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# A Novel Catalytic Ability of γ-Glutamylcysteine Synthetase of *Escherichia coli* and Its Application in Theanine Production

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**γ-Glutamylcysteine synthetase (γGCS, EC 6.3.2.2) catalyzes the formation of γ-glutamylcysteine from L-glutamic acid (Glu) and L-cysteine (Cys) in an ATP-dependent manner. While γGCS can use various amino acids as substrate, little is known about whether it can use non-amino acid compounds in place of Cys. We determined that γGCS from *Escherichia coli* has the ability to combine Glu and amines to form γ-glutamylamides. The reaction rate depended on the length of the methylene chain of the amines in the following order: *n*-propylamine > butylamine > ethylamine ≫ methylamine. The optimal pH for the reaction was narrower and more alkaline than for the reaction with an amino acid. The newly found catalytic ability of γGCS was used in the production of theanine (γ-glutamylethylamine). The resting cells of *E. coli* expressing γGCS, in which ATP was regenerated through glycolysis, synthesized 12.1 mM theanine (18 h) from 429 mM ethylamine.**

**Key words:** γ-glutamylcysteine synthetase; theanine; γ-glutamylamide; amine

Theanine (γ-glutamylethylamide) is an amino acid found in green tea that enhances the umami taste.[1)] Besides its graceful flavor, many physiological functions of it have been reported, including mind relaxation,[2)] improvement in sleep disturbances,[3)] suppression of blood pressure elevation,[4)] anti-oxidation of LDL cholesterol,[5)] modulation of neurotransmission,[6)] neuroprotection,[7)] anti-hypertension,[8)] enhancement of antitumor activity,[9)] and improved recognition.[10)]

In the tea plant, theanine is synthesized by theanine synthetase (EC 6.3.1.6), with Glu, ethylamine, and ATP as substrates,[11)] but it is very labile, so it cannot be used in industrial theanine production. Various methods have been employed for theanine production, including extraction from tea leaves, chemical synthesis, and enzymatic synthesis. The enzymatic methods can be classified into two groups. One starts from L-glutamine (Gln) using γ-glutamyltransferase activity, and the other starts from Glu using γ-amidation activity. Yamada *et al.*[12)] have reported theanine synthesis from Gln and ethylamine (Fig. 1a) using the γ-glutamyl transfer activity of the glutaminase of *Pseudomonas nitroreducens*. Since glutaminase also catalyzes the hydrolysis of the substrate, Gln, a high concentration of ethylamine and highly alkaline pHs are necessary. Improvements in the reaction conditions have been reported.[13)] γ-Glutamyltranspeptidase can be also used in theanine synthesis. Suzuki *et al.*[14,15)] explained that the γ-glutamyltranspeptidase from *E. coli* synthesized theanine from Gln and ethylamine at pH 10. γ-Amidation of Glu can be achieved with glutamine synthetase (GS) and with related enzymes (Fig. 1b). γ-Amidation of Glu needs ATP for the reaction, but Glu is stable and lower-priced than Gln. GS originating from *Micrococcus glutamicus* has been found to synthesize theanine from Glu and ethylamine in the presence of ATP.[16)] GS from *Pseudomonas taetrolens* Y-30 has also been reported to have the same activity.[17)] Susana *et al.*[18)] explained that IpuC (γ-glutamylamide synthetase) of *Pseudomonas* sp. shared homology with GS and retained theanine synthesizing activity through γ-amidation of Glu. Recently, γ-glutamylmethylamide synthetases (EC 6.3.4.12) from *Methylophaga* sp. AA-30 and from *Methylovorus mays* No. 9 were found to be efficient theanine-producing enzymes.[19,20)] The amino acid sequence of the latter enzyme showed significant identity to GS.[21)]

γ-Glutamylcysteine synthetase (γGCS, EC 6.3.2.2) catalyzes the ATP-dependent synthesis of γ-glutamylcysteine from Glu and Cys, the first step in the reaction of glutathione formation.[22,23)] The *gshA* gene encoding γGCS has also been identified in a variety of organism, including *E. coli*.[24)] Biochemical studies have revealed that γ-GCS can γ-glutamylate Cys and also many other kinds of amino acids, such as alanine and α-aminobutyrate,[25)] but little is known about whether the enzyme can accept other non-amino acid compounds.

