Enzymatic Hydrolysis of Theanine and the Related Compounds

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Some strains of *Pseudomonas* were found capable of utilizing L-theanine and D-theanine as a sole nitrogen and carbon source. L-Theanine and D-theanine were hydrolyzed by the enzyme from *Pseudomonas aeruginosa* to yield stoichiometrically L-glutamate and D-glutamate, respectively, and ethylamine, which were isolated from the reaction mixture and identified. The theanine hydrolase was purified approximately 200-fold. It was shown that the activities of L-theanine hydrolase, D-theanine hydrolase and the heat-stable L-glutamine hydrolase and D-glutamine hydrolase are ascribed to a single enzyme, which may be regarded as a γ-glutamyltransferase from the point of view of the substrate specificity and the properties. This theanine hydrolase catalyzed the transfer of γ-glutamyl moiety of the substrates and glutathione. L-Glutamine and D-glutamine were hydrolyzed by theanine hydrolase and also the heat-labile enzyme whose properties resembled the common glustaminase.

INTRODUCTION

L-Theanine was isolated from tea leaves as an important component of Japanese green tea by Sakato[1] in 1950 and the structure was comfirmed as N-ethyl-γ-L-glutamine, $HOOCCH(NH_2)CH_2CH_2CONHC_2H_5$. The compound was synthesized from L-glutamic acid and ethylamine. Since the discovery of theanine, reports have appeared concerning the isolation and the identification of many derivatives of glutamine and asparagine in which the substituents other than those represented by the common α-amino acids occur at the amide nitrogen atom[3-12]. Information on metabolism of these amide compounds has never been obtained.

Glutaminase (L-glutamine amidohydrolase) and asparaginase (L-asparagine amidohydrolase) which catalyze the hydrolysis of amide groups of L-glutamine and L-asparagine, respectively, are widely distributed in animals, plants and microorganism. Studies on these enzymes have been reviewed by several workers[13-16]. General properties of bacterial glutaminase[17,18] and asparaginase[19-22] were studied well and discussed. It has been believed so far that the substrate specificity of these enzymes is high from the point of view of the structure and also of the optical activity.

In the present paper, the enzymatic hydrolysis of L-theanine, D-theanine and the related amide compounds, the identification of the products and the properties of the enzyme will be described. The studies on the transfer reaction of the γ-

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glutamyl group of the substrates to hydroxylamine to form the hydroxamates will be also presented. The enzyme which catalyzes the hydrolysis of theanine will be named "theamine hydrolase" tentatively.

EXPERIMENTAL

Materials

L-Theanine, D-theanine and D-glutamine were kindly supplied by Dr. Y. Sakato of Shizuoka University and the late Dr. Y. Tsuchiya of Ajinomoto Co. Inc.. Hydroxylapatite was prepared according to the method of Tiselius et al.[23]. The amino acids, DEAE-cellulose (Serva) and the other chemicals were obtained commercially.

Microbiological Methods

The bacteria were, unless otherwise stated, cultivated on the L-glutamate medium composed of 10.0 g monosodium L-glutamate, 1.0 g KH_2PO_4, 2.0 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$ and 0.05 g $FeSO_4 \cdot 7H_2O$ per liter of tap water. The pH was adjusted to 7.0 by the addition of sodium hydroxide.

The cultures were grown at 30°C in 500 ml flasks or 2 l flasks containing 100 ml or 500 ml of medium respectively, under shaking for 18 hours. The cells were harvested by centrifugation and washed twice with 0.85 per cent sodium chloride solution.

Preparation of Partially Purified Enzyme

The washed cells were suspended in 0.05 M phosphate buffer, pH 7.4, containing 0.01 per cent 2-mercaptoethanol, and subjected to a 19 Kc Kaijo Denki sonic oscillater for 5 minutes. The intact cells and debris were centrifuged off at $10,000 \times g$ for 30 minutes. The supernatant was dialyzed against 0.001 M phosphate buffer, pH 7.4, containing 0.01 per cent 2-mercatpoethanol for about 15 hours. The dialyzed solution was used as a cell-free extract.

The partially purified enzyme was prepared as follows. To the cell free extract, was added 2 ml of 1 per cent protamine sulfate solution per 100 mg of protein under stirring. The mixture was centrifuged and the bulky inactive precipitate was discarded. The supernatant was adjusted to pH 7.4 with 10 per cent ammonium hydroxide, and brought to 50 per cent saturation by the addition of solid ammonium sulfate. After standing for 30 minutes, the precipitate was removed by centrifugation. To the supernatant, was added solid ammonium sulfate to bring 80 per cent saturation and the precipitate was collected by centrifugation. The precipitate was dissolved in a small volume of distilled water and dialyzed against 0.002 M phosphate buffer, pH 7.4, containing 0.01 per cent 2-mercaptoethanol for 15 hours. The inactive precipitate formed during dialysis was removed by centrifugation. All operations were carried out at 0—5°C. The dialyzed supernatant was employed as a partially purified enzyme, which was purified about ten-fold from the cell-free extract.

Analytical Methods

Amino acids were determined according to the methods described previously[24] as follows. Amino acids were separated by circular paper chromatography, in which n-butanol: acetic acid: water (4 : 1 : 1) system was employed. The paper was dried after development, and sprayed with 0.5 per cent ninhydrin solution in 75 per cent ethanol. After heating at 50 °C for 30 minutes, the colored zone of the paper was cut off and extracted with 75 per cent ethanol containing 0.005 per cent copper sulfate. The intensity of the color of the extract was measured at 500 mμ.

Hydroxamic acids were determined according to the method of Lipmann *et al.*[25] as follows. One ml of the freshly neutralized 2 M hydroxylamine solution and 3 ml of ferric reagent were added to 2 ml of the reaction mixture after incubation. After protein was filtered off from the mixture, the color intensity developed was measured at 540 mμ. Authentic succinylhydroxamic acid and aspartylhydroxamic acid were employed as the standard for glutamylhydroxamic acid and aspartylhydroxamic acid formed enzymatically, respectively.

