Metabolism of L-Theanine in Tea Leaves

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L-Theanine (γ-glutamylethylamide), a principal component of the taste of green tea, is synthesized from glutamate and ethylamine by theanine synthetase[3,4] in the root of tea, and the amount of which comes to 0.5 to 2% in tea leaves. It was clarified that the ethylamine moiety of theanine was well incorporated into catechins in tea seedlings[1]. This suggests that theanine is not an end product but it is involved in the biosynthesis of other compound in tea plants. Theanine also appears to be involved in the storage or transport of nitrogen in a non-toxic form in tea plants. However, little is known about the physiological significance and decomposition mechanism of theanine in tea plants. This paper summarized the some properties of the two enzymes taking part in the metabolism of theanine in tea leaves and presented the outline of the route of theanine metabolism in tea leaves.

An enzyme hydrolyzing L-theanine in tea leaves[5]

Theanine hydrolase and glutaminase activities in tea leaves were assayed by measuring enzymatically released ethylamine or ammonia from L-theanine or L-glutamine. The *o*-phthalaldehyde derivatives of ethylamine (OPT-ethylamine) and ammonia (OPT-ammonia) were measured by a reverse phase high performance liquid chromatography (HPLC) and recorded with a spectrofluorometric detector[6].

Since tea leaves contained an amine oxidase, it was necessary to add an amine oxidase inhibitor in the reaction mixture. Potent inhibitors of amine oxidase (hydroxylamine, semicarbazide, and ipronizide) protected the released alkylamine from enzymatic oxidation.

The pH optima for glutaminase and theanine hydrolase activity, extracted from acetone powder of tea leaves, are shown in Fig. 1. Both activities had pH optima at 8.5. Glutaminase activity had another pH optimum at 6.5, but theanine hydrolase activity did not show a clear peak at pH 6.5.

Crude enzyme solution extracted from acetone powder was put on a DEAE-cellulose column, and theanine hydrolase and glutaminase were eluted with a linear concentration gradient of KCl. A summary of the chromatography is given in Table 1. The theanine hydrolase activity could not be separated from the glutaminase. Since the yields of the enzyme activities were not the same value, theanine hydrolytic enzyme may be a different enzyme from glutaminase.

The theanine hydrolytic activity was inhibited by

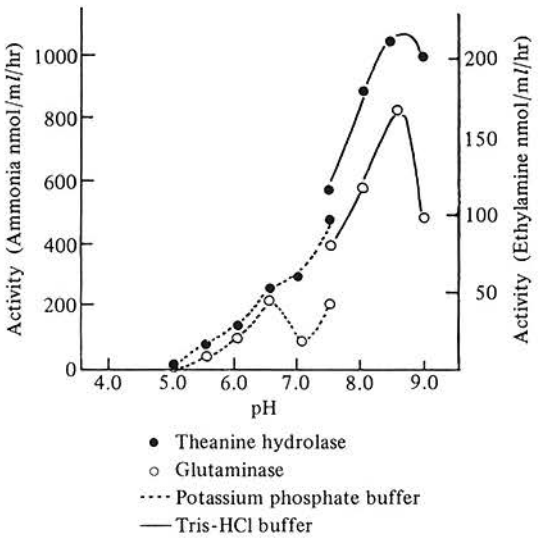

Fig. 1. Optimum pH of theanine hydrolase and glutaminase in tea leaves

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Table 1. Specific activities and yields of theanine hydrolase and glutaminase in tea leaves purified by DEAE-cellulose column chromatography

Enzyme	Sephadex G-25			DEAE-Cellulose		
	To. act. (units)	Sp. act. (units/mg)	Yield (%)	To. act. (units)	Sp. act. (units/mg)	Yield (%)
Theanine hydrolase	1248	13.6	100	925	62.5	74
Glutaminase	8062	87.8	100	2832	192.6	35

Table 2. Hydrolysis of various substrates by the theanine hydrolase in tea leaves

Substrate (83.3 mM)	Activity (nmol/mg/hr)	(%)
GMA[a)]	14.8	77
Theanine	19.3	100
GPA[b)]	27.9	144
GBA[c)]	53.3	276
GiBA[d)]	26.4	136
GAA[e)]	40.7	211
N-Methylpropionamide	0	0
N-Ethylpropionamide	0	0

a) γ-glutamylmethylamide, b) γ-glutamylpropylamide, c) γ-glutamylbutylamide, d) γ-glutamylisobutylamide, e) γ-glutamylamylamide.

acidic amino acids and L-alanine, and stimulated by L-malic acid.

