reagent. Thiamine disulfide was estimated by determination of thiamine after treatment with cysteine.

3. Reaction of thiamine-analogues with caffeic acid

Oxythiamine (OHB₃), hydroxyethylthiamine (HET), and hydroxypropylthiamine (HPT) in equivalent moles of thiamine (5 mg) were incubated with caffeic acid (2 mg) in the same manner as thiamine, and the remaining analogues were determined by the thiochrome method using ferric chloride and alkali and, in the case of OHB₃, by the colorimetric method of Prebulda.

4. Detection of decomposition products of thiamine

Paper partition chromatography was applied for the detection of decomposition products of thiamine. Toyoroshi No. 50 was used. n-Butanol: methanol: water (2:1:1) was used for the developing solvent at 25°C for 8 hr. The spots were detected by fluorescence, or the spots appeared after reaction with bromocyanide or ferric cyanide and alkali and by the orange color formed the reaction with Drangendorff’s Reagent. Rf values of these spots were compared with those of standard samples. The curve of the UV spectrum of a spot of the extracted solution corresponding to 2-methyl-4-amino-5-aminomethyl-pyrimidine (Pm–CH₂–NH₂) was also compared with that of the standard.

RESULTS

1. Distribution of the thermostable thiamine-inactivating factors

About 50 kinds of plants including ferns were tested to investigate the activity of the thermostable thiamine-inactivating factors. The results are shown in Tables 2 and 3. From these tables it is seen that some of the plants—shiitake, okra, butterbur flower stalk (fukinoto), black tea, and coffee—have factors which primarily produce the oxidized form of thiamine (thiamine disulfide) from thiamine. Extracted factors from ferns showed some ability to produce thiamine disulfide (in amounts from 2 to 27% of the thiochrome negative form of thiamine).

2. Thiamine-decomposing activity of caffeic acid

The results of experiment under different conditions are shown in Tables 4 and 5 and Figs. 1, 2, and 3. From the data in the Tables and Fig. 1, it is obvious that production of thiamine disulfide is not consistently large. As shown in Fig. 3,
Table 2. Distributions of thermostable thiamine-inactivating factors (I).

| Samples                          | Thiochrome negative form (%) | SSB<sup>†</sup> (%) |
|----------------------------------|------------------------------|---------------------|
| 1. Urajiro (Gleichenia glauca Hook) | 26                           | 4                   |
| 2. Koshida (Gleichenia dichotoma Hook) | 15                           | 27                  |
| 3. Tamashida (Nephrolepis cordifolia Presl) | 51                           | —                   |
| 4. Shinobu (Davallia Mariesii Moore) | 49                           | 11                  |
| 5. Inugankuso (Pentarthizidium orientale) | 27                           | 15                  |
| 6. Geijigishida (Dryopteris decursivelpinnata O. Kuntze) | 34<sup>b</sup> | 27<sup>b</sup> |
| 7. Komochishida (Woodwardia orientalis Swartz) | 8                            | 7                   |
| 8. Inuwarabi (Athyrium niponicum Hance) | 58<sup>b</sup>             | 2<sup>b</sup>       |
| 9. Bracken (Pteridium aquilinum Kuhn) | 47                           | 6                   |
| 10. Royal fern (Osmunda japonica Thunb) | 18                          | 79                  |
| 11. Fresh shiitake (Lentinus edodes Sing) | 53                            | 13                  |
| 12. Dried shiitake (Lentinus edodes Sing) | 14                          | 99                  |
| 13. Okra (H. esculentus L.) | 30                           | 67                  |
| 14. Black tea | 73<sup>b</sup> | 30<sup>b</sup> |
| 15. Coffee | 67<sup>b</sup> | 7<sup>b</sup> |
| 16. Butterbur flower stalk (Petasites japonicus' stalk [Fukinoto]) | 24 | 40 |

<sup>a</sup> % to the amounts of thiamine in thiochrome negative form.
<sup>b</sup> ×20 extraction: The rest of the samples ×10 extraction.