L-Glutamic acid was determined manometrically with L-glutamic acid decarboxylase of the acetone dried *Escherichia coli* cells.

Ethylamine was determined according to the modified Conway's micro-diffusion method.[26]

Protein was determined by the method of Lowry *et al.*[27].

Measurement of Enzyme Activity

The activity of hydrolase was assayed by measuring the amount of the amino acid formed from the substrate. The standard reaction mixture contained 40 μ moles of the substrate, 100 μmoles of phosphate buffer, pH 8.0, and the enzyme in a final volume of 2.0 ml. After incubation was carried out at 37 °C for 30 minutes, 0.2 ml of 50 per cent trichloroacetic acid was added to the mixture. A 10 μl alipuot of the deproteinized supernantant was used for amino acid analysis. Specific activity was expressed as micromoles of the amino acid formed from the substrate per mg of protein per hour.

The activity of transferase was determined by measurement of the amount of the hydroxamic acid formed. The standard reaction mixture consisted of 40 μmoles of the substrate, 400 μmoles of freshly neutralized hydroxylamine, 60 μmoles of phosphate buffer, pH 7.0, and the enzyme in a final volume of 2.0 ml. After incubation at 37 °C for 30 minutes, hydroxamic acid produced was determined as described above. Specific activity was expressed as micromoles of hydroxamic acid formed per mg of protein per hour.

RESULTS

Utilization of Theanine and Hydrolysis of Theanine by Bacteria

Growth of several strains of bacteria on a medium containing L-theanine as a sole carbon and nitrogen source was tested. As shown in Table 1, it was found that

Table 1. Growth of bacteria on L-theanine medium.

The medium contained L-theanine instead of sodium L-glutamate in the basal medium. Growth of the organisms was expressed as turibidity at 470 mμ of the medium incubated.

Strain	Incubation period	
	12 h.	24 h.
Pseudomonas fluorescens	0.120	0.790
Pseudomonas fragi	0	0
Pseudomonas riboflavinus	0	0
Pseudomonas strifaciens	0	0
Pseudomonas aeruginosa	0.827	1.237
Pseudomonas sp. T-1	0.232	1.092
Escherichia coli	0.064	0.074
Aerobacter aerogenes	0	0

Pseudomonas aeruginosa, *Pseudomonas fluorescens* and *Pseudomonas sp. T*-1 which had been isolated from the air, utilized L-theanine satisfactorily, while the other strains of *Pseudomonas*, *Escherichia coli* and *Aerobacter aerogenes* could not at all or little grow on the L-theanine medium. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas sp. T*-1 were shown to be able to utilize D-theanine as well as the L-enantiomorph.

L-Theanine hydrolase activity and D-theanine hydrolase activity of the cell-free extracts of the bacteria grown on the media containing peptone as a nitrogen source and sodium L-glutamate as a sole nitrogen and carbon source respectively, were assayed. As demonstrated in Table 2, the highest activity was observed in the cell-free extracts from *Pseudomonas aeruginosa* and *Pseudomonas polycolor* grown on

Table 2. Theanine hydrolase activity of the cell-free extract of various strains.

The cell-free extract was employed and assay of the a enzyme activity was carried out under the standard condition. Peptone medium was composed of 1.0 per cent peptone, 1.0 per cent glycerol, 0.4 per cent K_2HPO_4, 0.2 per cent KH_2PO_4, 0.2 per cent yeast extract and 0.02 per cent $MgSO_4 \cdot 7H_2O$.
Basal medium: See the text. Specific activity is shown in the table.

Strain	Peptone medium		Basal medium	
	Substrate		Substrate	
	L-The*	D-The*	L-The*	D-The*
Ps. fluorescens	1.76	1.83	0.53	0.60
Ps. fragi	1.63	1.53	1.17	1.60
Ps. riboflavinus	0.36	0.47	0.84	1.15
Ps. polycolor	1.09	1.29	5.26	4.09
Ps. aeruginosa	1.16	1.20	10.90	6.71
Ps. strifaciens	0.24	0.47	1.20	1.13

* The abbreviation of theanine

the L-glutamate medium and also the cell-free extracts of the strains which failed to grow on the L-theanine medium showed some extent of activity. *Pseudomonas aeruginosa* was employed in the following experiments.

In the extracts of these organisms, glutamic acid racemase, theanine racemase and D-glutamic acid—pyruvic acid transaminase activities could not be detected. This fact suggests that D-theanine may be hydrolyzed directly to D-glutamic acid, which may be converted to L-glutamic acid or other L-amino acids via certain unknown process.

Isolation and Identification of Reaction Products

A large amount of the reaction mixture whose content was described in the methods, was incubated at 37°C for 3 hours. Theanine in the mixture was hydrolyzed completely. The mixture was deproteinized by the addition of 50 per cent trichloroacetic acid and excess trichloroacetic acid was removed by continuous extracting with ether for 10 hours. The solution was passed through Amberlite IR-120 (H^+ form) column (2.3×25 cm). The column was eluted with 2 M ammonium hydroxide at a flow rate of 30 ml per hour. The effluent containing glutamic acid was concentrated *in vacuo*, and glutamic acid was crystallized from aqueous ethanol. Recrystallization was repeated twice.

Analysis		C%	H%	N%
Calcd. for	$C_5H_9O_4N$	40.81	6.16	9.52
Found	Product from L-theanine	40.66	6.25	9.73
	Product from D-theanine	40.41	6.32	9.35

Both of these preparations produced from L-theanine and D-theanine were also identified as glutamic acid by a comparison of their Rf values with those of authentic glutamic acid in a several solvent systems and the admixture of the preparations with an authentic sample showed one spot.

Optical activity was determined by manometric method with L-glutamic acid decarboxylase (*Escherichia coli* acetone powder) and by Rudolph photoelectric spectropolarimeter Model 200 S.