The substrate specificity of the enzyme is given in Table 2. When the substrate concentration was 83.3 mM, the activities of hydrolysis of γ-glutamylpropylamide (GPA), γ-glutamylbutylamide (GBA), and γ-glutamyl-isobutylamide (GiBA) were higher than that of theanine. *N*-Methylpropionamide and *N*-ethylpropionamide were not hydrolyzed by the enzyme.

Theanine hydrolase activity increased slightly during the first 10 hr after plucking but thereafter decreased gradually. In contrast, glutaminase activity in tea leaves decreased constantly, and was almost lost 48 hr after plucking. This suggests that theanine hydrolytic enzyme and glutaminase are different enzymes.

It was known that the theanine content of tea leaves slightly decreases after plucking[8)], and also 75% of C^{14}–theanine supplied to tea seedlings was metabolized to other compounds during 60 days of cultivation[2)]. Theanine appears to be metabolized by the enzyme described in this report.

Ethylamine content in tea leaves[6)]

Since it was clarified that the theanine hydrolase in tea leaves releases ethylamine from theanine, the content of ethylamine in tea leaves was measured by HPLC according to the pre-column derivatization method.

Fig. 2. shows the isocratic separation of OPT-amines in tea leaves-extract. The peak of OPT-ethylamine was observed at the retention time of 7.0 min, but other peaks were not detected clearly. Although ethylamine is one of the volatile amines, its content in tea leaves did not change during steaming and lyophilisation. The contents of ethylamine prepared from the leaves steamed and lyophilised, and from non-steamed fresh leaves were 10.3 ± 1.0 and 10.4 ± 0.9, respectively. It is said that the lower aliphatic monoamines are widely distributed in the plant kingdom. In general, their levels are not as high as the levels of free amino acid. As the content

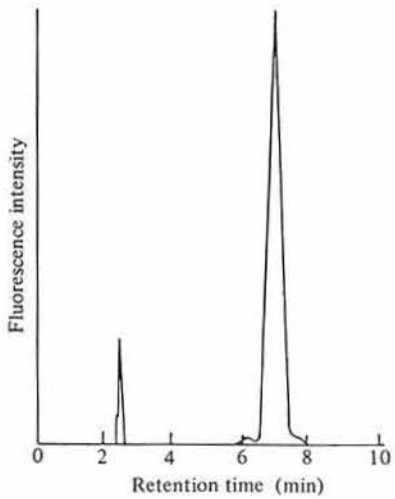

Fig. 2. HPLC isocratic separation of OPT-ethylamine in tea shoots extract

Table 3. Changes in the content of ethylamine in the shoots of cultivar Yabukita after plucking

Date of plucking	Treatment	Ethylamine content (μmol g^{-1})				
		Time after plucking (hr)				
		0	12	24	48	72
May 1	Dark	12.76	8.07	3.55	2.28	1.10
May 7	Dark	8.55	2.83	1.31	1.24	0.62
May 11	Dark	6.70	1.72	1.66	0.97	0.43
May 11	Light	6.70	1.59	0.78	0.51	0.30