The fact that both GS and γGCS form amide bonds using the γ-carboxy group of Glu prompted us to try to determine whether γGCS can react with ethylamine to yield theanine. In this study, we found that γGCS can synthesis theanine with ethylamine. Our results also indicate that certain amines are preferred substrates for γGCS.

## Materials and Methods

*Materials.* General biochemical reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). The amines were from Tokyo Chemical Industry (Tokyo). *E. coli* K12 W3110 strain (GenBank Accession no. AP009048) was from NITE Biological Resource Center (NBRC, Tokyo). *E. coli* K12 DH5α strain was from Toyobo (Osaka). Various restriction enzymes and Taq polymerases for PCR and *E. coli* BL21 strain were from Takara Bio (Ohtsu, Japan).

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*Abbreviations*: γGCS, γ-glutamylcysteine synthetase; Glu, L-glutamic acid; Cys, L-cysteine; Gln, L-glutamine; GS, glutamine synthetase

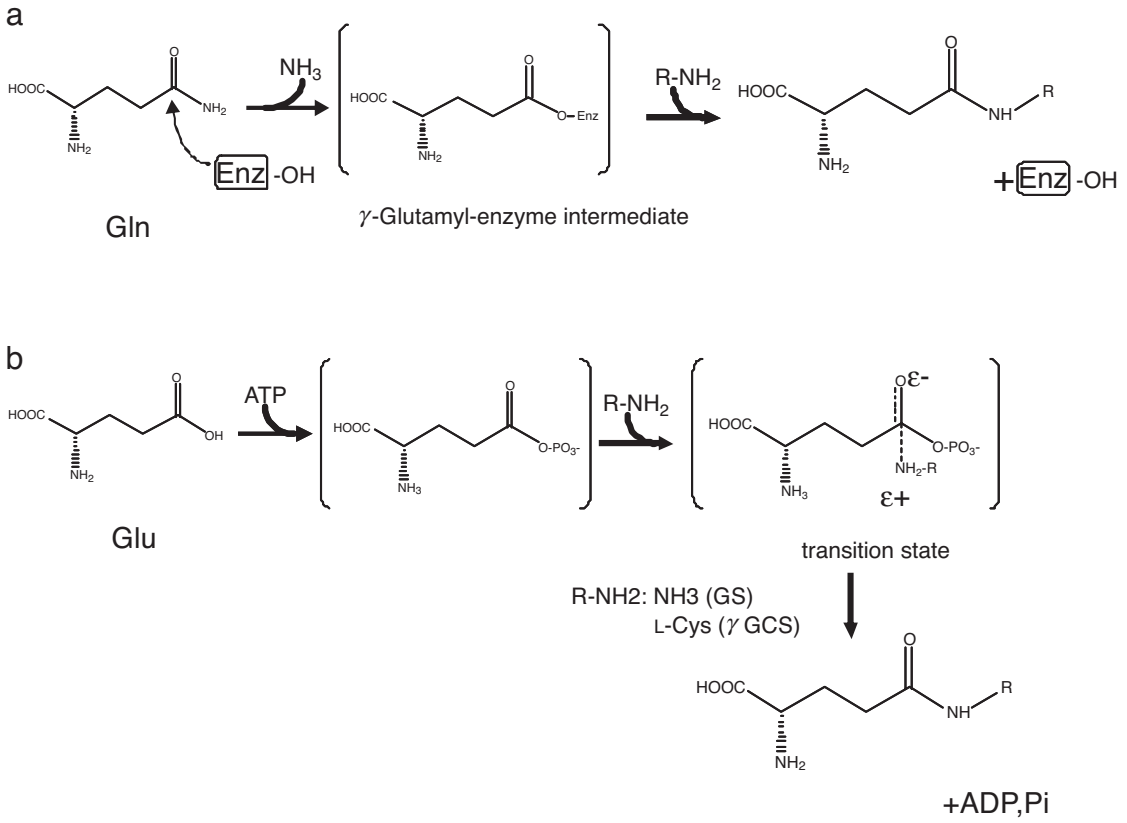


**Fig. 1.** Reaction Mechanisms of $\gamma$-Glutamylamide Bond-Producing Enzymes.
a, $\gamma$-glutamyl transfer reaction of glutaminase or $\gamma$-glutamyltranspeptidase. b, $\gamma$-amidation of Glu by GS and by $\gamma$GCS.

