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Purification and Characterization of Glutamine Synthetase of *Pseudomonas taetrolens* Y-30: An Enzyme Usable for Production of Theanine by Coupling with the Alcoholic Fermentation System of Baker's Yeast

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Concentrated cell-extract of *Pseudomonas taetrolens* Y-30, isolated as a methylamine-assimilating organism, formed γ-glutamylethylamide (theanine) from glutamic acid and ethylamine in a mixture containing the alcoholic fermentation system of baker's yeast for ATP-regeneration. Glutamine synthetase (GS), probably responsible for theanine formation, was isolated from the extract of the organism grown on a medium containing 1% methylamine, 1% glycerol, 0.5% yeast extract, and 0.2% polypepton as carbon and nitrogen sources. The molecular mass was estimated to be 660 kDa by gel filtration and 55 kDa by SDS-polyacrylamide gel electrophoresis, suggesting that *Ps. taetrolens* Y-30 GS consists of 12 identical subunits. The enzyme required Mg^{2+} or Mn^{2+} for its activity. Under the standard reaction condition for glutamine formation (pH 8.0 with 30 mM Mg^{2+}), GS showed 7% and 1% reactivity toward methylamine and ethylamine respectively of that to ammonia. Reactivity to the alkylamines varied with optimum pH of the reaction in response to divalent cation in the mixture: pH 11.0 was the optimum for the Mg^{2+}-dependent reaction with ethylamine, and pH 8.5 was the optimum for the Mn^{2+}-dependent reaction. In a mixture of an optimum reaction condition with 1000 mM ethylamine (at pH 8.5 with 3 mM Mn^{2+}), reactivity increased up to 7% of the reactivity to ammonia in the standard reaction condition. The isolated GS formed theanine in the mixture with the yeast fermentation system.

Key words: theanine; glutamine synthetase; *Pseudomonas taetrolens* Y-30; coupled fermentation with energy transfer; baker's yeast

Theanine, γ-glutamylethylamide, is an amino acid found by Sakato in Japanese green tea leaves (*Camellia sinensis*).[1] It is synthesized by theanine synthetase with glutamic acid, ethylamine, and ATP as the substrates.[2,3] The content of theanine is 1.5–3% of the dry weight of the tea leaves, and it renders the gracefully sweet taste.[4] Recently, various favorable physiological functions of theanine have been reported,[5–10] suggesting that demand for theanine might become great not only as a taste-enhancing additive but also as a supplement to improve and/or maintain human health.

Several methods have been reported for producing theanine including extraction of tea leaves, use of tea callus,[11–13] chemical synthesis,[14,15] *etc.*, but these methods might be impractical due to complexity with low yield and/or low purity.

Biochemical synthesis has also been investigated using crude enzyme,[16,17] which catalyzes a similar reaction to that of theanine synthetase, but this was on an experimental scale leaving out of consideration supply of the enzyme and ATP. Recently, enzymatic production was accomplished by γ-glutamyl transfer reaction of bacterial glutaminase[18] or γ-glutamyl transpeptidase[19] with glutamine and ethylamine as the substrates. The method might be satisfactory to some extent, but the process has certain shortcomings with respect to supply of glutamine as well as side reactions lowering theanine yield on glutamine: hydrolysis of glutamine and formation of other γ-glutamyl derivatives. These might be resolved by the use of an enzyme similar to theanine synthetase if ATP can be regenerated efficiently, because glutamic acid is supplied more easily than glutamine and the reaction is endergonic.

We have reported glutamine production using the reaction of glutamine synthetase (GS; L-glutamate:ammonia ligase, ADP-forming, E.C.6.3.1.2) of *Corynebacterium glutamicum* (*Micrococcus glutamicus*) or *Brevibacterium flavum*,[20,21] which was coupled with the sugar fermentation reaction of dried baker's yeast cells. This process was an application of "coupled fermentation with energy transfer," in which the yeast fermentation of glucose functioned as an ATP-regenerating system.[22–25] During the course of the investigation, we found that a small amount of theanine was formed when ammonia, one of the substrates of GS, was replaced by ethylamine.[26] But production of theanine in high concen-

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Amino acid is L-isomer unless otherwise stated.

Table 1. Effect of Growth Conditions on GS Activity in Cells

Exp. A

Medium	Addition (%)				Growth*2	GS activity	
						SA*3	TA*4
1	1% methylamine		0.3% YE*1		0.8	0.015	1.2
2	1% glucose		0.3% YE		1.0	0	0
3	1% glycerol		0.3% YE		0.8	0	0
4	1% methylamine	1% glycerol	0.3% YE		2.7	0.011	6.7
5	1% methylamine	2% glycerol	0.3% YE		2.9	0.009	5.6
6	2% methylamine	1% glycerol	0.3% YE		2.0	0.005	2.7
7	2% methylamine	2% glycerol	0.3% YE		2.2	0.004	2.2
8	1% methylamine	1% glycerol	0.1% YE		0.7	0.017	1.0
9	1% methylamine	1% glycerol	0.5% YE		4.7	0.009	9.9
10	1% methylamine	1% glycerol	1.0% YE		5.5	0.000	0.2
11	1% methylamine	1% glycerol	0.5% YE	0.2% polypepton	5.9	0.009	11
12	1% methylamine	1% glycerol	0.5% YE	0.4% polypepton	7.0	0.003	3.4

Exp. B

Cultivation (d)	Growth*2	GS activity	
		SA*3	TA*4
1	3.5	0.000	0.4
2	5.5	0.003	5.0
3	5.7	0.008	7.1
4	5.5	0.009	8.7
5	5.1	0.015	15
6	3.6	0.010	10

Exp. A: Carbon and nitrogen sources in Medium B were changed as indicated in the Table, and *Ps. taetrolens* Y-30 was grown for 3 d.

Exp. B: The organism was grown on an optimum medium for GS production, which consisted of 1.0% methylamine·HCl, 1.0% glycerol, 0.5% yeast extract, 0.2% polypepton, 1.0% NaCl, 0.02% KCl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.005% KH_2PO_4, 0.005% K_2HPO_4, and 1×10^{-7}% cyanocobaramine.

Cultivation was carried out at 30 °C with 1 liter medium in a 2-liter Sakaguchi flask. Cell-free extract was used to measure GS activity (γ-glutamylhydroxamate-forming reaction).