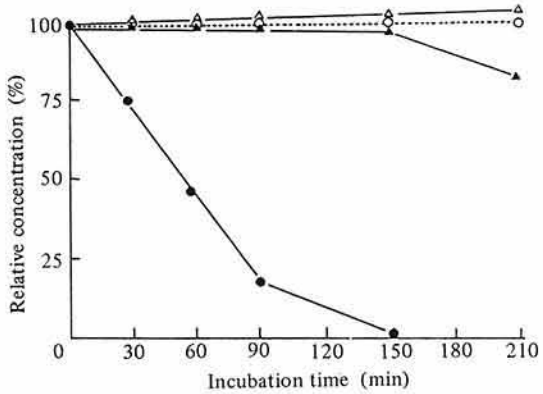

Fig. 3. Effects of polyvinylpyrrolidone and inhibitors of amine oxidase on the changes in the concentration of ethylamine in fresh tea shoots extract during incubation at 37°C
○: Extract prepared without polyvinylpyrrolidone
●: Extract prepared with polyvinylpyrrolidone
▲: 1 mM hydroxylamine was added to the extract prepared with polyvinylpyrrolidone
△: 1 mM iproniazide was added to the extract prepared with polyvinylpyrrolidone

of ethylamine in tea leaves was about 1.5 to 3.0% of the total free amino acid, it was thought that the occurrence of relatively high levels of ethylamine is one of the characteristics of tea plants.

Ethylamine levels in plucked tea leaves declined rapidly, as shown in Table 3. In mature leaves, plucked on May 11, the levels declined more rapidly than in younger leaves, plucked on May 1. When the shoots were illuminated, the level declined slightly more rapidly than in those which were not illuminated.

Fig. 3 shows the changes of ethylamine concentration in the extract from fresh leaves. It declined rapidly in the extract obtained from fresh leaves by homogenizing with polyvinylpyrrolidone (Polyclar AT), but when hydroxylamine and iproniazide (potent inhibitors of amine oxidase) were added, the decline was completely depressed. However, in the extract prepared without Polyclar AT, the ethylamine concentration did not decline during incubation. This result showed that the amine oxidase in the extract was inhibited by phenolics. The amine oxidase may give rise to a decline in the level of ethylamine in the leaves.

Some properties of tea leaf amine oxidase[7]

Since ethylamine levels in tea leaves decreased markedly after plucking, and the decrease was considered to be caused by amine oxidase, the purification and some properties of tea leaf amine oxidase were investigated.

The results of the purification are summarized in Table 4. The total activity increased six-fold with the salting out procedure. Finally, the enzyme was purified about 55-fold from the salting out fraction with a yield of 41.9%.

The purified amine oxidase gave one protein band on disc gel electrophoresis. This protein band showed amine oxidase activity. On SDS disc electrophoresis, a single protein band was obtained.

Some properties of tea leaf amine oxidase were summerized in Table 5. The molecular weight was calculated to be 162,000 by the method of gel filtration. The molecular weight of the subunit deter-

Table 4. Summary of purification of tea leaf amine oxidase

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	6134	12.13	0.002	
Salting out	1647	67.99	0.041	100.0
DEAE-cellulose	482	81.13	0.168	119.3
Sephadex G-200	251	63.21	0.253	93.0
CM-cellulose	165	52.81	0.319	77.7
AH-Sepharose 4B	54	31.89	0.594	46.9
DEAE-cellulose	13	28.45	2.254	41.8

Table 5. Some properties of tea leaf amine oxidase

Property	Value
Optimum pH	7.0
Molecular weight (MW)	162000
MW of subunit	81000
Number of subunit	2
Isoelectric point	5.0
Inhibitor	Copper (Cu^{2+}) binding reagent, Carbonyl reagent (semicarbazide, hydroxylamine, etc.), Iproniazide