Expression vector pTrS33 was previously reported in our patent.[26] The plasmid purification kit and pQE80L vector were from Qiagen (Hilden, Germany).

*Expression and purification of* E. coli *(His)$_6$-tagged $\gamma$GCS.* The *gshA* gene (KEGG Accession no. JW2663 http://www.genome.jp/kegg/) was amplified from *E. coli* W3110 genome by PCR using primer A, 5′-CGCGGATCCATGATCCCGGACGTATCACAGGCG-CTG-3′, and primer B, 5′-GCACCCAAGCTTTGCAGGCGTGTTTTT-CCAGCCACACCGCA-3′. Primer A was added to the *Bam*HI recognition sequence, and the initiation codon for the gene, which begins with TTG, was altered to ATG in expectation of improved translation efficiency. Primer B was added to the *Hin*dIII recognition sequence. Expression vector pQE80L and the PCR product were digested with *Bam*HI and *Hin*dIII. Both DNA fragments were ligated, and the resulting plasmid was introduced into *E. coli* DH5$\alpha$. Recombinant *E. coli* DH5$\alpha$ was cultivated in LB medium (10 g/l Bacto-tryptone, BD (New Jersey, USA), 5 g/l yeast extract, BD, and 5 g/l NaCl) containing 100 mg/l of ampicillin at 30 °C overnight with shaking. The culture was transferred to fresh LB medium and incubated at 25 °C. Four h after transfer, 0.2 mM IPTG (isopropyl-$\beta$-D-thiogalactopyranoside) was added, and cultivation was continued for 16 h. Cells were collected by centrifugation, and the cell pellet was suspended in 50 mM phosphate buffer (pH 7.8) and disrupted by sonication at 4 °C. The cellular debris was removed by centrifugation and the supernatant was subjected to further purification using HiTrap™HP (GE Healthcare, Sweden). The protein concentration was determined using the Quick Start™ Bradford Dye Reagent (Bio-Rad, California, USA) with bovine serum albumin as the standard.

*Construction of a strain expressing $\gamma$GCS for theanine synthesis.* A strain expressing $\gamma$GCS was constructed by cloning *gshA* by the following method: First, *gshA* was amplified from the *E. coli* W3110 genome by PCR using primer C, 5′-CGGGAGAAGCTTATGATCCC-GGACGTATCACAG-3′, and primer D, 5′-TTTTCTGGATCCTTA-GGCGTGTTTTTCCAGCC-3′. Primer C was added to the *Hin*dIII recognition sequence, and the initiation codon in this primer, TTG, was altered to ATG. Primer D was added to the *Bam*HI recognition sequence. The *lac* promoter region was amplified from expression vector pUC19 by PCR using primer E, 5′-GGAAGAGAATTCAA-TACGCAAACCGCCTCT-3′, which was added to the *Eco*RI recognition sequence, and Primer F, 5′-GGTCATAAGCTTTTCCTGTGT-GAAATTGTTAT-3′, which was added to the *Hin*dIII recognition sequence. Each of the PCR products was digested with restriction enzymes, and the 1.6-kb *gshA* fragment and the 0.3-kb *lac* promoter fragment were respectively recovered using the Geneclean Kit (Funakoshi, Tokyo). Expression vector pTrS33 was double digested with *Bam*HI and *Eco*RI. *GshA*, the *lac* promoter fragment, and the 3.16-kb vector fragment were subjected to ligation using a Ligation kit (Takara Bio) at 16 °C for 16 h. *E. coli* DH5$\alpha$ was transformed using the ligation reaction mixture by standard methods, and the resulting transformant was spread on LB agar medium containing 50 µg/ml of ampicillin and cultured overnight at 30 °C. The recombinant plasmid was extracted from a colony of the transformant, and it was confirmed that the plasmid contained the inserted *lac* promoter region and *gshA* by sequence analysis. The expression vector was designated pGSK1. The structure of pGSK1 is shown in Fig. 2. The *E. coli* DH5$\alpha$ carrying the plasmid was designated *E. coli* DH5$\alpha$/pGSK1.