*1: yeast extract *2: turbidity at 610 nm *3: specific activity (unit/mg protein) *4: total activity (units)

trations was not successful due to the low reactivity of the GS to ethylamine and to difficulty in controlling the pH of the reaction mixture. The optimum pH for theanine synthesis and the yeast fermentation was 10 and 7 respectively.[27] Precise pH control of the mixture was impossible due to large amounts of yeast cells in the mixture.[26] These findings suggested the need to search for a new GS with high reactivity to ethylamine at neutral pH range.

It is known that some bacteria, which grow on methylamine as a sole carbon and nitrogen source, generate γ-glutamylmethylamide (GMA) as an intermediate in their metabolism of methylamine.[28,29] Synthesis of GMA in the organisms is performed with glutamic acid, methylamine, and ATP as the substrates by GMA synthetase (GMAS; L-glutamate: methylamine ligase, ADP-forming, EC 6.3.4.12). Differentiation of GMAS from GS might be difficult because the reactions catalyzed by the enzymes are very similar. But GMAS has been reported to be active to methylamine rather than to ammonia, and also to show high reactivity to ethylamine,[30] suggesting that the enzyme might be usable for theanine production by coupled fermentation with energy transfer if it is stable. But the enzyme was reported to be unstable.[30]

We found that concentrated cell-free extract of a bacterium, which was isolated as a methylamine-assimilating organism, formed theanine from glutamic acid and ethylamine in a reaction mixture of coupled fermentation with energy transfer. An enzyme responsible for theanine formation, tentatively indicated as GS, was isolated from the cell-free extract and characterized mainly in respect to reactivity for theanine synthesis.

Materials and Methods

Isolation of methylamine-assimilating bacteria. Soil samples were suspended in sterilized water and 0.1 ml of the upper phase was inoculated into 5 ml of Medium A-1 or Medium A-2 in a test tube, which contained methylamine as a carbon and a nitrogen source. Medium A-1 was designed according to the medium for a marine methylotroph,[29] with a small modification, and contained 0.15% methylamine·HCl, 2.5% NaCl, 0.02% KCl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.005% KH_2PO_4, 0.005% K_2HPO_4, 4×10^{-6}% each of $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 5H_2O$, $NiSO_4$, $Na_2MoO_4 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, and $CoSO_4 \cdot 7H_2O$, and 1×10^{-7}% cyanocobalamine (pH 7). Medium A-2 was a simplified Medium A-1 with a higher concentration of methylamine and a lower concentration of NaCl: 1.0% methylamine·HCl, 0.2% NaCl, 0.02% KCl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.005% KH_2PO_4, 0.005% K_2HPO_4, 0.03% yeast extracts, and 1×10^{-7}% cyanocobalamine (pH 7). After 10–14 d of cultivation at 30 °C on a shaker (200 rpm), part of the turbid culture was transferred into fresh Medium A-1 or A-2 and cultivated further for 10–14 d. This procedure was repeated 3–5 times, then the culture was streaked on an agar plate containing 0.5% glucose, 1.0% polypepton, 0.5% yeast extract, 0.5% NaCl, and 2.0% agar. Bacterial colonies that appeared after incubation at 30 °C for 1–2 d were purified and then isolated. The isolates were

cultivated for 8 d in Medium A-1 and/or A-2, and the methylamine-assimilating microorganisms, which gave turbidity at 610 nm of more than 0.25, were submitted for screening experiments.

The methylamine-assimilating organisms usually started to grow 3–5 d after inoculation. Growth was repressed (slightly or remarkably) by increasing the concentration of methylamine to 1%, and the repressed growth was recovered and stimulated by the addition of 0.05–0.1% yeast extract: early start as well as increase in the growth. The addition of 1% glycerol further increased the growth of many organisms.

Selection of microorganism to form an enzyme suitable for theanine production. The selection was made with two screening experiments. The first was dependent on the GS activity of the cell-free extract. The isolated organism was cultivated for 3 d on 1 liter of Medium B in a Sakaguchi flask. Medium B was defined by several preliminary experiments to increase microbial growth without remarkable decrease of GS activity (see Table 1). The medium contained 1.0% methylamine·HCl, 1.0% glycerol, 0.3% yeast extract, 1.0% NaCl, 0.02% KCl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.005% KH_2PO_4, 0.005% K_2HPO_4, and 1×10^{-7}% cyanocobalamine. The cells were collected by centrifugation ($12{,}000 \times g$, 4 °C, 20 min), and washed twice with 10 mM potassium phosphate buffer (pH 6.0). The washed cells were suspended in the same buffer and disrupted with a sonicator (Nihonseiki, 20 khz for 20 min at 0–15 °C). The centrifugal supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 6.0) and used to measure GS activity.

In the second screening, the cell-free extract of the organisms selected in the first screening was concentrated with ammonium sulfate (30–70% saturation; see legend to Fig. 1), and examined in respect to theanine-forming activity in a reaction mixture of the coupled fermentation with energy transfer.

Assay of GS activity. The activities of two reactions were measured.

Glutamine-forming reaction: Activity was determined according to the previous method,[31] with a small modification: a reaction mixture consisting of 50 mM sodium glutamate, 25 mM ammonium chloride, 7.5 mM ATP, 30 mM $MgCl_2$, 100 mM imidazole buffer (pH 8.0), and a suitable amount of the final GS preparation (see Table 2) was incubated at 30 °C, and the amount of inorganic phosphate formed was determined.