Table 6. Substrate specificity of tea leaf amine oxidase

Substrate (4 mM)	Relative activity (%)
Methylamine	24.5
Ethylamine	100.0
n-Propylamine	97.5
n-Butylamine	75.0
n-Amylamine	85.2
n-Hexylamine	71.9
iso-Butylamine	41.9
Benzylamine	87.2
Tyramine	0.1
Histamine	0.7
Serotonin	0.0
Ethanolamine	57.0
Cysteamine	0.0
β-Alanine	0.0
Lysine	0.0
Dimethylamine	0.0
Trimethylamine	0.0
Ethylenediamine	0.4
1,3-Diaminopropane	0.2
1,4-Diaminobutane	0.1
Spermidine	0.0

mined on SDS disc electrophoresis was 81,000. Therefore, the tea leaf amine oxidase was deduced to be a dimer composed of two identical subunits. The isoelectric point of this enzyme determined by the density gradient column method, using 1.45% ampholine pH 3–10, was pH 5.0. And the pH optimum for ethylamine was around 7.0. Copper binding reagents such as diethyldithiocarbamate, cuprizone and 8-hydroxyquinoline inhibited the enzyme activity, but neocuproine, which is known as a specific chelater of cupprous copper, did not inhibit the activity. This result shows that the tea leaf amine oxidase contains copper in the cupric state. Carbonyl reagents such as semicarbazide, hydroxylamine and hydrazine also strongly inhibited the enzyme at 1.0 mM. Iproniazid, known as a potent inhibitor of amine oxidase, also inhibited the activity completely.

The activity of the enzyme on various substrates is shown in Table 6. Ethylamine, *n*-propylamine, *n*-butylamine, *n*-amylamine, *n*-hexylamine, ethanolamine and benzylamine were all oxidized. But the oxidation rate for methylamine was low compared to those for other alkylamines. Diamines such as ethylenediamine, 1,3-diaminopropane and 1,4-diaminobutane were oxidized very slowly. Dimethylamine and trimethylamine were not oxidized. Tyramine and histamine were also oxidized slightly, but cysteamine, β-alanine, lysine and serotonin were not oxidized.

The Michaelis constant, as calculated by the method of Lineweaver and Burk, is shown in Table 7. The Km value for ethylamine was 8.8×10^{-5} M. The Km value for *n*-hexylamine was higher than those for the others.

Tea leaves contained about 15 μmol of ethylamine per gram of dried leaves[6]. Since the acetone powder of tea leaves contained 0.162 units per gram of amine

Table 7. Km values of tea leaf amine oxidase for alkylamines

Substrate	Km ($\times 10^{-5}$ M)
Methylamine	7.1
Ethylamine	8.8
n-Propylamine	4.5
n-Butylamine	4.3
n-Amylamine	9.7
n-Hexylamine	83.0

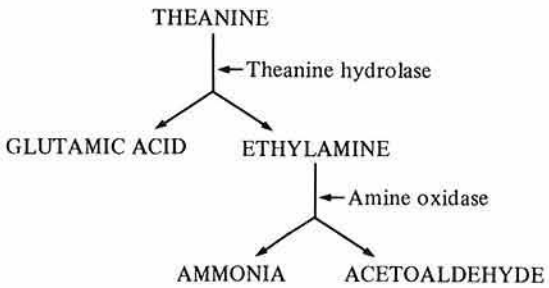

Fig. 4. Suggested route of theanine metabolism in tea leaves

oxidase[7], it was estimated that ethylamine contained in tea leaves was oxidized completely in 90 min by the enzyme in tea leaves. From these results the amine oxidase reported in this paper is considered to be the main enzyme oxidizing the ethylamine in tea leaves.

Conclusion

Up to the present, there has been no attempt to clarify the enzymes taking part in the metabolism of theanine. It was disclosed that the theanine hydrolase described in this report plays an important role to this metabolism. As shown in Fig. 4, at first, theanine is hydrolyzed to glutamic acid and ethylamine by the theanine hydrolase in tea leaves. Then the released ethylamine was oxidized to ammonia and acetoaldehyde by amine oxidase. This released ammonia is suggested to be used as a source of nitrogen in tea leaves.

As described previously, theanine, a predominant amino acid of tea, has been considered to be characteristic of and exclusive to the tea plant. However, the physiological roles of theanine in tea plants were unknown. The results described in this paper are the strong pieces of evidence supporting the conjecture that theanine is one of the means for storage of nitrogen in tea plants.

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