*Production of theanine using a treated culture as the enzyme source.* Recombinant *E. coli* DH5$\alpha$/pGSK1 was inoculated into 40 ml of LB medium containing 100 mg/l ampicillin and subjected to shaking culture in a 300-ml Erlenmeyer flask at 30 °C overnight. After culture completion, the cells recovered by centrifugation of the culture were suspended in 100 mM/l Tris–HCl (pH 8.0), and the cell concentration was adjusted so that the OD660 was 70 as measured with a spectrophotometer, and this was designated the cell-containing solution. To the cell-containing solution was added xylene at a concentration of 10 ml/l, and the mixture was vortexed for 15 min to obtain treated cells. To 100 µl of the treated cells, 5 g/l $MgSO_4$, 3.5 g/l $K_2SO_4$, 143 mg/l $NAD^+$, 66 mg/l FMN, 12.1 g/l ATP, 2.5 g/l monosodium L-glutamate monohydrate (MSG), and 20 g/l ethylamine hydrochloride were added, and the resulting reaction mixture (total volume, 200 µl) was reacted at 37 °C for 60 min. After completion of

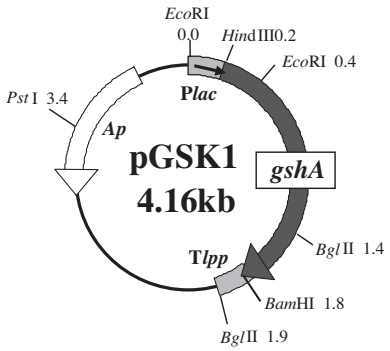


**Fig. 2.** Structure of γGCS Expression Vector pGSK1.
*gshA*, γGCS gene origin of *E. coli* W3110; P*lac*, *lac* operon promoter region; T*lpp*, terminator region of lipoprotein gene from *E. coli* W3110; *Ap*, ampicillin resistance gene.

the reaction, theanine was detected and quantitatively determined by HPLC under the following conditions: The mobile phase was 10% acetonitrile aqueous solution containing 2 g/l sodium 1-heptanesulfonate (adjusted to pH 2.0 with phosphoric acid). The column was a Develosil ODS-HG5 (Nomura Chemical, Tokyo, 4.6 × 250 mm), the temperature was 40 °C, and the flow rate was 0.9 ml/min. Products were detected by absorption at 210 nm.

Next, recombinant plasmid pGSK1 was introduced into *E. coli* BL21 by a standard method. BL21/pGSK1 was spread on LB agar medium containing 100 mg/l ampicillin and this was subjected to static culture at 30 °C overnight. The cells that grew on the medium were inoculated into 300 ml of a pre-culture medium (6% corn steep liquor, 1.15% MSG, 0.2% lactic acid, 200 mg/l casamino acid, 5 mg/l vitamin B1, pH 7.2) in a 2 liters Erlenmeyer flask, and cultured at 28 °C at 220 rpm for 20 h. The culture obtained (2.25 ml) was inoculated into one liter of a seed medium (2% corn steep liquor, 0.5% soybean peptide, (SMS, Fuji Oil, Osaka), 1.5% dipotassium hydrogen phosphate, 0.1% sodium chloride, 0.6% ammonium sulfate, 0.1% glycine, 0.06% arginine hydrochloride, 4.95 mg/l ferrous sulfate, 4.4 mg/l zinc sulfate, 1.97 mg/l copper sulfate, 360 μg/l manganese chloride, 440 μg/l sodium borate, 185 μg/l ammonium molybdate, 5 mg/l vitamin B1, 5 mg/l nicotinic acid, 20 mg/l leucine, 20 mg/l threonine, 20 mg/l tryptophan, 0.01% LG109 (Asahi Denka, Tokyo), 1% glucose, 0.05% magnesium sulfate, and 100 mg/l ampicillin (pH 6.5) in a 2 liters jar, and cultured with aeration at a rate of 1 l/min and agitation at a speed of 800 rpm at 30 °C for 8 h.