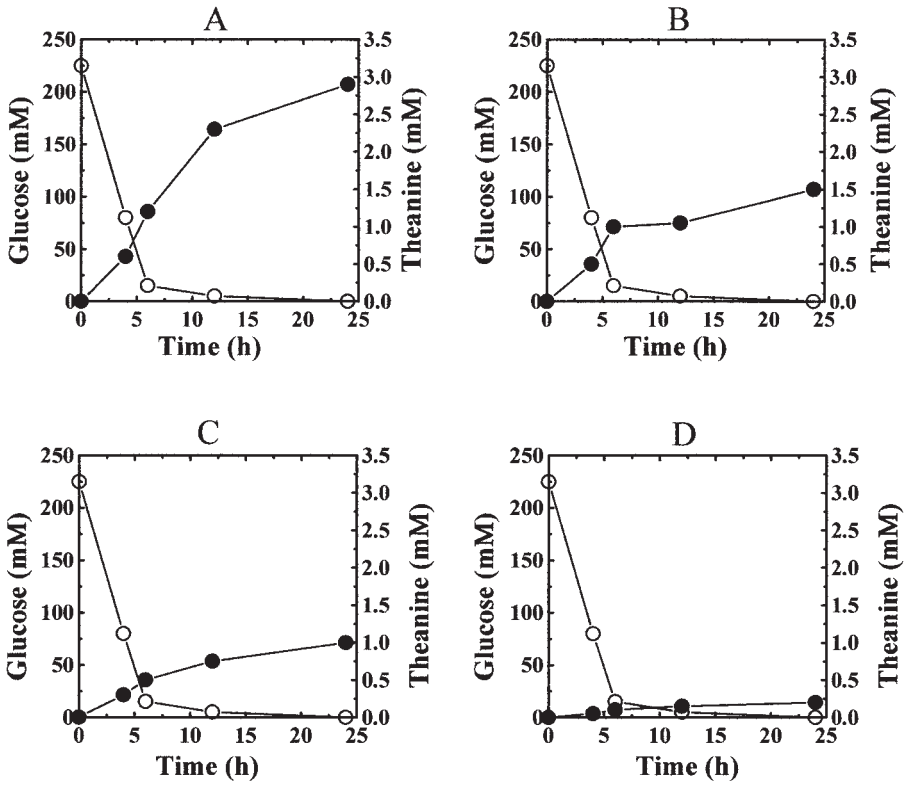

Fig. 1. Formation of Theanine by Coupling of Concentrated Cell-free Extract of Methylamine-assimilating Bacteria with Dried Yeast Cells.

Ammonium sulfate was added to the cell-free extract to 30% saturation with adjusting pH at 7, and then ammonium sulfate was added to the centrifugal supernatant to 70% saturation. The precipitate was collected by centrifugation, dissolved in a small volume of 10 mM potassium phosphate buffer (pH 6.0) and dialyzed against the same buffer. The concentrated extract, corresponding to 1 unit/ml of GS, was added to a temporary reaction mixture for coupled fermentation with energy transfer ("Materials and Methods"). The reaction was carried out at 30 °C for 24 h with shaking. Theanine was not formed in control mixtures (without yeast cells, data not shown; without GS preparation, see Fig. 4(A)). (A), strain Y-30; (B), strain Z-10; (C), strain Z-76; (D), *B. flavum*. Symbols: ○, glucose; ●, theanine.

Table 2. Purification of *Ps. taetrolens* Y-30 GS

Purification step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)
Cell-free extract	160,000	0.015	2,400
Ammonium sulfate	37,600	0.052	1,955
DEAE-cellulofine	1,850	0.68	1,258
Butyl-Toyopearl	850	0.96	816
Sepharose CL-4B	450	1.17	526

The mixture was the optimum for *Ps. taetrolens* GS and it was used for general characterization of the isolated enzyme. The condition is sometimes indicated as "the standard reaction condition" in the text.

γ-Glutamylhydroxamate-forming reaction: A reaction mixture consisting of 50 mM sodium glutamate, 15 mM hydroxylamine, 7.5 mM ATP, 30 mM $MgCl_2$, 100 mM imidazole buffer (pH 8.0), and GS preparation was incubated at 30 °C. After incubation, 2 volumes of a mixture of 0.2 M $FeCl_3 \cdot 6H_2O$, 0.12 M trichloroacetic acid, 0.25 N HCl, and water (8:2:1:13) was added to stop the reaction. The developed color of γ-glutamylhydroxamate was determined spectrophotometrically at 540 nm. One unit of GS was defined as the amount of enzyme forming 1 μmol of γ-glutamylhydroxamate per min.

This method was used in the experiments for selection of the organism, for estimation of the cultivation conditions, and for purification of the enzyme.

Dried yeast cells and the mixture for coupled fermentation with energy transfer. Baker's yeast was supplied by Oriental Yeast (Tokyo, Japan). It was air-dried according to the previous method.[24] A reaction mixture for theanine production was defined temporarily according to the mixture for production of glutamine[21] and GMA.[26] It contained 200 mM glucose, 50 mM potassium phosphate buffer (pH 7.0), 200 mM sodium glutamate, 300 mM ethylamine, 25 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM AMP, 40 mg/ml dried yeast cells, and GS preparation. The mixture (1 ml in a test tube) was incubated at 30 °C with shaking (200 rpm). The reaction was terminated by immersing the reaction tube in boiling water for 3 min. The centrifugal supernatant of the mixture (1000 rpm, 10 min) was submitted to the assay.

Purification of GS. All operations were carried out at 0–10 °C and 10 mM potassium phosphate buffer (pH 6.0), containing 10% glycerol, 1 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, was used unless otherwise specified.

Cell-free extract: The selected strain Y-30, tentatively named *Pseudomonas taetrolens* Y-30, was grown at 30 °C for 5 d on an optimum medium (1 liter in 2-liter Sakaguchi flask), as described in "Results and Discussion" (see Table 1).

The washed cells from 170-liter medium were suspended in the buffer and stored at −20 °C until use. The frozen cells were thawed and disrupted with the sonicator (20 khz for 20 min at 0–15 °C). The cell debris was centrifuged off, and the supernatant was used as the starting material.

Ammonium sulfate fractionation: To the cell-free extract, ammonium sulfate was added to achieve 30% saturation, with adjusting pH at 7 with 12% ammonium hydroxide. The mixture was stirred for 30 min at 4 °C and the precipitate was centrifuged off. Ammonium sulfate was added to the supernatant to 60% saturation. After stirring for 30 min at 4 °C, the precipitate was collected by centrifugation and dissolved in the buffer. The solution was dialyzed against the buffer.

DEAE-cellulofine column chromatography: The dialyzed solution was put on a DEAE-cellulofine column (11.8 × 22 cm) equilibrated with the buffer. After washing with the buffer, the column was developed with the buffer containing 0.25 M, 0.45 M, and 1.0 M NaCl. The enzyme was eluted with the buffer containing 0.45 M NaCl. The active fractions were combined, and then concentrated with ammonium sulfate (70% saturation). The precipitate formed was collected by centrifugation and dissolved in a small volume of the buffer. The solution was dialyzed against the buffer.

Butyl-Toyopearl 650M column chromatography: To the dialyzed solution, ammonium sulfate was added to achieve 30% saturation. The solution was put on a Butyl-Toyopearl 650M column equilibrated with the buffer containing 30% ammonium sulfate. After washing with the same buffer, the column was eluted by decreasing the concentration of ammonium sulfate in the buffer linearly. The active fractions, eluted at 20–10% ammonium sulfate, were combined and concentrated with ammonium sulfate (70% saturation). The precipitate formed was collected by centrifugation and dissolved in a small volume of the buffer.