		CO_2 Out put	$[\alpha]_{589}^{20}$*
Calcd.		5.0 μmoles	
Found	Authentic L-glutamic acid	4.8	+32.4
	Product from L-theanine	4.8	+32.5
	Authentic D-glutamic acid	0.0	−32.8
	Product from D-theanine	0.08	−32.7

*(1 per cent solution in 1 N HCl)

These results demonstrate that L-glutamic acid and D-glutamic acid were produced enzymatically from L-theanine and D-theanine respectively.

Isolation and Identification of Ethylamine

Ethylamine was isolated as the ethylamine picrolonate. A reaction mixture in which phosphate buffer, pH 6.4 was employed instead of the same buffer, pH 8.0 in the standard mixture, was incubated at 37°C for 2 hours. The enzyme preparation used in this experiment was dialyzed thoroughly to be free of ammonium ion. After incubation, protein was removed by the addition of 50 per cent trichloroacetic acid. Ammonium was not detected in the deproteinized supernatant by Nessler's reaction. The supernatant solution which was made alkaline with 40 per cent sodium hydroxide solution, was steam-distilled in a Kjeldahl apparatus for 15 minutes. The tip of the condenser was dipped into 6 ml of ethanol containing picrolonic acid equivalent to ethylamine to be formed. The picrolonate was crystallized. Recrystallization was repeated twice from aqueous ethanol. Authentic ethylamine picrolonate was prepared from pure ethylamine in the same way as described above.

Analysis		C%	H%	N%
Calcd. for	$C_{12}H_{15}O_5N_5$	46.60	4.89	22.64
Found	Authentic ethylamine picrolonate	46.61	4.98	22.58
	Product from L-theanine	46.39	4.95	22.76
	Product from D-theanine	46.56	4.92	23.07

Both preparations of the picrolonate produced from L-theanine and D-theanine showed the same pattarn in infrared spectrum as that of the authentic ethylamine picrolonate (Figure 1).

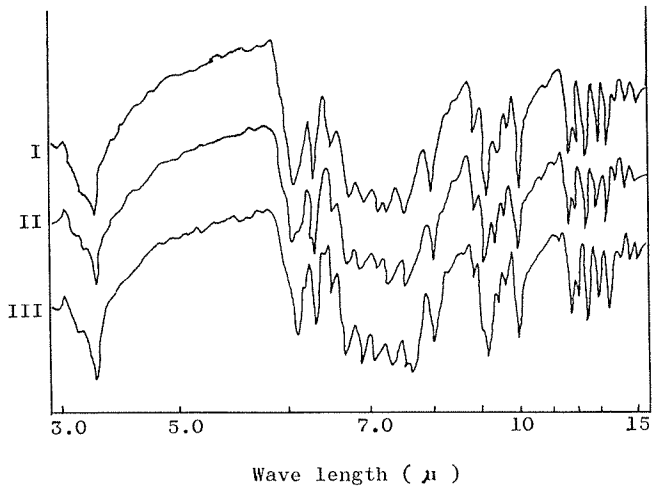

Fig. 1. Infrared spectra of ethylamine picrolonate.
I: Authentic ethlamine picrolonate, II: Picrolonate of the product from L-theanine, III: Picrolonate of the product from D-theanine.

Stoichiometry of Hydrolysis Reaction

The formation of glutamic acid and ethylamine from theanine proceeded stoichiometrically as indicated in Figure 2.

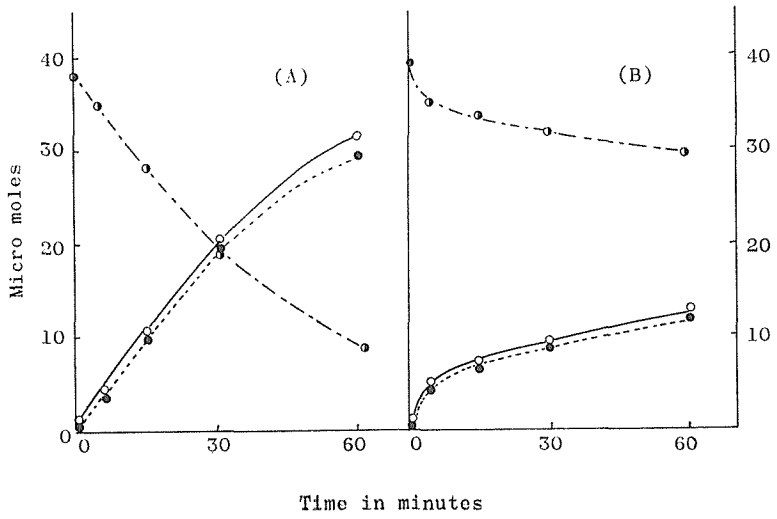

Fig. 2. Theanine hydrolysis reaction.
The reaction mixture consisted of 40 μmoles of L-theanine in (A) or D-theanine in (B), 100 μmoles of phosphate buffer, pH 6.4 and the enzyme (500μg) in a final volume of 2.0 ml. Incubation was carried out at 37°C.
--◑--◑--: Theanine, —○—○—: D-Glutamic acid in (A) and D-glutamic acid in (B), ---●---●---: Ethylamine.

Purification of Enzyme

The enzyme was purified from the extract of *Pseudomonas aeruginosa* as described below. All operations, unless otherwise stated, were carried out at 0—5°C.

Step 1. Extraction. The washed cells were suspended in 0.05 M phosphate buffer, pH 7.4, containing 0.01 per cent 2-mercaptoethanol and sonicated in a 19 Kc Kaijo Denki sonic oscillator for 10 minutes. The intact cells and debris were removed by centrifugation at 10,000×g for 40 minutes.

Step 2. Treatment with protamine. To the supernatant obtained was added 1 ml of 1.5 per cent protamine sulfate solution per 100 mg of enzyme protein under stirring. The bulky inactive precipitate was centrifuged off. The increase in total activity during this procedure suggests that some inhibitory factor may be removed by protamine treatment.

Step 3. Heating treatment. After 1 M acetate buffer, pH 5.3, was added to the supernatant solution to bring the final concentration to 0.04 M, the solution was heated in a water bath of 70°C until the temperature of the solution reached 55°±1°C. The enzyme solution was kept at this temperature for 5 minutes. The enzyme solution became quite milky during this process. The solution was cooled rapidly to 4°C and centrifuged at 10,000×g for 15 minutes.