Table 3. Distributions of thermostable thiamine-inactivating factors (II).

| Samples<sup>a</sup> | Thiochrome negative form (%) | SSB<sup>†</sup> (%) |
|----------------------|------------------------------|---------------------|
| 1. Lettuce | 47 | 4 |
| 2. Salad leaf (Beta vulgaris L. var. Cicla L.) | 50 | 10 |
| 3. Japanese honewort (Cryptotaenia canadensis D.C.) (Mitsuba) | 43 | 6 |
| 4. Carrot (reddish) (Ducus Carota L. var. sativa D.C.) | 47 | 30 |
| 5. Carrot (yellowish) | 7 | 21 |
| 6. Spinach (Spinacia oleracea L.) | 29 | 30 |
| 7. Great burdock (Arctium lappa L.) | 31 | 11 |
| 8. Chrysanthemum (yellow) | 57 | 12 |
| 9. Chrysanthemum (white) | 30 | 13 |
| 10. Green tea | 26 | 0 |
| 11. Horsetail (Equisetum arvense L.) | 25 | 5 |
| 12. Tojisha (Beta vulgaris L. var. Cicla L.) | 21 | 10 |
| 13. Apple | 10 | 12 |
| 14. Celery (Apium graveolens L.) | 20 | 0 |
| 15. Japanese radish | 7 | 0 |
| 16. Azuki beans (Phaseolus angularis W. F. Wight) | 6 | 0 |
| 17. Pumpkins (Cucurbita maxima Duchesne) | 7 | 6 |

<sup>a</sup> % to the amount of thiamine in thiochrome negative form.
<sup>b</sup> ×10 extraction.

Samples in which the factor was not detected are as follows:
- Tomato, cucumber, taro, broccoli, nanohana, yam, sweet potato, flammulinaw velutips, mushroom “shimeji,” onion, sweet pepper, red cabbage, asparagus, radish, soybeans, peanuts, sesame seed, tangerine, grape.
the course of the thiamine decomposition estimated by the thiochrome method is almost identical with that estimated by the diazo method. This means that the decomposition mechanism of thiamine is not deamination of pyrimidine moiety of thiamine by caffeic acid. If the value estimated by the diazo method on the reaction mixture of thiamine and caffeic acid is higher than that by the thiochrome method, it may be possible that deamination has occurred in the pyrimidine moiety of thiamine.

3. Ability of caffeic acid to destroy thiamine-analogues

Reactions of OHB₃, HET, and HPT with caffeic acid were compared with that of thiamine as described above. As shown in Fig. 4, thiamine is more sensitive to decomposition with caffeic acid than are the analogues tested.

4. Thiamine-decomposing activities of other factors

1) Catechol. Catechol in molar ratios to thiamine of 0.1 to 1.0 was tested

| Expt. No. | pH | Tem. (°C) | Hours | Thiochrome negative form (A) (%) | SSBr/A (%) |
|-----------|----|-----------|-------|---------------------------------|------------|
| 1         | 6.0| 20        | 1     | 4.0                             | —          |
| 2         | 7.7| 37        | 18    | 79.0                            | —          |
| 3         | 7.7| 37        | 18    | 83.6                            | 5.6        |
| 4         | 7.7| 37        | 15    | 79.0                            | 10.3       |
| 5         | 7.7| 37        | 21    | 98.0                            | 11.6       |

Thiamine 5 µg and caffeic acid 2 µg with phosphate buffer (total 3 ml) were incubated.

| Expt. No. | pH | Tem. (°C) | Hours | Thiochrome negative form (A) (%) | SSBr/A (%) |
|-----------|----|-----------|-------|---------------------------------|------------|
| 1         | 7.7| 37        | 44    | 30.0                            | —          |
| 2         | 7.7| 37        | 48    | 60.0                            | —          |
| 3         | 7.7| 45        | 792   | 70.0                            | 16.1       |
| 4         | 7.7| 45        | 91    | 60.0                            | 18.0       |
| 5         | 7.7| 45        | 193   | 99.0 [3.0]                      | 14.9       |
| 6         | 7.7| 45        | 47    | 88.2                            | 13.1       |
| 7         | 7.7| 45        | 120   | 99.9 [1.5]                      | 1.5        |
| 8         | 7.7| 45        | 23    | 49.2                            | 21.6       |
| 9         | 7.7| 45        | 46    | 95.2                            | (5.2)      |
| 10        | 7.7| 45        | 69    | 99.1 [1.6]                      | 2.6        |

[ ], Thiochrome; (), control, without caffeic acid.

