DETECTION OF THIOTHIAMINE IN NATURAL FOODSTUFFS¹

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Thiothiamine (I), an intermediary product in the industrial synthesis of thiamine, is produced by aeration from an alkaline solution of thiamine. Thiothiamine is converted into thiamine by an oxidant, and a dilute solution of thiothiamine is oxidized into thiamine by the oxygen dissolved in the solution (1). The thiothiamine in natural foodstuffs, if any,

may be oxidized to thiamine during zeolite treatment. The existence of thiothiamine in the onion has been proved by Kawasaki and Horio (2) by paper chromatography. Yurugi *et al.* (3) have reported the formation of thiothiamine by heating the ethanol extract of the onion together with thiamine at pH 8.

The authors have studied the assay method of thiothiamine in onions, especially in the presence of thiamine in order to test the occurrence of thiothiamine in natural foodstuffs.

EXPERIMENTAL

1. Preparation of Extract

Twenty grams of a vegetable was directly or after heating in a boiling water-bath for 5 minutes, homogenized in a glass homogenizer and 1 N HCl was added up to 100 ml. In all the experiments, the distilled water was boiled to remove free chlorine.

2. Assay of Thiothiamine

Benzoyl Peroxide Method—Thiothiamine is oxidized with alkaline benzoyl peroxide solution and the thiochrome thus formed is extracted with isobutanol and determined fluorometrically (3). Under these conditions, no thiamine is produced.

Oxidation with BrCN—Cyanogen bromide method has also been reported for determining thiothiamine (4), but thiochrome is also produced from thiamine, and the fractionation of both substances is difficult. Kawasaki, Suhara and Horio² have found that thiochrome was converted to thiamine by the reaction with

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² Unpublished.

cyanogen bromide at an acidic reaction below pH 4, whereas thiamine failed to react with cyanogen bromide under this condition, but reacted after addition of alkali. The reaction of thiothiamine with cyanogen bromide at an acidic reaction is promoted by heating. The authors have adopted the following condition. Three ml of 3% BrCN was added to 1–3 ml of the sample solution (pH 3.4). After standing at 37° for 30 minutes, the excess BrCN was removed by aeration, and the thiochrome thus formed was extracted with isobutanol, followed by fluorometric estimation using the solution without BrCN treatment as the control. Besides this, a recovery test was made by adding 20 µg thiothiamine (1 µg per g tissue) to the homogenized solution. As shown in Table I, the values determined by benzoyl peroxide method agreed practically with those determined by the above method. Since the values for thiamine remained unchanged after BrCN treatment, thiamine is considered to remain unchanged by BrCN treatment.

TABLE I

Comparison of the Values for Thiamine and Thiothiamine in Vegetables

- A, cold extract of the sample was used directly. Thiamine was determined by ferricyanide method and thiothiamine by benzoyl peroxide method.
 B, the extract was treated with BrCN at pH 3.4. After removal of BrCN, thiamine was determined by ferricyanide method and thiothiamine was determined from the thiochrome formed by BrCN treatment.

	A		B	
	Thiamine	Thiothiamine	Thiamine	Thiothiamine
	µg/100 g	µg/100 g	µg/100 g	µg/100 g
Cabbage	32.4	14.6	32.4	12.6
" + 100 µg thiothiamine	32.4	119.8	32.4	118.2
Cabbage	49.5	15.1	49.9	14.5
" (microbioassay ^a)	46.8		44.2(45.6)	
" + 100 µg thiothiamine		119		123.4
Onion	51	5.9		
" + 100 µg thiothiamine	51	108.8		115.3
Onion	34.6	7.3	32.3	5.1
" (microbioassay ^a)	31.2		31.0 (30.8)	
" + 100 µg thiothiamine		110.6		116

^a With *L. fermenti*.

The parenthesized figures represent the values of the extract after BrCN treatment without aeration.

In the recovery test of thiothiamine, the solution reacted with BrCN for 30 minutes showed spectrophotometrically the absorption maximum at 360 mµ, specific for thiochrome and the value of $E_{1\text{cm}}^{1\%}$ rose by 0.01 after addition of 100 µg thiothiamine per 100 ml.