In this chromatography, GS was detected with a γ-glutamyl-transferring reaction because the γ-glutamylhydroxamate-forming reaction is interfered with ammonium sulfate in the eluate. Ammonia is a substrate for the glutamine-forming reaction. The mixture for the transferring reaction contained 35 mM glutamine, 15 mM hydroxylamine, 0.4 mM ADP, 1 mM $MnCl_2$, 10 mM arsenate, 100 mM imidazole–HCl buffer (pH 7.5), and the enzyme solution. The reaction was carried out at 30 °C and stopped, as was the γ-glutamylhydroxamate-forming reaction, followed by determination of γ-glutamylhydroxamate.

Sepharose CL-4B column chromatography: The concentrated enzyme solution was filtered through a column of Sepharose CL-4B (2.8 × 130 cm) equilibrated with the buffer. The active fractions were combined and used as a final preparation.

N-terminal amino acid sequence analysis. The GS protein on a gel of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was blotted onto a poly-vinylidene difluoride (PVDF) membrane with a

semidry-blotting apparatus (AE-6670, Atto, Tokyo, Japan). The protein on the PVDF membrane was then stained with ponsew. The stained protein on the membrane was cut off to analyze the N-terminal amino acid sequence with a peptide sequencer (PPSQ-10S, Shimadzu), according to the method of Edman.[32] The sequence was compared with the BLAST2 protein database.

Assay. Protein was determined by Lowry's method[33] with egg albumin as a standard. In column chromatography, it was monitored with absorbance at 280 nm. Glucose was assayed according to the method of Somogyi,[34] or enzymatically with glucose oxidase. Inorganic phosphate was determined by the method of Fiske and Subbarow.[35] Theanine formed in the reaction mixture was assayed by paper chromatography and ninhydrin colorimetry according to the method described previously.[27]

Reagents. Glucose oxidase (glucose C II-test wako) was supplied by Wako Pure Chemical. The theanine was from Tokyo Kasei. The other reagents were highest-grade commercial products.

Results and Discussion

Selection of methylamine-assimilating bacteria

Among the methylamine-assimilating isolates that grew on Medium A-1 or A-2 containing methylamine as a carbon and nitrogen source, five strains were selected in the first screening, depending on GS activity in the cell-free extract (data not shown).

In the second screening, the theanine-forming activity of the concentrated extract was examined in the mixture of coupled fermentation with energy transfer. Figure 1 shows some of the results. These indicate that extracts of strains Y-30, Z-10, and Z-76 formed theanine with a decrease in glucose, an energy source for ATP-regeneration. The amounts were larger, especially that with strain Y-30 extract, than the amount in the mixture with the extract of *B. flavum*, which has been used for glutamine production.[21]

Strain Y-30 was chosen for further experiments. The organism is a rod-shaped gram-negative bacterium, and the other physiological properties suggested that it belongs to *Pseudomonas*. The sequence of 16S ribosomal DNA showed 98% similarity to that of *Pseudomonas taetrolens*. The organism is tentatively designated *Pseudomonas taetrolens* Y-30 in this paper.

Effect of cultivation conditions on GS level in Ps. taetrolens Y-30

Experiment A of Table 1 summarizes the activities of GS in cells grown under various nutritional conditions. GS activity was barely detected when *Ps. taetrolens* Y-30 was grown in the medium containing glucose or glycerol (media 1, 2, and 3), indicating that the enzyme was formed inductively by methylamine. The addition of 1% glycerol (medium 4) to the medium containing 1% methylamine and 0.3% yeast extract (medium 1) was effective in increasing cell growth without significant decrease of GS activity, bringing about a considerable increase in total activity, but the addition of it at higher concentrations was not as effective (medium 5). The addition of 1% glucose repressed microbial growth considerably (data not shown).

Yeast extract significantly affected cell growth and GS level in the cells (media 4, 8, 9, and 10). It stimulated cell growth whereas it decreased the GS level, especially at higher concentrations, and a concentration of 0.5% was the most favorable for enzyme production. The addition of 0.2% polypepton (media 9, 11, and 12) increased cell-growth significantly with a certain increase in enzyme production.

On the bases of the above results, an optimum medium for GS production was defined. It is indicated in the legend to Exp. B in Table 1. The cells under 5 d of cultivation gave the highest GS activity.

Purification of GS

As summarized in Table 2, GS of *Ps. taetrolens* Y-30 was purified from the extract of cells grown on the optimum culture medium (about 80-fold purification with an overall yield of 22%). SDS-PAGE indicated that the final preparation was homogeneous (Fig. 2).

The apparent molecular mass of the enzyme was estimated to be 660 kDa by gel filtration with a Sepharose CL-4B column standardized with catalase (232 kDa), ferritin (440 kDa), and thyrogloblin (669 kDa), and to be 55 kDa by SDS-PAGE (Fig. 2). These results indicate that the enzyme might consist of 12 identical subunits (MW: 55 kDa), like the enzymes of several gram-negative bacteria including *Escherichia coli*.[36]

The N-terminal amino acid sequence was SKSVQLIKDXDVKWI (X, not determined). This has 93% similarity to that of *Pseudomonas syringae* GS.

Stability

The GS of *Ps. taetrolens* Y-30 was stable at pH 6.0. Activity was retained after 10 min of incubation at 55 °C and pH 6.0, and lowered to 60% at 65 °C.

About 95% of the initial activity was maintained for 30 d at 5 °C in a state of ammonium sulfate precipitate (70% saturation), and full activity continued for 2 months at −20 °C in a frozen state.

Substrate and divalent cation specificity

Table 3 shows the reactivity of *Ps. taetrolens* Y-30 GS to various substrates and that with several divalent cations in the standard reaction condition.