The culture obtained (28 ml) was inoculated into 1 liter of a production medium (2.25% corn steep liquor, 0.55% SMS, 1.68% dipotassium hydrogen phosphate, 0.115% sodium chloride, 0.68% ammonium sulfate, 5.57 mg/l ferrous sulfate, 4.95 mg/l zinc sulfate, 2.21 mg/l copper sulfate, 405 μg/l manganese chloride, 495 μg/l sodium borate, 208 μg/l ammonium molybdate, 5.6 mg/l vitamin B1, 5.6 mg/l nicotinic acid, 22 mg/l leucine, 22 mg/l threonine, 22 mg/l tryptophan, 0.018% LG109, 1.26% glucose, 0.08% magnesium sulfate, and 100 mg/l ampicillin (pH 6.5)) in a 2 liters jar and this was cultured with aeration at a rate of 1 l/min and agitation at a speed of 800 rpm at 30 °C controlling the pH to 6.5 with 28% aqueous ammonia. During culture, 340 ml of a sugar solution (57.7% glucose and 0.188 g/l calcium chloride) for feeding was added at a fixed flow rate. Culturing was terminated after 30 h, and 7.5 ml/l xylene was added, followed by agitation for 10 min to obtain treated matter of the culture. To 700 ml of the treated matter were added 4.4 g/l of magnesium sulfate, 3 g/l of potassium sulfate, 438 mg/l of ATP, 62.5 mg/l of $NAD^+$, 27.5 mg/l of FMN, 81 g/l of glucose, 35 g/l of ethylamine hydrochloride, and 70 g/l of MSG, and the mixture was aerated at a rate of 0.7 ml/min with agitation at 950 rpm at 34 °C for 18 h controlling the pH to 7.2 with a sodium hydroxide solution. The reaction product was analyzed by HPLC under the conditions described above.

*Substrate specificities of γGCS for amines.* The substrate specificity of γGCS was assayed as follows: The 0.2 ml reaction mixtures contained 15 mM ATP, 100 mM $MgSO_4$, 5 g/l MSG, 2 or 10 g/l amines, and 100 mg/l $(His)_6$-tagged γGCS in 0.1 M Tris–HCl buffer (pH 8.5). The reaction was carried out at 37 °C for 60 min, and was stopped by boiling at 100 °C for 5 min. The reaction mixture was analyzed by HPLC under the conditions described above, except that the mobile phase was 8–18% acetonitrile aqueous solution containing 2 g/l sodium 1-heptanesulfonate (adjusted to pH 2.0 with phosphoric acid).

The phosphate released from ATP was monitored by spectrophotometric assay (P-Test Wako, Wako Pure Chemical Industries, Tokyo) as an indicator of ATP-dependent synthetic activity. Background rates were determined from samples containing everything but γGCS.

*Identification of γ-glutamylamide structure by MS and NMR.* The reaction products from ethylamine, propylamine, and allylamine were purified as follows: The 1 ml reaction mixtures contained 60 mM ATP, 100 mM $MgSO_4$, 5 g/l MSG, 10 g/l amines and 100 mg/l $(His)_6$-tagged γ-GCS in 0.1 M Tris–HCl buffer (pH 8.0). The reaction was carried out at 37 °C for 60 min, and was stopped by boiling at 100 °C for 5 min. After completion of the reaction, the contents of the reaction mixture were detected by HPLC analysis under the following conditions: The mobile phase was 3–5% acetonitrile aqueous solution containing 1 g/l sodium heptafluorobutyric acid (adjusted to pH 2.0 with phosphoric acid). A Develosil ODS-HG5 (Nomura Chemical, Tokyo, 4.6 × 250 mm) column was used. The temperature was 40 °C and the flow rate was 0.9 ml/min. Products were detected by absorption at 210 nm. The products were purified by HPLC and collected from the fractions of interest, and then the solution was evaporated using a Centrifugal Concentrator VC-360 (Taitec, Koshigaya, Japan). The samples obtained were analyzed by MS and NMR. Mass spectrometry analysis was performed on two types of equipment, a ZMD 2000 (Waters, MA) and a LTQ Orbitrap (Thermo Fisher Scientific, MA), operated in positive electrospray ionization mode. On the former single-quadrupole mass spectrometer, each sample was dissolved in water and analyzed by flow injection analysis-mass spectrometry (FIA-MS) to confirm the molecular weight. The pump was set to a flow rate of 0.25 ml/min, and the mobile phase was 50% acetonitrile containing 0.1% v/v formic acid. The latter spectrometer was used to obtain high-resolution mass spectra. Each sample solution was diluted with 50% acetonitrile containing 0.1% v/v formic acid, and was injected with an automatic flow syringe at a flow rate of 10 μl/min. The NMR spectra were recorded at 500.13 MHz for $^1H$ and at 125.76 MHz for $^{13}C$ on a Bruker DMX 500 spectrometer (Bruker Biospin, Karlsruhe, Germany) in $D_2O$ at 30 °C. The structures of the γ-glutamylamides were identified by analysis of the NMR spectra ($^1H$, $^{13}C$, $^1H$–$^1H$ COSY, NOESY, HSQC, and HMBC). Data processing was carried out using the program XWINNMR (Bruker Biospin).