Step 4. Ammonium sulfate fractionation. The clear supernatant solution obtained was adjusted to pH 7.4 by the addition of 10 per cent ammonium hydroxide solution and brought to 70 per cent saturation with ammonium sulfate. After standing for 30 minutes, the precipitate formed was removed by centrifugation at 10,000×g for 15 minutes. The supernatant was adjusted to 90 per cent saturation with solid ammonium sulfate. After allowing the mixture to equilibrate for 30 minutes, the precipitate was collected by centrifugation at 10,000×g for 20 minutes, dissolved in a small volume of 0.01 M phosphate buffer pH 7.0, and dialyzed against the same buffer containing 0.01 per cent 2-mercaptoethanol for 15 hours. The inactive precipitate formed during dialysis was centrifuged off.

Step 5. DEAE-Cellulose chromatography. The dialyzed enzyme solution was placed on a column of DEAE-cellulose (2×18 cm) equilibrated with 0.01 M phosphate buffer, pH 7.0. Elution was carried out stepwise with 0.01 M phosphate buffer, pH 7.0 containing various concentrations of sodium chloride. The flow rate was 20 ml per hour and 10 ml aliquots of eluate were collected. The elution of the protein was followed by measuring the absorbancy at 280 mμ. Figure 3 shows the typical elution pattern of the enzyme. The major protein peaks are designated by letters A to D in the figure. L-Theanine hydrolase activity was found only in fraction C, while L-glutamine hydrolase activity was separated clearly into fractions

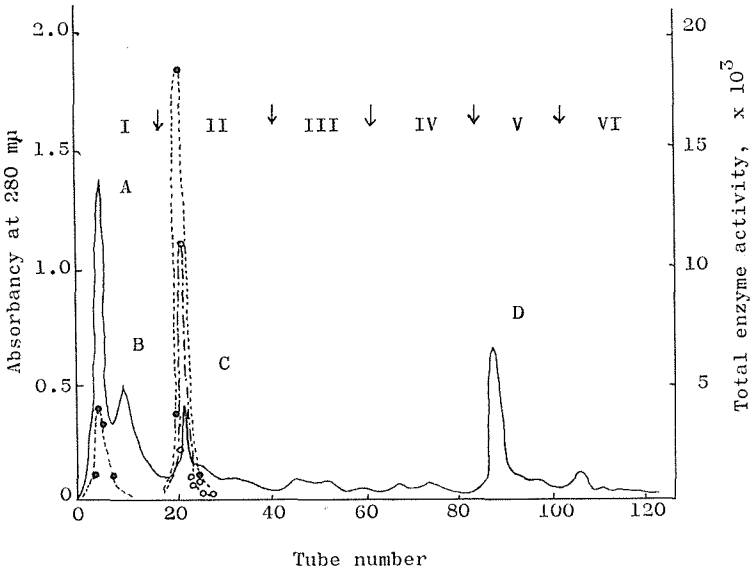

Fig. 3. Chromatography of the enzyme on DEAE-cellulose column.
The column (2.0×18.0 cm) was charged with 89.3 mg of enzyme protein, Elution was carried out by the following buffer containing various concentrations of sodium chloride; I: 0.01 M phosphate buffer pH 7.0, II: I+0.03 M sodium chloride, III: I+0.06 M sodium chloride, IV: I+0.1 M sodium chloride, V: I+0.2 M sodium chloride, VI: I+0.3 M sodium chloride. The total activities of L-theanine hydrolase (—○—·○—), and L-glutamine hydrolase (---●---●---) were indicated.

A and C. The finding is in agreement with the result of the heat stability of the partially purified enzyme to be described later, providing the evidence that glutaminase activity in the crude preparation is due to the two different enzymes. In the subsequent studies on the enzyme purification and the properties of theanine hydrolase and glutamine hydrolase, the fraction C was employed unless otherwise noted, although glutamine hydrolase in fraction A was also investigated as described later.

The fractions containing theanine hydrolase activity were pooled and concentrated by the dialysis against solid polyethyleneglycol.

Step 6. Hydroxylapatite chromatography. The concentrated enzyme solution was subjected to hydroxylapatite chromatography. Hydroxylapatite was packed into a column (2×4 cm) and equilibrated with 0.003 M phosphate buffer, pH 7.0. The enzyme solution was introduced into the column and eluted stepwise with increasing concentration of phosphate buffer, pH 7.0. The buffer was allowed to flow at a rate of 2 ml per hour and eluted fractions of 5 ml were collected. The elution pattern of the enzyme is shown in Figure 4. Theanine hy-

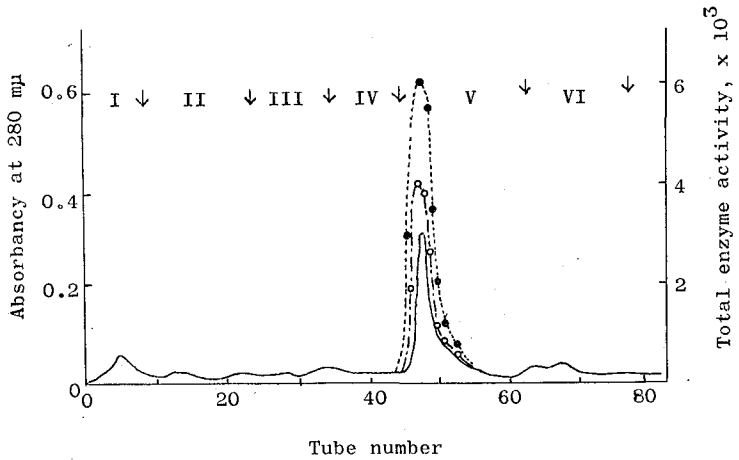

Fig. 4. Chromatography of the enzyme on hydroxylapatite column.
The column (2×4.0 cm) of hydroxylapatite was charged with 10.9 mg of enzyme protein, which had been previously dialyzed against the same buffer at 0 °C for 12 hours. Elution was carried out by the phosphate buffers (pH 7.0) of the following concentration; I: 0.003 M, II: 0.01 M, III: 0.03 M, IV: 0.05 M, V: 0.1 M, VI: 0.15 M. Total activities of L-theanine hydrolase (—-○—-○—) and L-glutamine hydrolase (---●---●---) are shown.

drolase activity and glutamine hydrolase activity were found in the same pattern and the protein peak was in parallel with the enzyme activities. It is suggested that the enzyme protein may be chromatographically homogenous.