The enzyme specifically required L-glutamic acid, and utilized hydroxylamine, methylamine, and ethylamine with 32, 7, and 1% reactivity of that to ammonia. GTP and ITP were substituted for ATP with 26 and 18% reactivity. The substrate specificity was not specific as

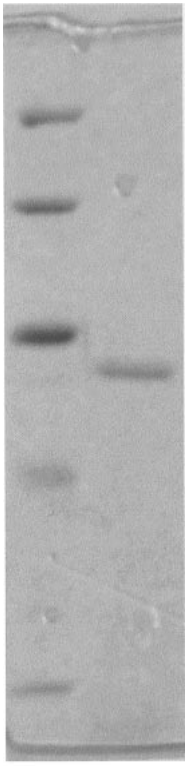

Fig. 2. SDS-PAGE of *Ps. taetrolens* GS.
The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular weight marker: myosin ($Mr = 200{,}000$), β-galactosidase (116,250), albumine (66,200), ovalbumine (45,000). Lane 2, GS.

Table 3. Substrate Specificity of *Ps. taetrolens* GS

Substrate	Relative activity (%)	Divalent cation (mM)	Relative activity (%)
L-glutamic acid	100	Mg^{2+} (30)	100
D-glutamic acid	0	Mg^{2+} (3)	5
L-aspartic acid	0	Mn^{2+} (30)	0
D-aspartic acid	0	Mn^{2+} (3)	11
Ammonia	100	Fe^{2+} (30)	0
Hydroxylamine	32	Co^{2+} (30)	0
Methylamine	7	None	0
Ethylamine	1		
ATP	100		
GTP	26		
ITP	18		
CTP	0		

The reaction mixture described in "Materials and Methods" (the standard reaction condition) was used with a 0.034 unit/ml enzyme preparation. One of the substrates was replaced with a substrate analog, or 30 mM $MgCl_2$ was replaced with another divalent cation. The reaction was carried out at 30 °C for 10 min, and the amount of inorganic phosphate formed was determined. The activity was expressed relatively to that in the standard reaction mixture: 100% = 0.76 mM inorganic phosphate/10 min.

compared with those of GS from many sources.

As for the divalent cation, Mg^{2+} and Mn^{2+} were effective for GS activity whereas the others were not under the present condition. Activity increased responding to the increase in Mg^{2+} concentration with an optimum of 15 mM, and did not vary at excess concentrations (data not shown). In the case of Mn^{2+}, maximum GS activity was obtained in the mixture with 3–4 mM, and decreased in the mixture with higher concentrations of Mn^{2+} (data not shown). The optimum concentration of divalent cation varied responding to the concentration of ATP in the mixture (optimum ratios: Mg^{2+}:ATP = 2:1; Mn^{2+}:ATP = 1:2). These are typical properties observed with GS from various sources.[31,37,38]

The low reactivity of the enzyme to methylamine and ethylamine (Table 3) suggested that the *Ps. taetrolens* Y-30 enzyme was GS but not GMAS, and indicated that the enzyme is unusable for theanine production. But it should be noted that the standard reaction condition was the optimum for the glutamine-forming reaction with 30 mM Mg^{2+}. The reactivity of GS toward substrates, especially ammonia analogs, frequently varied, responding to the divalent cation used and to the reaction pH.[27,36]

Effect of pH on the reactivity of GS to ammonia, methylamine and ethylamine with Mg^{2+} or Mn^{2+} as a divalent cation

Figure 3 compares the pH profiles of the GS reaction with ammonia (A, B), methylamine (C, D), and ethylamine (E, F) as a substrate in a mixture containing 30 mM Mg^{2+} (A, C, E) or 3 mM Mn^{2+} (B, D, F). The concentration of Mg^{2+} and Mn^{2+} was optimum for the glutamine-forming reaction under the standard reaction condition.

With ammonia as a substrate, the optimum pH was 8.0 in the mixture containing 30 mM Mg^{2+} (A), and was 7.5 with 3 mM Mn^{2+} (B). Reactivity with Mg^{2+} was about 10-fold higher than with Mn^{2+}, similarly to findings with enzymes from other bacteria,[36] but differently from findings with the *C. glutamicum*[27] or *B. flavum* enzyme.[39]

The optimum pH for the reaction with methylamine was 8.5 in the mixture with 30 mM Mg^{2+} (C), and 8.0 with 3 mM Mn^{2+} (D). The pH profiles were similar to those for GS of *C. glutamicum*[27] and GMAS of a methylotroph, *Methylophaga* sp. AA-30[30] (pH 8.5 and pH 7.5–8.0 respectively).

In the mixture with ethylamine, the Mg^{2+}-dependent reaction proceeded optimally at pH 11.0 (E) and the Mn^{2+}-dependent reaction at pH 8.5 (F). The optimum pH of 8.5 for the Mn^{2+}-dependent reaction was noticeable for theanine production by coupling with sugar fermentation of the yeast. The optimum pH of the reaction by *C. glutamicum* GS was 10.0,[27] and the reaction could not be coupled with yeast fermentation.[26]

Table 4 summarizes the K_m values for the substrates in the reactions with ammonia, methylamine, and ethylamine under optimum pH conditions with 30 mM Mg^{2+} or 3 mM Mn^{2+}, and indicates that the K_m for the substrates were variable except for ATP.