## Results

*Formation of theanine by γGCS*

The *gshA* gene encoding γGCS was amplified from the *E. coli* W3110 genome by PCR and was ligated with expression vector pQE80L. The resulting plasmid was introduced into *E. coli* DH5α. Recombinant *E. coli* DH5α was cultivated in LB medium and disrupted by sonication. Cellular debris was removed by centrifugation, and the supernatant was subjected to further purification using HiTrap™HP. His-tagged γGCS protein was eluted using 500 mM imidazole buffer, and was confirmed to be a single band on SDS–PAGE analysis (Fig. 3). γGCS activity was confirmed by checking γ-glutamylcysteine and γ-glutamylaminobutyrate-forming ability using Glu and Cys and Glu and α-aminobutyrate respectively (Fig. 4a, b).

The His-tagged γGCS was incubated with Glu, ethylamine, and ATP. HPLC analysis revealed a new peak that had the same retention time as theanine (Fig. 4g). Reactions without the enzyme and without ATP did not yield peak (data not shown). The peak was fractionated and subjected to MS and NMR analysis. From a high-resolution ESI mass spectrum, $[M + H]^+$ was measured at $m/z$ 175.1086, corresponding to the

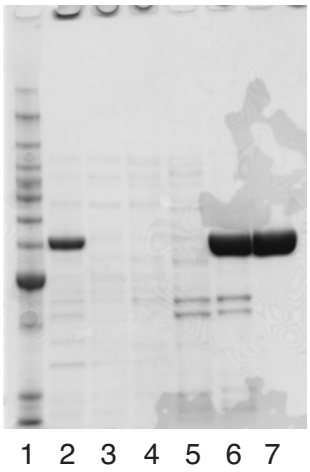


**Fig. 3.** SDS-Polyacrylamide Gel Electrophoresis Analysis of Cell Lysate and Purified Enzyme Fractions.

Lane 1, marker; lane 2, IPTG induced cell extracts; lanes 3, 4, 5, 6, and 7, fractions eluted from the nickel column with 50, 100, 200, 350, and 500 mM of imidazole respectively.

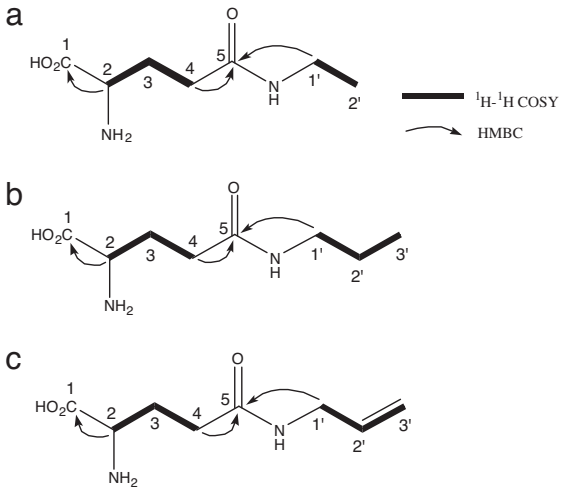


**Fig. 5.** Summary of NMR Spectral, COSY, and HMBC Data.

Reaction products from ethylamine (a), *n*-propylamine (b), and allylamine (c).