In Table 3, a summary of a purification of the enzyme was demonstrated. The over-all purification from the extract to DEAE-cellulose chromatography fractions was approximately 200-fold. Since the hydroxylapatite chromatography showed

Table 3. Summary of purification of enzyme.

Step	Total protein	Total activity	Specific activity	Yield
	mg			%
1. Extraction	3520	39,150	11.1	(100)
2. Protamine treatment	3240	45,080	13.9	115
3. Heat treatment	1315	41,900	30.5	107
4. Ammonium sulfate fractionation	89.3	16,800	188.7	43.1
5. DEAE-Cellulose chromatography	6.1	13,360	2210	34.1

Enzyme activity was expressed as L-theanine hydrolase activity.

no effect to increase the specific activity of the enzyme, the result of this procedure was not described in the table.

Table 4 shows a summary of comparison of four enzyme activities, i.e. L-

Table 4. Comparison of enzyme activity.

Step	L-GHA / D-THA	D-GHA / D-THA	D-GHA / L-THA	D-THA / L-THA
1. Extraction	7.56	6.19	0.76	0.77
2. Protamine treatment	6.89	6.21	0.82	0.74
3. Heat treatment	6.54	6.10	0.75	0.72
4. Ammonium sulfate fractionation	1.85	2.26	0.74	0.91
5. DEAE-Cellulose chromatography	1.67	2.10	0.63	0.74
6. Hydroxylapatite chromatography	1.62	2.05	0.74	0.83

The abbreviation THA and GHA are for theanine hydrolase activity and glutamine hydrolase activity, respectively.

theanine hydrolase, D-theanine hydrolase, L-glutamine hydrolase and D-glutamine hydrolase, during the purification procedures. The ratio of the hydrolase activities of L-theanine and L-glutamine to those of the D-enantiomorphs remained approximately constant and the ratio of L-glutamine hydrolase activity to L-theanine hydrolase activity or of D-glutamine hydrolase activity to D-theanine hydrolase activity decreased remarkably after ammonium sulfate fractionation and thereafter remained almost constant. These facts suggest that L-theanine and D-theanine may be hydrolyzed by a single enzyme or very similar enzymes and that hydrolysis of L-glutamine and D-glutamine may be catalyzed by at least two different enzymes, which were able to be separated by ammonium sulfate fractionation, and also DEAE-cellulose chromatography as illustrated in Figure 3.

Properties of Enzyme. The linear relationship between the amount of the enzyme protein and the amount of L-glutamate or D-glutamate produced from L-theanine or D-theanine, respectively, was obtained under the conditions employed. The formation of glutamate from theanine by theanine hydrolase proceeded as a function of time within 60 minutes under the standard condition.

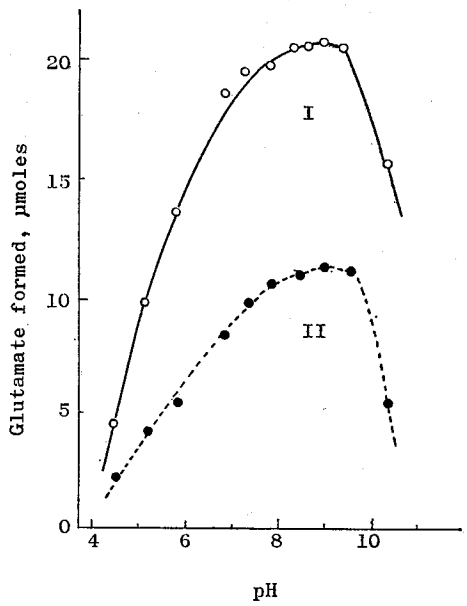

Fig. 5. Effect of pH on the hydrolysis of L-theanine (I) and D-theanine (II).

The buffers used were acetate for pH 4.6, phosphate for pH 5.25 to 8.0, veronal for pH 8.5 to 9.6 and borate for pH 10.4.

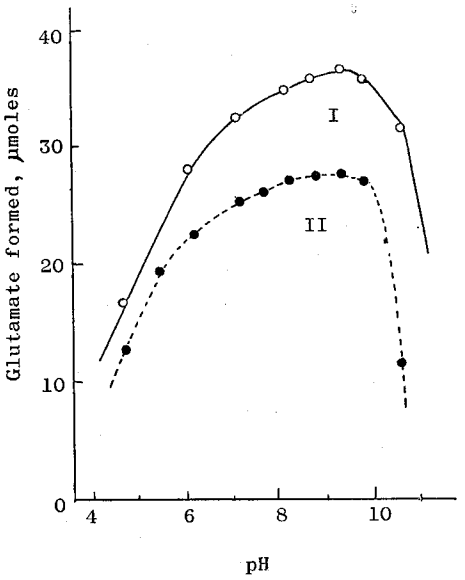

Fig. 6. Effect of pH on the hydrolysis of L-glutamine (I) and D-glutamine (II).

The buffers used were acetate for pH 4.6, phosphate for pH 5.25 to 8.0, veronal for pH 8.5 to 9.6 and borate for pH 10.4.

Effect of pH on Theanine Hydrolase Activity and Glutamine Hydrolase Activity

The effect of pH on the enzymatic hydrolysis of L-theanine, D-theanine, L-glutamine and D-glutamine by the purified enzyme was demonstrated in Figures 5 and 6. Both of these pH-activity curves exhibited a similar tendency and the optimal pH values for these four hydrolase reactions were all in the range of 8.5 to 9.0.