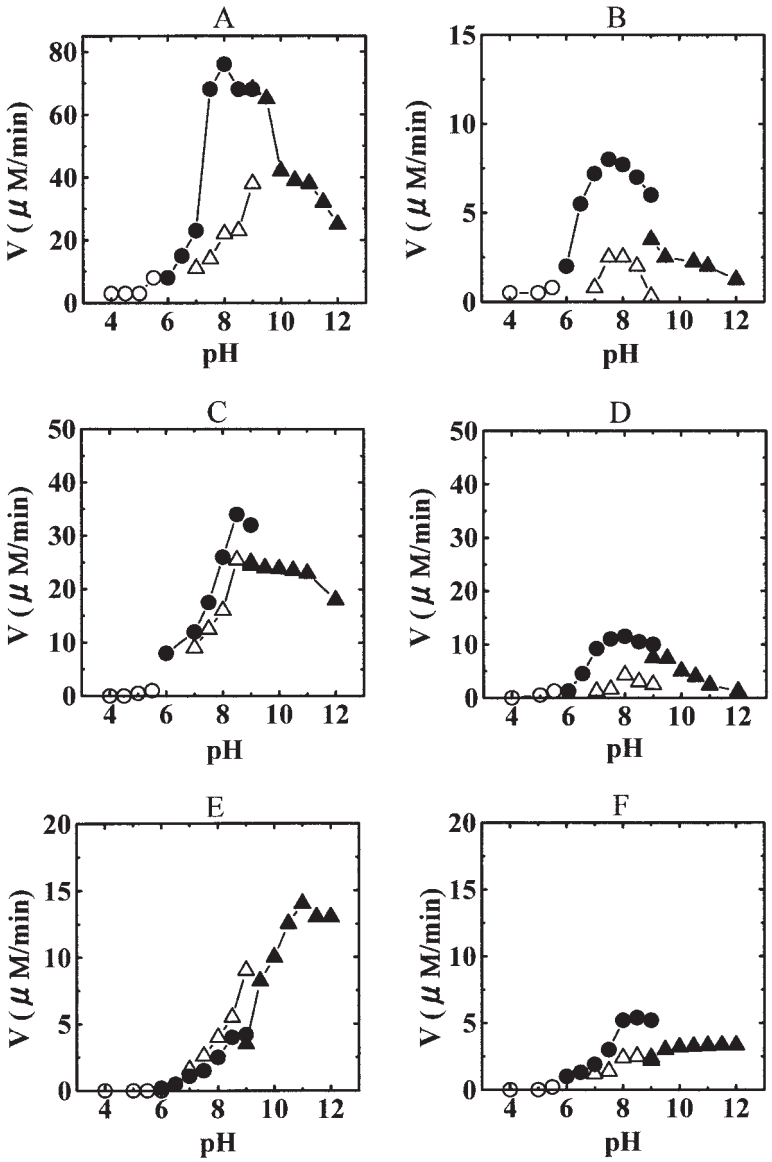

Fig. 3. Effects of pH and Divalent Cation on the GS Reaction with Ammonia, Methylamine or Ethylamine.

(A, B) The reaction mixture contained 50 mM sodium glutamate, 25 mM ammonium chloride, 7.5 mM ATP, 30 mM $MgCl_2$ (A) or 3 mM $MnCl_2$ (B), 100 mM buffer, and 0.034 units/ml GS. The mixture with 30 mM $MgCl_2$ and imidazole buffer (pH 8.0) was the standard reaction condition. (C, D) The reaction mixture contained 50 mM sodium glutamate, 500 mM methylamine, 7.5 mM ATP, 30 mM $MgCl_2$ (C) or 3 mM $MnCl_2$ (D), 100 mM buffer, and 0.034 units/ml GS. The concentration of 500 mM for methylamine was defined by a preliminary experiment on the effect of its concentration to the activity. (E, F) The reaction mixture contained 50 mM sodium glutamate, 1000 mM ethylamine, 7.5 mM ATP, 30 mM $MgCl_2$ (E) or 3 mM $MnCl_2$ (F), 100 mM buffer, and 0.034 units/ml GS. The concentration of 1000 mM for ethylamine was defined by a preliminary experiment. The reaction was carried out at 30 °C for 10 min and the amount of inorganic phosphate formed was determined. Symbols: ○, acetate buffer; ●, imidazole–HCl buffer; △, tris–HCl buffer; ▲, borate–NaOH buffer.

The change in reaction velocity in response to the concentration of glutamic acid was biphasic in all the reactions. There was higher affinity for glutamic acid at its lower concentrations. Similar observation was made for the Mn^{2+}-dependent reactions of *E. coli* GS[40] and *B. flavum* GS.[41] The value also varied in response to divalent cation in the mixture.

The K_m values for ammonia, methylamine, and ethylamine varied in different ways responding to the divalent cation in the mixture. The K_m for ammonia was not influenced, and those for methylamine and ethylamine varied significantly with variations in optimum pH. It was notable that the affinity of the enzyme toward methylamine or ethylamine with 3 mM Mn^{2+} was higher than that with 30 mM Mg^{2+}.

Table 4 also compares the reactivity of the enzyme under the conditions defined to be optimum on the bases of the kinetic parameters. The reactivity to ethylamine in the mixture containing 3 mM Mn^{2+} (at pH 8.5) was 7% of that to ammonia with 30 mM Mg^{2+} (at pH 8.0), suggesting its participation in theanine synthesis in the mixture of coupled fermentation with energy transfer.

Certain divalent cations have been reported to induce unique structural changes of GS protein, both of

Table 4. K_m Values for Substrates and Relative Activities in the Reaction with Various Amines

Exp	Reaction conditions			K_m value (mM) for				Relative activity (%)
				Glutamic acid				
	Ammonia or alkylamine	Divalent cation	pH	Lower conc.	Higher conc.	ATP	Ammonia and amine	
A	Ammonia	30 mM Mg^{2+}	8.0	0.65	2.5	2.0	0.56	100
B	Ammonia	3 mM Mn^{2+}	7.5	1.4	5.6	1.5	0.59	11
C	Methylamine	30 mM Mg^{2+}	8.5	0.5	2.0	1.4	100	45
D	Methylamine	3 mM Mn^{2+}	8.0	0.5	2.5	1.4	20	14
E	Ethylamine	30 mM Mg^{2+}	11.0	0.67	4.0	1.3	700	18
F	Ethylamine	3 mM Mn^{2+}	8.5	0.22	1.1	1.5	500	7

The reaction mixture for each experiment contained:
A: 50 mM sodium glutamate, 15 mM ammonium chloride, 7.5 mM ATP, 30 mM Mg^{2+}, 100 mM imidazole buffer (pH, in the Table), and 0.068 unit/ml GS. This mixture is for the glutamine-forming reaction, and was designated the standard reaction condition.
B: 50 mM sodium glutamate, 15 mM ammonium chloride, 7.5 mM ATP, 3 mM Mn^{2+}, 100 mM imidazole buffer (pH, in the Table), and 0.068 unit/ml GS.
C: 50 mM sodium glutamate, 500 mM methylamine, 7.5 mM ATP, 30 mM Mg^{2+}, 100 mM imidazole buffer (pH, in the Table), and 0.068 unit/ml GS.
D: 50 mM sodium glutamate, 500 mM methylamine, 7.5 mM ATP, 3 mM Mn^{2+}, 100 mM imidazole buffer (pH, in the Table), and 0.068 unit/ml GS.
E: 50 mM sodium glutamate, 1000 mM ethylamine, 7.5 mM ATP, 30 mM Mg^{2+}, 100 mM imidazole buffer (pH, in the Table), and 0.068 unit/ml GS.
F: 50 mM sodium glutamate, 1000 mM ethylamine, 7.5 mM ATP, 3 mM Mg^{2+}, 100 mM imidazole buffer (pH, in the Table), and 0.068 unit/ml GS.
K_m values were obtained by varying the concentration of a substrate with fixed concentrations of the other substrates. Reactivities are expressed relative to that of the glutamine-forming reaction (Exp. A).