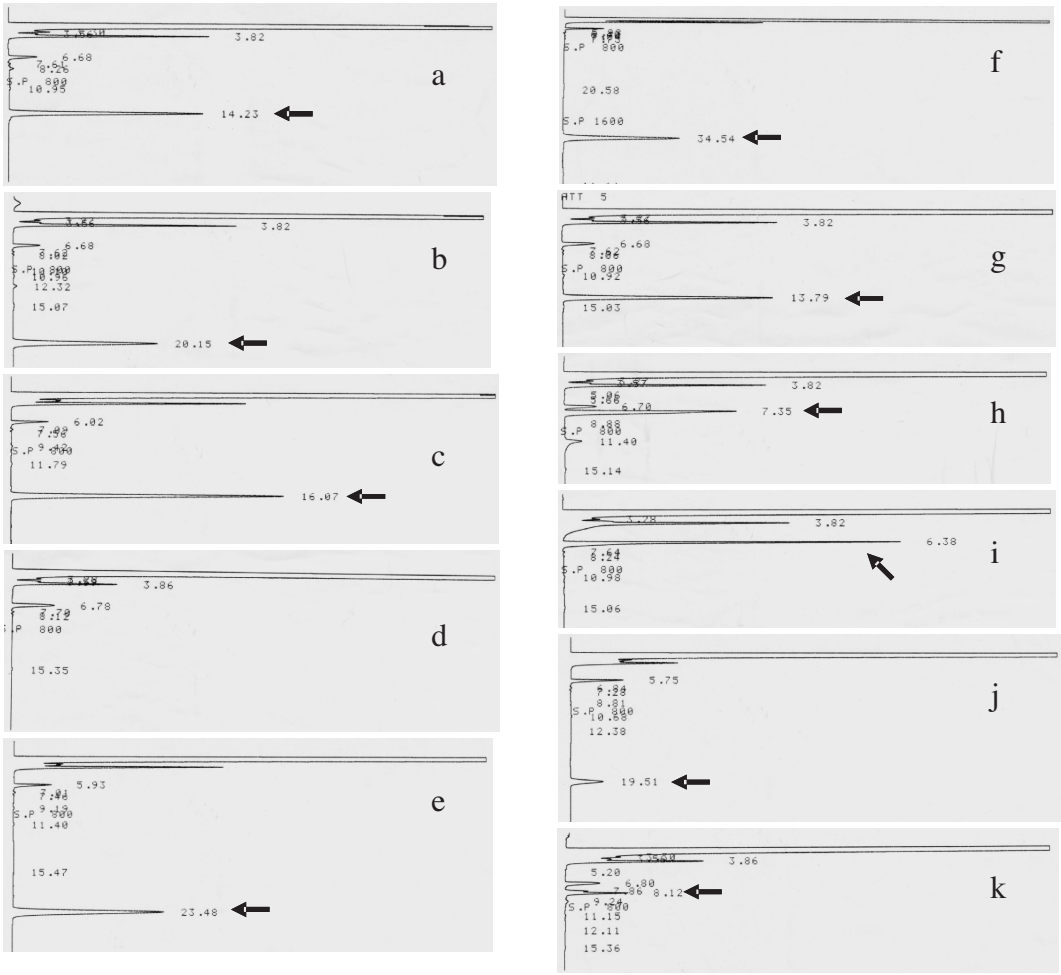


**Fig. 4.** HPLC Analysis of the Reaction Samples of γGCS with Glu and Various Amines.

a, cysteine; b, α-aminobutyrate; c, allylamine; d, allylamine; same as (c) but no added γGCS. e, *n*-propylamine; f, *n*-butylamine; g, ethylamine; h, 2-amino-1-butanol; i, ethanolamine; j, isopropylamine; k, methylamine. Arrows indicate reaction products that cannot be found in the absence of γGCS.

molecular formula $C_7H_{14}N_2O_3$. NMR spectra ($^1$H, $^{13}$C, $^1$H–$^1$H COSY, NOESY, HSQC, and HMBC) showed that ethylamine was connected to the γ-carboxyl unit of Glu (Fig. 5a, Table 1). These results clearly demonstrate that the compound formed by the reaction was γ-glutamylethylamide (theanine), indicating that γGCS from *E. coli* can synthesize theanine from Glu, ethylamine, and ATP.

**Table 1.** $^{1}$H and $^{13}$C-NMR Data for the γGCS Product Using Amines as Substrates

$^{1}$H-NMR

| | Ethylamine (ppm) | | J (Hz) | Propylamine (ppm) | | J (Hz) | Allylamine (ppm) | | J (Hz) |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 4.09 | 1H (t) | 6.4 | 4.10 | 1H (t) | 6.5 | 4.01 | 1H (t) | 6.4 |
| 3 | 2.24 | 2H (m) | | 2.23 | 2H (m) | | 2.23 | 2H (m) | |
| 4 | 2.48 | 2H (m) | | 2.48 | 2H (m) | | 2.54 | 2H (m) | |
| 1′ | 3.21 | 2H (q) | 7.3 | 3.14 | 2H (t) | 6.9 | 3.83 | 2H (dt) | 5.1, 1.7 |
| 2′ | 1.12 | 3H (t) | 7.3 | 1.51 | 2H (m) | | 5.89 | 1H (ddt) | 17.5, 10.4, 5.1 |
| 3′ | — | | | 0.88 | 3H (t) | 7.4 | 5.21 | 1H (ddt) | 17.5, 1.6, 1.7 |
| | | | | | | | 5.18 | 1H (ddt) | 10.4, 1.6, 1.7 |