Microbiological Assay—Thiothiamine cannot be determined with *Lactobacillus fermenti*. However, it can be assayed after conversion of thiothiamine to thiamine as follows.

Sulfurous acid water (containing 24.1 mg SO₂), 1 ml, was added to 50 ml of the solution under test, and the pH was adjusted to 4.5. The solution was filled with water up to 100 ml, followed by incubation at 38° overnight. The excess sulfurous acid was removed by aeration at pH 1 until no blue reaction was observed on an

iodine-starch paper. The thiamine thus formed is determined by the conventional microbioassay technique using *L. fermenti*. The value for thiothiamine was calculated by subtracting the value of the untreated solution from that of the tested. Thiamine was shown not to be decomposed by this treatment; 100 ml of the solution containing 15 or 20 μg thiamine was treated with sulfurous acid and 100% of the added thiamine was recovered. The values obtained by this method agreed well with those obtained by benzoyl peroxide method using the cold extract of onions. Following figures were obtained in the case of onions (parenthesized figures represent the values obtained by benzoyl peroxide method): 15 (15), 13 (15), 19 (18), 17 (18), 18 (20), 16 (14), 18 (29), 18 (20), 18 (28), 13 (13), 13 (11), 40 (29), 15 (16), 20 (29), 15 (15); average $\pm$ standard deviation was 19 ± 7 (19 $\pm$ 6).

3. Assay of Thiamine and Thiothiamine in Onions

Each 20 g was taken from both the external and internal parts of an onion and the cold extracts were prepared. The thiamine and thiothiamine values of the extracts were determined with and without zeolite treatment with the results as shown in Table II. Thiamine was assayed by both ferricyanide method and microbiological method using *L. fermenti*, whereas thiothiamine by both benzoyl peroxide method and microbiological method. Thiamine values of the onion determined by ferricyanide method were 38 μg /100 g in the interior part and 17 μg /100 g in the exterior part, whereas those by microbioassay 42 and 24 μg /100 g respectively. Both values agreed pretty well. Thiothiamine values determined by benzoyl peroxide method were 23 μg /100 g in the interior part and 19 μg /100 g in the exterior part. Both values agreed pretty well with those determined by microbioassay. Considering only the cases with zeolite treatment, the thiamine values obtained by zeolite treatment were, in both chemical and microbiological assays, higher and the thiothiamine values lower than

TABLE II
Thiamine and Thiothiamine Content in Onions

Interior part				Exterior part			
Thiamine		Thiothiamine		Thiamine		Thiothiamine	
Ferri- cyanide method	Micro- bioassay	BPO ^b method	Micro- bioassay	Ferri- cyanide method	Micro- bioassay	BPO ^b method	Micro- bioassay
$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$
19	28	17		14	23	15	15
32	30	14		19	23	12	14
32	36	15		19	25	15	
28(43) ^a	42(52)	17(0)	15	19	37(38)	17(0)	15
56(50)	45(44)	34(0)	25	15	29(41)	24(0)	24
32(38)	31(60)	17(2)	25	23	31(52)	12(0)	21
51(50)	50(52)	28(5)		5(6)	6	28(3)	
60(76)	64(76)	34(3)		23(23)	24	31(9)	
46(46)	49(51)	31(5)		17(33)	20	20(3)	
M 38	42	23		17	24	19	
M' 46.51	47.54	21.3		16.17	32.44	18.3	

^a The figures in parentheses represent the values of zeolite method.

^b Benzoyl peroxide method.

M, average values.

M', averages of the values determined after zeolite treatment.

those obtained without zeolite treatment. This can be explained by the finding that thiothiamine is partially converted to thiamine in the course of zeolite treatment (6).

In order to test a question if the thiamine in onion homogenate may be converted into thiothiamine in the course of time, the following experiment was undertaken. 30–350 μg thiamine per 100 g was added to the onion. A part was allowed to stand as it is and the other part was heated in a boiling water-bath for 10 minutes. Then each part was homogenized and allowed to stand at pH 6 and 38° for 24 hours. They were analyzed for thiothiamine by both benzoyl peroxide method and microbioassay. As shown in Table III, the conversion of thiamine into thiothiamine was not observed.