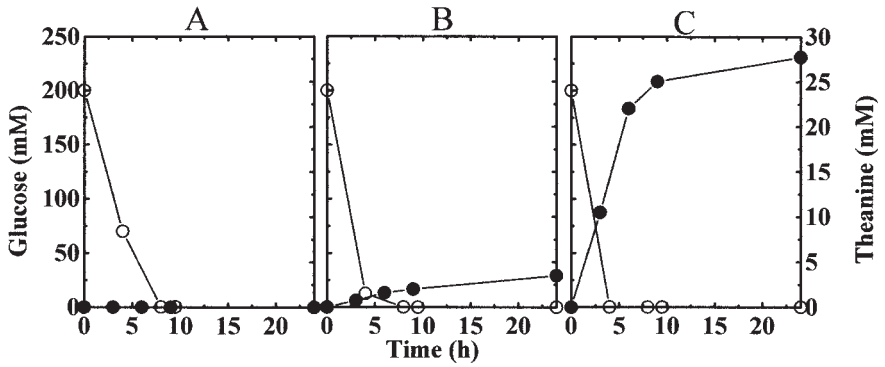

Fig. 4. Formation of Theanine by Coupling of Dried Yeast Cells and Purified GS.
The temporary reaction mixture was described in "Materials and Methods." The amounts of GS were null (A), 1 (B), and 10 (C) units/ml. Incubation was done at 30 °C with shaking (200 rpm). Symbols: ○, glucose; ●, theanine.

intrasubunit structure and subunit interaction. In addition, they brought about variation of GS-properties such as the pH-activity profile, reactivity to the substrates, K_m values for substrates, *etc.* These changes in the structure and properties of GS are perhaps related to one another.[36,42] The observations summarized in Fig. 3 and Table 4 might be caused by divalent cations similarly to those described above, but detailed experiments were not made in this study.

Synthesis of theanine by GS in a mixture with the yeast fermentation system

Figure 4 shows that isolated GS formed theanine in the temporary mixture of coupled fermentation with energy transfer. The amount in (B) with 1 unit/ml GS was almost the same as that formed with the concentrated cell-extract corresponding to 1 unit/ml GS (Fig. 1A), suggesting that the formation was done by this enzyme. The amounts of theanine formed were increased with increase in GS to 10 unit/ml (C). These findings indicate that *Ps. taetrolens* Y-30 GS might be usable for theanine production, and that production in high concentrations can be achieved, as in the examples of "coupled fermentation with energy transfer,"[20,25] by improving the reaction mixture with larger amounts of GS. This will be reported soon.

References

1) Sakato, Y., The chemical constituents of tea: III. A new amide theanine. *Nippon Nōgeikagaku Kaishi* (in Japanese), **23**, 262–267 (1949).
2) Sasaoka, K., Kito, M., and Inagaki, H., Studies on the biosynthesis of theanine in tea seedlings synthesis of theanine by the homogenate of tea seedlings. *Agric. Biol. Chem.*, **27**, 467–468 (1963).
3) Sasaoka, K., Kito, M., and Onishi, Y., Some properties of the theanine synthesizing enzyme in tea seedling. *Agric. Biol. Chem.*, **29**, 984–988 (1965).
4) Arai, M., Matsuura, T., Kakuda, T., Sasaki, K., and Yamanaka, T., Variation of the yields and the chemical