Thiamine 5 mg and caffeic acid 2 mg with phosphate buffer (total 3 ml) were incubated.
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Fig. 1. Decomposition percentages of thiamine with caffeic acid.

Test at pH 7.7, 45°C

| Decompo. (%) |
|--------------|
| 0            |
| 25           |
| 50           |
| 75           |
| 100          |

| hr     |
|--------|
| 23     |
| 46     |
| 69     |

Fig. 2. Decomposition percentages of thiamine with caffeic acid. Thiamine 5 mg/0.1 ml, caffeic acid 2 mg/2 ml, phosphate buffer (pH 7) 0.9 ml were incubated at 45°C.

at pH 7, 35°C and pH 7, 45°C. The results are shown in Figs. 5 and 6. 0.2 mole equivalent of catechol to thiamine destroyed nearly 100% of thiamine at pH 7, 45°C. However, about 10–16% reversed to thiamine with cysteine. At 37°C the rate of reaction was much slower.

2) 6, 7, 4'-Trihydroxyisoflavone. One of the isoflavones, which was isolated (10) from the fermented soybeans (tempeh) as Factor 2 and has an o-dihydroxy group, 6, 7, 4'-trihydroxyisoflavone, was tested at pH 7.5, 45°C (Factor 2 was insoluble in water at pH 7.0). The results are shown in Fig. 7. About 70–100% of the thiochrome negative form of thiamine were reversed to thiamine
Fig. 3. Decomposition percentages of thiamine with caffeic acid. Thiamine 5 mg/0.1 ml, caffeic acid 2 mg/2 ml, phosphate buffer (pH 7) 0.9 ml were mixed and incubated at 45°C.

Fig. 4. Decomposition percentages of thiamine and its analogue with caffeic acid. Reaction conditions are the same as those shown in Fig. 3. Control value was subtracted from each test.

with cysteine. Thus, the reaction mechanism of thiamine with Factor 2 was entirely different from that with caffeic acid or catechol.

3) Pyrogallol, quercetin and rutin. Pyrogallol, quercetin or rutin was tested at pH 7, 45°C with equimolar amounts of thiamine. The results are shown in Table 6.

5. Detection of the decomposition products of thiamine with caffeic acid, catechol, and 6, 7, 4'-trihydroxyisoflavone (Factor 2)

Paper partition chromatograms of the reaction mixture of thiamine with caffeic acid, and Factor 2 are shown respectively as 1, 2, 3 and 4, and the standards
Fig. 5. Decomposition percentages of thiamine with catechol (I). Thiamine 5 mg/0.1 ml, catechol 0.1–1 mole in 2 ml equivalent to thiamine, phosphate buffer (pH 7) 1.9 ml were incubated at 37°C.

Fig. 6. Decomposition percentages of thiamine with catechol. Reaction conditions are the same as those shown in Fig. 5, except temperature was at 45°C.

Fig. 7. Thiochrome negative percentages of thiamine with Factor 2. Factor 2 4 mg/2 ml, B1 5 mg/0.1 ml, buffer solution (pH 7.5, 45°C) 1.9 ml, B1: Factor 2 (1:1), thiochrome negative % (A), SSB1 % to (A).

As seen in the chromatograms of reaction mixtures of thiamine with caffeic acid or catechol, the thiazole moiety of thiamine was not detected, but the pyrimidine moiety was indicated by a spot with an Rf value identical with that of 2-methyl-4-amino-5-aminomethyl-pyrimidine (Pm-CH2-NH2), not with 2-methyl-4-amino-5-hydroxymethyl-pyrimidine (PM-CH2OH). In the case of the reaction mixture with Factor 2, however, a major spot of thiamine disulfide was detected, but neither a pyrimidine moiety nor a thiazole moiety was detectable. This result corresponded with evidence for the formation of thiamine...
disulfide in the reaction mixture of thiamine and Factor 2 shown in Fig. 7. The parts of the paper strip corresponding to the spot with Rf 0.53 of the reaction mixture of thiamine and caffeic acid or catechol and that of Pm-CH₂-NH₂ as a standard were extracted with acidified water and the UV spectra measured at pH 2 and pH 9. The extracts from the reaction mixtures showed almost identical curves with the standard at pH 2 and 9, respectively, as shown in Fig. 9.