TABLE III

Change of Thiamine Added to Onion Extract

Thiamine was added to 100 g of onions followed by homogenization, then A assayed immediately, B after incubation at 38° for 24 hours. Thiamine was determined microbiologically and thiothiamine by benzoyl peroxide method. The figures in parentheses represent the values determined after addition of thiamine, heating and homogenization.

Thiamine added	A		B		
	Thiamine	Thiothiamine (BPO method)	Thiamine	Thiothiamine (micro-bioassay)	Thiothiamine (BPO method)
μg	μg	μg	μg	μg	μg
350	391 (312)	11 (23)	370 (325)	9 (11)	11 (24)
300	271 (333)	29 (24)	319 (372)	-2 (16)	23 (32)
35	70 (83)	11 (17)	74 (78)	5 (12)	31 (23)
30	80 (83)	15 (12)	34 (42)	3 (9)	13 (17)

4. Detection of Thiothiamine in Vegetables

The test for thiothiamine was made in various vegetables by benzoyl peroxide method and in those positive for thiothiamine the determination of thiamine and

TABLE IV

Thiothiamine Contents in Vegetables Determined by Benzoyl Peroxide Method and Microbioassay

Sample	No. of assay	Thiamine (microbioassay)	Thiothiamine (microbioassay)	Thiothiamine (BPO method)
		$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$
Udo (<i>Aralia cordata</i>)	1	59	-1	6
Lettuce	1	102	0	0
Sprouting bean	1	148	-6	4
Parsley	1	67	0	0
Potato	1	105	0	6
Celery	1	108	-4	6
Pea	1			6
Spinach	1	130	-2	0
Stone-leek	1	66	-35	6
Welsh onion	4	61	-33	31
Scallion	2	44	+23	40
Garlic	6	283	-134	11
Cabbage	4	55	-25	29
Sprouting cabbage	4	101	-40	76

thiothiamine was made microbiologically. As summarized in Table IV, thiothiamine was negative by benzoyl peroxide method in the lettuce, parsley, celery and spinach, and almost negative in the pimiento, cucumber, tomato and pea. Thiothiamine was markedly detected by benzoyl peroxide method in cabbages, sprouting cabbages, Welsh onions, scallions and garlies and they were analyzed carefully for thiothiamine. In microbiological assay, thiothiamine was scarcely detected except for garlies and in many of them thiamine was decreased by sulfite treatment. Consequently, hydrogen peroxide was tried instead of sulfite.

5. Oxidation of Thiothiamine with Hydrogen Peroxide

Hydrogen peroxide solution containing 500 mg H_2O_2 , was used as an oxidant instead of sulfurous acid water. Other procedures were the same as given above. First, cabbage was used as a sample and the recovery test was carried out with the results given in Table V. The amounts of thiamine determined after H_2O_2 treatment following addition of 50 μg thiothiamine per 100 ml were 50–90 % of the values to be expected, and those following addition of 50 μg thiamine per 100 ml were 10–70 % of those to be expected. These findings show that thiamine is partially destroyed by H_2O_2 treatment and the H_2O_2 treatment can not be a satis-

TABLE V
*Treatment of Hydrogen Peroxide and Recovery Test of
Thiamine or Thiothiamine in Cabbage Extract*

- A The cold extract was directly treated, thiamine was determined microbiologically and thiothiamine by benzoyl peroxide method.
B Thiothiamine or thiamine was added and after H_2O_2 treatment the extract was treated as above.
In the recovery tests, 50 μg of thiothiamine or thiamine was added.

Recovery test of thiothiamine					
Sample	A		B (Thiothiamine added)		
	Thiamine	Thiothiamine	Thiamine	Expected value	Recovery
	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	per cent
D	26.5	40	105	132	80
D ₂	42.5	40	98	147	63
E ₁	56.0	40	115	161	72
E ₂	58.0	40	90	164	55
G interior	73.5(63.6)	11	75.6(64.5)	86	88
G interior	73.5(63.6)	11	101.2(96.8)	157	64
G exterior	51.5(42.4)	11	54 (40)	64	84
G exterior	51.5(42.4)	11	71.4(65.8)	135	53
Recovery test of thiamine					
Sample	A		B (Thiamine added)		
	Thiamine	Thiothiamine	Thiamine	Expected value	Recovery
	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	per cent
F interior	129	88	100.3	231	57
F interior	129	88	94.5	281	34
F exterior	53.5	55	36.6	118	31
F exterior	53.5	55	16.0	168	9.5
G interior	73.5	11	94 (100)	136	69
G exterior	51.5	11	82.5(75.6)	119	69

The parenthesized figures indicate the values determined with ferricyanide.