composition in leaves on nutriculture of tea plant. *Nippon Dojo Hiryogaku Zasshi* (in Japanese), **60**, 157–159 (1989).
5) Yokogoshi, H., Kato, Y., Sagesaka, M. Y., Mastuura, T. T., Kakuda, T., and Takeuchi, N., Reduction effect of theanine of blood pressure and brain 5-hydroxyindoles in spontaneously hypertensive rats. *Biosci. Biotechnol. Biochem.*, **59**, 615–618 (1995).
6) Kimura, R., and Murata, T., Influence of alkylamides of glutamic acid and related compounds on the central nervous system: II. Synthesis of amides of glutamic acid and related compounds, and their effects on the central nervous system. *Chem. Pharm. Bull.*, **19**, 1301–1307 (1971).
7) Kakuda, T., Nozawa, A., Unno, T., Okamura, N., and Okai, O., Inhibiting effects of theanine on caffeine stimulation evaluated by EEG in rat. *Biosci. Biotechnol. Biochem.*, **64**, 287–293 (2000).
8) Kobayashi, K., Nagato, Y., Aoi, N., Juneja, L. R., Kim, M., Yamamoto, T., and Sugimoto, S., Effect of L-theanine on release of α-brain waves in human volunteers. *Nippon Nōgeikagaku Kaishi* (in Japanese), **72**, 153–157 (1998).
9) Yokogoshi, H., Mochizuki, M., and Saitoh, K., Theanine-induced reduction of brain serotonin concentration in rats. *Biosci. Biotechnol. Biochem.*, **62**, 816–817 (1998).
10) Yokogoshi, H., Kobayashi, M., Mochizuki, M., and Terashima, T., Effect of theanine, γ-glutamylethylamide, on brain monoamines and striatal dopamine release in conscious rats. *Neurochem. Res.*, **23**, 671–677 (1998).
11) Matsuura, T., and Kakuda, T., Effects of precursor, temperature, and illumination of theanine accumulation in tea callus. *Agric. Biol. Chem.*, **54**, 2283–2286 (1990).
12) Matsuura, T., Kakuda, T., Kinoshita, T., Takeuchi, N., and Sasaki, K., Theanine formation by tea suspension cells. *Biosci. Biotechnol. Biochem.*, **56**, 1179–1181 (1992).
13) Matsuura, T., Sakane, I., Kakuda, T., Kitada, T., Kitada, T., and Takeuchi, N., Effect of plant growth regulators and carbon sources on theanine formation in callus cultures of tea (*Camellia sinensis*). *Biosci. Biotechnol. Biochem.*, **58**, 1519–1521 (1994).
14) Lichtenstein, N., Preparation of γ-alkylamides of glutamic acid. *J. Am. Chem. Soc.*, **64**, 1021–1022 (1942).
15) Furuyama, T., Yamashita, T., and Senoh, S., The synthesis of L-theanine. *Bull. Chem. Soc. Jpn.*, **37**, 1078 (1964).
16) Sasaoka, K., Kito, M., and Onishi, Y., Synthesis of theanine by pea seed acetone powder extract. *Agric. Biol. Chem.*, **28**, 318–324 (1964).
17) Sasaoka, K., Kito, M., and Onishi, Y., Synthesis of theanine by pigeon liver acetone powder extract. *Agric. Biol. Chem.*, **28**, 325–330 (1964).
18) Tachiki, T., Yamada, T., Mizuno, K., Ueda, M., Shiode, J., and Fukami, H., γ-Glutamyl transfer reactions by glutaminase from *Pseudomonas nitroreducens* IFO 12694 and their application for the syntheses of theanine and γ-glutamylmethylamide. *Biosci. Biotechnol. Biochem.*, **62**, 1279–1283 (1998).
19) Suzuki, H., Izuka, S., Miyakawa, N., and Kumagai, H., Enzymatic production of theanine, an "umami" component of tea, from glutamine and ethylamine with bacterial γ-glutamyltranspeptidase. *Enzyme Microb. Technol.*, **31**, 884–889 (2002).
20) Tachiki, T., Suzuki, H., Wakisaka, S., Yano, T., and Tochikura, T., Glutamine production in high concentrations with energy transfer employing glutamine synthetase from *Micrococcus glutamicus*. *J. Gen. Appl. Microbiol.*, **29**, 355–363 (1983).
21) Wakisaka, S., Oshima, Y., Ogawa, M., Tochikura, T., and Tachiki, T., Characteristics and efficiency of glutamine production by coupling of a bacterial glutamine synthetase reaction with the alcoholic fermentation system of baker's yeast. *Appl. Environ. Microbiol.*, **64**, 2952–2957 (1998).
22) Kawaguchi, K., Kawai, H., and Tochikura, T., Production of sugar nucleotides by fermentation. *Methods Carbohydr. Chem.*, **3**, 261–269 (1980).
23) Tochikura, T., Production of nucleotide-related substances by yeast and utilization of fermentation energy to biosynthetic processes. *Hakko Kogaku Kaishi* (in Japanese), **56**, 508–526 (1978).
24) Tochikura, T., Kuwahara, M., Yagi, S., Okamoto, H., Tominaga, Y., Kano, T., and Ogata, K., Fermentation and metabolism of nucleic acid-related compounds in yeast. *J. Ferment. Technol.*, **45**, 511–529 (1967).
25) Tochikura, T., Kariya, Y., Yano, T., Tachiki, T., and Kimura, A., Studies on utilization of fermentation and respiratory energy to biosynthetic processes. I. Fermentative production of phosphoric acid anhydrides and phosphate esters. *Amino Acid Nucleic Acid* (in Japanese), **29**, 59–74 (1974).
26) Tachiki, T., Suzuki, H., Wakisaka, S., Yano, T., and Tochikura, T., Production of γ-glutamylmetylaminde and γ-glutamylethylamide by coupling of baker's yeast preparations and bacterial glutamine synthetase. *J. Gen. Appl. Microbiol.*, **32**, 545–548 (1986).
27) Tachiki, T., Wakisaka, S., Suzuki, H., Kumagai, H., and Tochikura, T., Variation of properties of *Micrococcus glutamicus* glutamine synthetase brought about by divalent cations. *Agric. Biol. Chem.*, **47**, 287–292 (1983).
28) Kung, H. F., and Wagner, C., γ-Glutamylmethylamide. A new intermediate in the metabolism of methylamine. *J. Biol. Chem.*, **244**, 4136–4140 (1969).
29) Kimura, T., Sugahara, I., Hayashi, K., Kobayashi, M., and Ozeki, M., Primary metabolic pathway of methylamine in *Methylophaga* sp. AA-30. *Agric. Biol. Chem.*, **54**, 2819–2826 (1990).
30) Kimura, T., Sugahara, I., Hanai, K., and Tonomura, Y., Purification and characterization of γ-glutamylmethylamide synthetase from *Methylophaga* sp. AA-30. *Biosci. Biotechnol. Biochem.*, **56**, 708–711 (1992).
31) Tachiki, T., Wakisaka, S., Kumagai, H., and Tochikura, T., Glutamine synthetase from *Micrococcus glutamicus*: effect of nitrogen sources in culture medium on enzyme formation and some properties of crystalline enzyme. *Agric. Biol. Chem.*, **45**, 1487–1492 (1981).
32) Edman, P., and Cooper, A. G., Amino acid sequence at N-terminal end of a cold agglutinin kappa chain. *FEBS Lett.*, **2**, 33–35 (1968).
33) Lowry, O. H., Rosebrought, N. J., Farr, A. L., and Randall, R.J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275 (1951).
34) Somogyi, M., Notes on sugar determination. *J. Biol. Chem.*, **195**, 19–23 (1952).

35) Fiske, C. H., and Subbarow, Y., The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**, 375–400 (1925).
36) Stadtman, E. R., and Ginsburg, A., The glutamine synthetase of *Escherichia coli*: structure and control. In "The enzymes" Vol. 10, ed. Boyer, P. D., Academic Press, New York, pp. 755–807 (1974).
37) Segal, A., and Stadtman, E. R., Effect of cobaltous ion on various catalytic parameters and on heterologous subunit interactions of *Escherichia coli* glutamine synthetase. *Arch. Biochem. Biophys.*, **152**, 356–366 (1972).
38) Stacy, G., Van Baalen, C., and Tabita, F. R., Nitrogen and ammonia assimilation in cyanobacteria: regulation of glutamine synthetase. *Arch. Biochem. Biophys.*, **194**, 457–467 (1972).
39) Sung, H. C., Tachiki, T., Kumagai, H., and Tochikura, T., Production and preparation of glutamine synthetase from *Brevibacterium flavum*. *J. Ferment. Technol.*, **62**, 371–376 (1984).
40) Denton, M. D., and Ginsburg, A., Some characteristics of the binding of substrates of glutamine synthetase from *Escherichia coli*. *Biochemistry*, **9**, 617–632 (1970).
41) Wakisaka, S., Tachiki, T., and Tochikura, T., Properties of *Brevibacterium flavum* glutamine synthetase "*in vivo*-like" conditions. *J. Ferment. Bioeng.*, **70**, 182–184 (1990).
42) Matsuoka, K., and Kimura, K., Conformational change in *Micobacterium smegmatis* glutamine synthetase induced by certain divalent cations. *J. Biochem.*, **97**, 1033–1042 (1985).