Effect of Heating on the Enzyme Activities

The partially purified enzyme preparation was heated at 55°C for a given period and after being cooled rapidly, the enzyme activities hydrolyzing L-theanine, D-theanine and L-glutamine were determined. As incidated in Figure 7, L-theanine

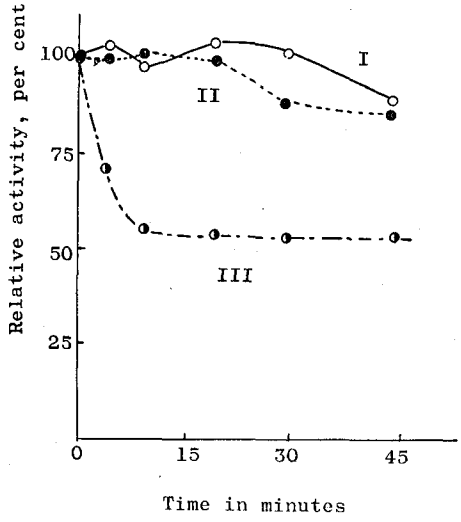

Fig. 7. Effect of heating on hydrolase activity.
Enzyme was heated at 55°C for indicated time before the reaction. Enzyme activity was assayed under the standard condition. I: L-theanine hydrolase activity; II: D-theanine hydrolase activity; III: L-glutamine hydrolase activity.

hydrolase and D-theanine hydrolase activities were stable against heating the enzyme under the condition employed here. L-Glutamine hydrolase activity, however, was reduced rapidly to a half of the original activity by heating for 10 minutes and thereafter the activity was no longer influenced by heating. This finding suggests that L-glutamine may be hydrolyzed by two different kinds of enzymes, both of which were contained together in the partially purified enzyme preparation and one of them is heat-stable and another is heat-labile. The result obtained in this experiment is consistent with the separation of the enzyme activity of hydrolyzing L-glutamine into two different fractions by DEAE-cellulose chromatography demonstrated in Figure 3.

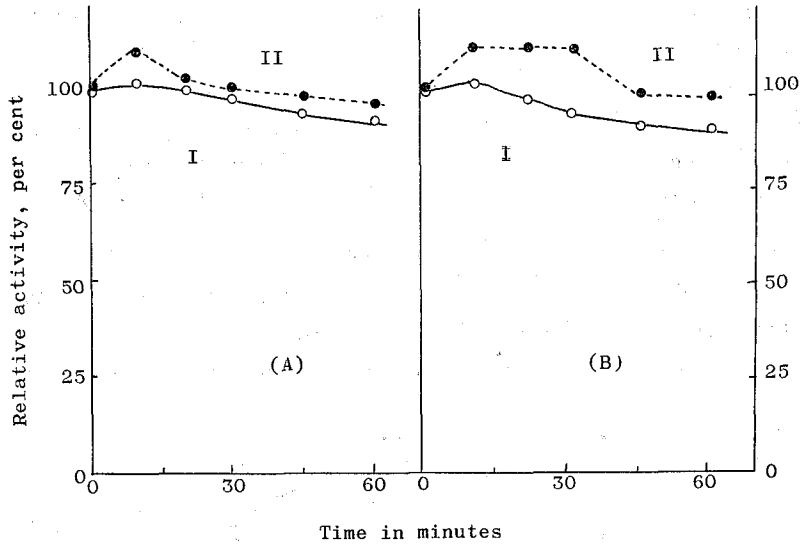

Fig. 8. Effect of heating period on purified enzyme.
Enzyme preparation was heated at 55 °C for indicated time before the reaction. Enzyme activity was assayed under the standard condition. I: L-theanine (A) or L-glutamine (B) hydrolase activity; II: D-theanine (A) or D-glutamine (B) hydrolase activity.

Figure 8 shows the effect of the period of heat treatment on the activities of the highly purified enzyme preparation. As indicated in Figure 8, these four kinds of the enzyme activities were all little affected by heating the enzyme at 55 °C for 60 minutes. Although the partially purified enzyme preparation may contain two different enzymes catalyzing the hydrolysis of L-glutamine as shown in Figure 7, the highly purified enzyme preparation has probably one enzyme hydrolyzing L-glutamine whose behaviour was similar to those of D-glutamine hydrolase, L-theanine hydrolase or D-theanine hydrolase of the purified preparation during heat treatment. The results obtained here and in Figure 7 indicate that fraction A in Figure 3 contained a heat-labile glutamine hydrolase and that in fraction C containing also L-theanine and D-theanine hydrolyzing enzyme activity, another glutamine hydrolase which is resistant to heating, was found.

Effect of Substrate Concentration on Enzyme Activity

The enzyme activities catalyzing hydrolysis of L-theanine and D-theanine with various concentrations of the substrate were investigated. The partially purified enzyme preparation was used in this experiment. The apparent Michaelis constants for L-theanine and D-theanine were calculated by the method of Lineweaver and Burk to be approximately 5.8×10^{-3} M and 9.2×10^{-3} M, respectively. The apparent Michaelis constants for L-glutamine and D-glutamine were shown to be 4.7×10^{-3} M and 6.4×10^{-3} M, respectively. It is concluded that affinity of the enzyme to these four substrates is almost the same.

Formation of γ-Glutamylhydroxamic acid by Transfer Reaction

In the course of these studies, it was observed that incubation of the highly purified enzyme preparation with theanine or glutamine in the presence of hydroxylamine led to significant formation of γ-glutamylhydroxamic acid. Neither metal ion nor nucleotide was required in this reaction. Figure 9 demonstrates relation-