TABLE VI
*Thiothiamine Contents in Vegetables Determined by
 Microbiological Assay Following Hydrogen Peroxide Treatment*

Sample	No. of determi- nation	Thiamine (microbioassay)	Thiothiamine (microbioassay)	Thiothiamine (BPO method)
		$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$	$\mu\text{g}/100\text{ g}$
Scallion	2	44	+33	40
Garlic	6	283	-46	11
Cabbage	4	71	+14	29

factory method for determining thiothiamine.

The results determined by this method in the scallion, cabbage and garlic are shown in Table VI. In scallions and garlies, formation of thiamine by H_2O_2 treatment was observed, showing the existence of thiothiamine.

DISCUSSION

The existence of thiothiamine in onions seems to be definite, since about the same values were obtained by three different methods, benzoyl peroxide method, BrCN-oxidation method and microbioassay.

It was also detected in scallions by benzoyl peroxide method and microbioassay, but the latter value was lower than the former. In cabbages, thiothiamine was also detected by both benzoyl peroxide and BrCN methods. However, the value for thiamine was decreased by the microbioassay following sulfite treatment. Since thiamine alone was not decomposed by sulfite treatment, but destroyed after addition of cabbage extract, a thiamine-destroying factor or a factor capable of destroying thiamine in the existence of sulfite seems to be present in cabbages. In cabbages, a little thiothiamine was detected by converting thiothiamine into thiamine by H_2O_2 treatment. However, the recovery of thiamine or thiothiamine was very poor after adding thiothiamine or thiamine, possibly due to the decomposition of thiamine by H_2O_2 treatment. The existence of thiothiamine in cabbages seems to be certain, as thiothiamine is definitely detected by three methods, *i.e.*, microbioassay following H_2O_2 treatment, benzoyl peroxide method and BrCN method. In garlies no increase of thiamine was detected by both sulfite and H_2O_2 treatments, but on the contrary the value fell. In case of garlies, however, detection of thiothiamine may be difficult owing to the formation of allithiamine and more detailed investigation is necessary.

Thiothiamine was detected by both chemical and microbiological methods in onions, cabbages and scallions. Thus the possibility of existence of thiothiamine in vegetables has been amply proved. However, alkylsulfide, alkyldisulfide and other sulfur compounds have been shown to be present in these vegetables and there is a possibility of producing thiothiamine from thiamine during homogenation. Yurugi (3) has reported that thiothiamine was formed from thiamine when the vitamin was allowed to react with the extract of onions, Welsh onions or chives at pH 8. In the author's experiment, onions were extracted in an acidic solution and the thiamine added to the onion homogenate did not change into thiothiamine. Therefore, the thiothiamine detected can not have been formed from thiamine during the procedure.

SUMMARY

For determination of thiothiamine in natural foodstuffs two chemical methods other than benzoyl peroxide method which had hitherto been used, have been described. One is an oxidation method by BrCN in an acidic medium and the other a microbiological method using *L. fermenti*, following conversion of thiothiamine into thiamine by sulfite. Applying these methods, thiothiamine contents in various vegetables were determined. Thiothiamine was detected in onions, Welsh onions, scallions, garlics, cabbages, sprouting cabbages by benzoyl peroxide method. In onions, thiothiamine was determined by benzoyl peroxide, BrCN and microbiological methods with the results agreeing fairly well one another. In scallions, thiothiamine was detected also microbiologically. However, the values were lower than those determined by benzoyl peroxide method, whereas in cabbages thiothiamine was not detected microbiologically. After converting thiothiamine into thiamine by H_2O_2 treatment, thiothiamine could be detected also in cabbages, but the recovery was poor, possibly due to the decomposition of thiamine by H_2O_2 . The existence of thiothiamine in natural foodstuffs needs further investigations.

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