6. Estimation of 2-methyl-4-amino-5-aminomethyl-pyrimidine in the reaction mixture of thiamine with caffeic acid or catechol

Known amounts of thiamine and Pm-CH₂-NH₂ were chromatogrammed and

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**Table 6. Percent of thiochrome negativity and formation of SSB₁ with some factors (pH 7.0, 45°C)**

| Factors   | Reaction time (hr) | Thiochrome neg. % (A) | SSB₁/(A) % |
|-----------|--------------------|------------------------|------------|
| Pyrogallol| 24                 | 59                     | 48         |
|           | 48                 | 71                     | 40         |
|           | 72                 | 76                     | 34         |
| Quercetin | 24                 | 39                     | 26         |
|           | 72                 | 72                     | 24         |
|           | 120                | 94                     | 22         |
| Rutin     | 24                 | 11                     | 59         |
|           | 72                 | 62                     | 25         |
|           | 96                 | 83                     | 18         |

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**Fig. 8. Paper partition chromatogram of reaction mixture with caffeic acid or Factor 2.**
standard curves shown in Fig. 10 were prepared from acidified water extract from parts of the paper strips corresponding to the spots of thiamine or the pyrimidine. Since thiamine and Pm-CH$_2$-NH$_2$ was difficult to separate with various developing solvents, values corresponding to the thiamine remaining estimated in the reaction mixture were subtracted from the value of samples determined from
the chromatograms. Extracted samples of paper strips subjected to the reaction mixture were compared with those of standard curves and the total amounts in the reaction mixture calculated. As shown in Table 7, around 10% thiamine disulfide and 70% Pm-CH₂-NH₂ and trace amounts of thiochrome were produced from thiamine with caffeic acid or catechol under the above reaction conditions. On the other hand, about 70% was thiamine disulfide in the reaction mixture of thiamine and Factor 2; Pm-CH₂-NH₂ was difficult to detect from the mixture on the paper partition chromatogram.

DISCUSSION

Water extracts of some plants, especially ferns, contain thermostable thiamine-inactivating factors. The identified active factors are found to be some flavonoids and other compounds with an o-diphenol group in the molecule.

Their reaction mechanisms differ depending on the reaction condition, amounts of oxygen dissolved, pH, temperatures, etc., and also upon the chemical structure of factors in plants. For a reaction of thiamine with each factor, there are many combinations of the reaction conditions.

In the present study, we have used distilled water solutions of thiamine buffered at pH 7.0–7.7 and 37° or 45°C to investigate the reaction mechanisms with several different factors. It was observed that around 10–20% of the thiochrome negative form was thiamine disulfide produced by the mechanism 2 in Fig. 11,

![Fig. 11. Possible pathway of thiamine decomposition with caffeic acid (C.A.)](image)

when thiamine was reacted with caffeic acid or catechol, and also that there was a breakdown of the thiazole moiety resulting in the formation of Pm-CH₂-NH₂.

MATSUKAWA et al. (11) reported that when thiamine hydrochloride in aqueous solution is heated, it is decomposed, forming Pm-CH₂-NH₂, γ-aceto-γ-mercapto-propyl alcohol, and formic acid by the mechanism 1 shown in Fig. 11. That we have detected Pm-CH₂-NH₂ by paper partition chromatography to the extent of about 70% of the thiamine decomposed in the reaction mixture of thiamine
and caffeic acid or catechol, strongly supports the presumption that the mechanism in this case is the same as that described by MATSUWA et al. (II). On the other hand, there are also many factors in nature, such as flavonoids, which accelerate mainly or partly the oxidation of thiamine into thiamine disulfide, as indicated in the case with 6, 7, 4'-trihydroxyisoflavone, quercetin and rutin.

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