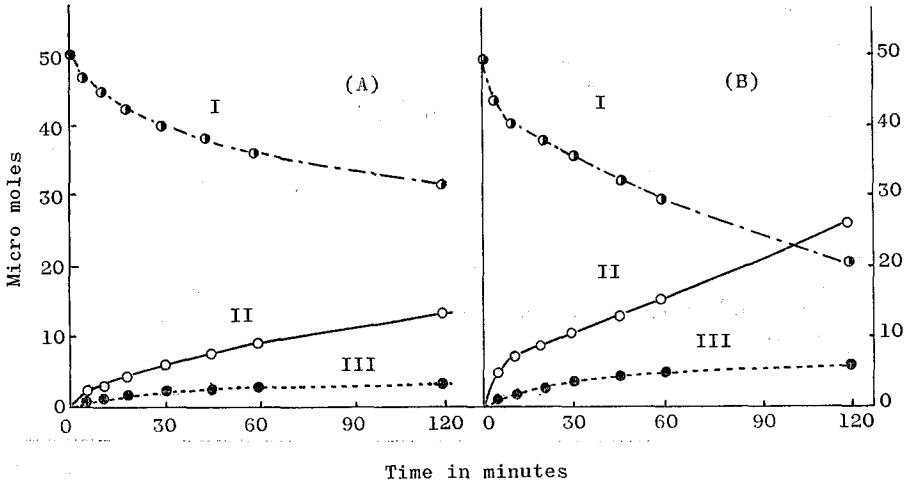

Fig. 9. Time course of hydrolysis and transfer reaction of L-theanine (A) and L-glutamine (B).

Reaction mixtures contained 50 μ moles of L-theanine or L-glutamine, 400 μ moles of hydroxylamine, 5 μ g of purified enzyme and 60 μ moles of phosphate buffer, pH 7.4, in a final volume 2.0 ml. Incubation for indicated time at 37°C. I: L-theanine (A) or L-glutamine (B); II: Glutamate; III: γ-Glutamylhydroxamic acid.

ship among the formation of glutamate by the hydrolase action, the production of γ-glutamylhydroxamic acid by transferase action, and the disappearance of L-theanine or L-glutamine at various incubation periods. D-Theanine and D-glutamine could be the substrate of the transfer reaction as well as their L-isomers as mentioned later. It is realized that this enzyme catalizes the hydrolysis reaction of both isomers of theanine and glutamine in the absence of hydroxylamine, and does also the transfer reaction of γ-glutamyl moiety of both isomers of theanine and glutamine to hydroxylamine to form γ-glutamylhydroxamate besides the hydrolysis reaction, in the presence of hydroxylamine. In the latter case, the rate of the formation of glutamic acid by hydrolysis reaction was as high as approximately three times that of the hydroxamic acid by the transfer reaction, and the total amount of these two products coming from both reactions was virtually the same as that of the substrate consumed.

Effect of Inhibitors on Hydrolase Activity and Transferase Activity

The effect of several inhibitors on the hydrolase activity and the γ-glutamyltransferase activity was investigated. Urea and *p*-chloromercuribenzoic acid

had no effect on both activities at a concentration of 10^{-3} M. Sodium fluoride (5×10^{-2} M) inhibited L-theanine transferase activity in the range of 10 to 20 per cent, but not L-theanine hydrolase activity at all. At a concentration of 10^{-2} M, ethylenediamine tetraacetic acid, on the contrary, caused a 30 per cent inhibition for the hydrolase activity, but no change for the transferase activity when L-theanine was employed as a substrate. Mercuric chloride (10^{-3}M), iodoacetic acid (2×10^{-3}M) and copper sulfate (10^{-2}M) inhibited both enzyme activities in the range of 50 to 80 per cent. The inhibion of the activities by iodoacetic acid was protected partially (about 30 per cent) by preincubation of the enzyme with 2-mercaptoethanol at a concentration of 2×10^{-2} M for 15 minutes.

Substrate Specificity

Table 5 indicates the substrate specificity of the two separate enzyme prepa-

Table 5. Substrate specificity of two enzyme preparations.

Enzyme activity was assayed under the standard condition. Experiment I; The enzyme of fraction A on DEAE-cellulose chromatography was used. Experiment II; The enzyme of fraction C on DEAE-cellulose chromatography was used.

Substrate	Experiment I		Experiment II	
	Hydroxamic acid formation	Hydrolysis of amide	Hydroxamic acid formation	Hydrolysis of amide
	μ moles	μ moles	μ moles	μ moles
L-Theanine	0	0	1.24	5.27
D-Theanine	0	0	0.31	4.10
L-Glutamine	2.62	7.20	1.92	6.60
D-Glutamine	2.22	3.85	1.01	6.09
L-Asparagine	0.86	3.32	0	0
D-Asparagine	3.31	4.15	0	0
Glutathione	0	–	1.16	–
Acetamide	0	–	0	–

rations, i.e. fraction A and fraction C, obtained by DEAE-cellulose chromatography as demonstrated in Figure 3. The hydrolysis of both isomers of asparagine and the formation of β-aspartylhydroxamic acid were catalyzed by fraction A which was not active for either isomer of theanine but for both isomers of glutamine. D-Asparagine was hydrolyzed and also transfered more rapidly by fraction A than its L-enantiomorph. Fraction C which was not active for L-asparagine and D-asparagine, however, catalyzed the hydrolysis of both isomers of theanine, and the formation of γ-glutamylhydroxamic acid from them. L-Gltuamine and D-glutamine could be hydrolyzed and their γ-glutamyl moiety was able to be transfered to hydroxylamine by either fraction A or C. The formation of the γ-glutamyl-hydroxamic acid from glutathione was catalyzed by fraction C, but not by fraction A at all. Acetamide was inert for both preparations.

DISCUSSION

It is well known that glutaminase and asparaginase catalyzed hydrolysis of the amide group of L-glutamine and L-asparagine, respectively. Groot *et al.*[19,28], described that neither asparaginase from *Pseudomonas fluorescens* nor that from mammalian liver hydrolyzed both N-ethyl-β-L-asparagine and N-methyl-β-L-asparagine.

It has been also reported that N-ethyl-γ-glutamine (L-theanine) and N-methyl-γ-glutamine are completely inert as a substrate in the enzymatic amide hydrolysis and transfer reactions[29-32].

The present studies show that some strains of *Pseudomonas* can utilize L-theanine and D-theanine as a sole nitrogen and carbon source, and that the cell-free extract catalyzes the hydrolysis of amide group of L- and D-theanine. It is of interest that D-theanine can be utilized by bacteria and hydrolyzed enzymatically to yield D-glutamic acid and ethylamine without conversion into L-theanine via a racemization system. As the enzyme catalyzes the stoichiometric formation of glutamic acid and ethylamine from theanine, it can be named theanine ethylamidohydrolase or theanine hydrolase in this respect.

The highly purified enzyme preparation from *Pseudomonas aeruginosa* which catalyzed the hydrolysis of L-theanine and D-theanine, and the formation of γ-glutamylhydroxamic acid from these compounds by γ-glutamyl transfer to hydroxylamine, was obtained. Failure to separate these enzyme activities by various procedures suggest that these four reactions may be catalyzed by a single enzyme. Besides L-theanine and D-theanine, L-glutamine and D-glutamine could be used as a substrate for the enzymatic hydrolysis and transfer reactions, but L-asparagine, D-asparagine and acetamide could not. Although the fact that both enantiomorphs of glutamine were potenter substrates than L-theanine and D-theanine appears to show that the enzyme should be considered as a glutaminase which has a wide substrate specificity, the properties of the enzyme in question differ from those of the enzymes of other bacterial and animal sources which have investigated so far. The optimal pH of this enzyme for glutamine hydrolysis was between 8.5 to 9.0, and those of the glutaminases from *Escherichia coli*[33], *Clostridium welchii*[17] and some *Pseudomonas*[22] were reported to be around 5.0 to 6.6. In this respect, the enzyme is likely to be close to the glutaminases from animal tissues[31,34-36], which optimal pH was found in the alkaline, but the present *Pseudomonas aeruginosa* enzyme differs from the animal glutaminases in many properties. The glutaminases from animal tissues were activated by phosphate ions and were inhibited completely by p-chloromercuribenzoic acid at 10^{-3} M, while this enzyme could not be activated by phosphate ions and was not influenced by *p*-chloromercuribenzoic acid at the concentration of 10^{-3} M. It is another remarkable difference of this enzyme of *Pseudomonas aeruginosa* from the usual glutaminases from other sources that the former is able to hydrolyze L-theanine and D-theanine, and to form the γ-glutamylhydroxamate from them and glutathione, and that the latter is inert for other γ-glu-

tamyl derivatives than glutamine.

An enzyme isolated from *Agaricus bisporus*[37] which catalyzes the irreversible hydrolysis of γ-glutamylhydrazide bond and the transfer of γ-glutamyl moiety of agaritine to hydroxylamine, was reported to hydrolyse L-theanine and L-glutamine, and to transfer their γ-glutamyl residue, but the common γ-glutamyl peptides e.g. glutathione, was inactive in these enzyme reactions.

The purified γ-glutamyltranspeptidase from hog kidney[38] which transfered γ-glutamyl residue of γ-glutamyl-*p*-nitroanilide, hydrolyzed L-glutamine, D-glutamine and L-glutamic acid γ-ester, and catalyzed the formation of γ-glutamylhydroxamate from both isomers of glutamine, glutathione, γ-ethylglutamate and γ-glutamylglycine besides γ-L-glutamyl-*p*-nitroanilide. L-Asparagine and L-homoglutamine were not used as a substrate in the hydrolysis and treansfer of the hog kidney enzyme and it is essential to involve γ-glutamyl residue in the substrate. Williams *et al.*[39] demonstrated that transamidase preparation from *Bacillus subtilis* catalyzed the transfer of γ-glutamyl moiety of glutamine, glutathione and other γ-glutamylpeptide to hydroxylamine.

The behavior of the enzyme in question of *Pseudomonas aeruginosa* for the inhibitors and the substrate specificity serve to differentiate it form an amidase of *Pseudomonas aeruginosa*[40] which is specific for the hydrolysis of several aliphatic amides and the transfer of the acyl moiety of such amides to hydroxylamine. The amidase and transferase activities of the aliphatic amides were reported to be inhibited by urea or potassium fluoride, while our enzyme of the same organisms was not influenced by such compounds and could not hydrolyze acetamide.

Thus, the enzyme in question from *Pseudomonas aeruginosa* seems to be more similar to γ-glutamyltransferases than glutaminases previously reported and is regarded as a γ-glutamyltransferase which has a wide substrate specificity in respect to the kind of the substituted moeity at the amide nitrogen atom of γ-glutamyl compounds and to the stereostructure. The hydrolytic activity of this enzyme can be considered its ability to transfer γ-glutamyl residue to water instead of hydroxylamine.

The fact that the glutamine amidohydrolase activity was separated completely to two different fractions by DEAE-cellulose column chromatography shows the occurrence of two kinds of enzyme catalyzing hydrolysis of glutamine in the cell-free extract of this organisms. One of them is the enzyme to catalyze the hydrolysis of theanine and the transfer reaction of theanine and glutathione as well as glutamine as discussed above. Another enzyme, whose fraction was designated as A in Figure 3 and activity was very unstable, catalyzes the hydrolysis of both enantiomorphs of asparagine and glutamine, and the transfer of β-aspartyl or γ-glutamyl residue of them to hydroxylamine, but none of L-theanine, D-theanine and glutathione was active in such enzymatic hydrolysis and transfer.

Recently, Ramadan *et al.*[22] reported that a highly purified enzyme preparation

from a strain of *Pseudomonas* which activity was inherently unstable, has both glutaminase and asparaginase activities and D-asparagine has about 20 per cent of the activity of the L-isomer. It was also demonstrated by Ehrenfeld *et al.*[32] that the enzyme isolated from *Azotobacter agilis* catalyzed hydrolysis of both isomers of glutamine and asparagine, and succinamic acid as well as the formation of hydroxamate from these amides, and that L-theanine, L-glutamic acid γ-ethyl ester, acetamide and other several compounds were not active in both enzymatic reactions.

Therefore, the enzyme found in Fraction A appears to bear the closest resemblance to the common enzyme known as glutaminase or asparaginase and widely distributed. It is of interest that both isomers of glutamine are hydrolyzed and transfered by different two enzymes in the same organisms and both kinds of enzymes have no stereospecificity for their substrates.

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