$^{13}$C-NMR

| | Ethylamine (ppm) | | Propylamine (ppm) | | Allylamine (ppm) | |
|---|---|---|---|---|---|---|
| 1 | 172.5 | s | 172.3 | s | 173.2 | s |
| 2 | 53.2 | d | 53.1 | d | 53.8 | d |
| 3 | 26.6 | t | 26.6 | t | 26.8 | t |
| 4 | 32.1 | t | 32.1 | t | 32.1 | t |
| 5 | 174.5 | s | 174.8 | s | 175.0 | s |
| 1′ | 35.4 | t | 42.1 | t | 42.4 | t |
| 2′ | 14.2 | q | 22.5 | t | 134.4 | d |
| 3′ | — | | 11.4 | q | 116.4 | t |

**Table 2.** Production of Theanine in *E. coli* DH5α/pGSK1

| Strain | Ethylamine·HCl (g/l) | MSG (g/l) | Theanine (mg/l) |
|---|---|---|---|
| DH5α/pGSK1 | 0 | 0 | — |
| DH5α/pGSK1 | 20 | 0 | 54 |
| DH5α/pGSK1 | 20 | 2.5 | 520 |
| DH5α | 20 | 2.5 | — |

—, not detected <5 mg/l

*Theanine production by resting cells of* E. coli *overexpressing γGCS*

Next we examined to determine whether *E. coli* cells overexpressing γGCS might be useful for theanine synthesis. *E. coli* DH5α/pGSK1 was cultivated and permeabilized by xylene treatment, as described in "Materials and Methods." The treated cells were mixed with ethylamine, Glu, and ATP, and incubated at 37 °C. After a 60-min reaction, the reaction mixture was analyzed by HPLC. As shown in Table 2, 3 mM of theanine was synthesized from 245 mM of ethylamine and 14.8 mM of Glu. Without pGSK1, no theanine was detected (see Table 2, column for DH5α alone), showing the necessity of overexpression of γGCS. DH5α/pGSK1 cells formed theanine without the addition of Glu (Table 2), probably due to the available intracellular pool of Glu. Since glucose was consumed by the xylene-treated resting cells (data not shown), we surmiseed that ATP might be regenerated through the sugar metabolism of the cells. To explore this possibility, we conducted a resting-cell reaction in the presence of glucose in a pH-controlled manner. *E. coli* BL21/pGSK1 was cultured in a 2-liter jar fermenter. After cultivation, xylene was added to the cultivation fluid for permeabilization, and 429 mM of ethylamine, 414 mM of Glu, 0.8 mM of ATP, and glucose were added. The pH condition was kept at 7.2 during the reaction. As shown in Fig. 3a, 12.1 mM (2.1 g/l) theanine was detected in the 18 h broth (Fig. 6a). Another HPLC peak was detected in the

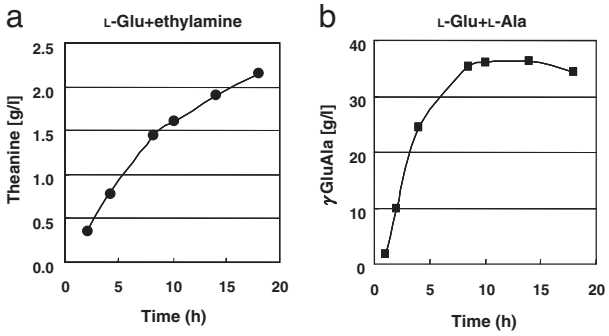


**Fig. 6.** Production of Theanine (panel a) and γ-Glutamylalanine (panel b) Using *E. coli* BL21/pGSK1 in a Jar Fermenter.

theanine broth (data not shown), and it was identified as 11.5 mM (2.5 g/l) γ-glutamylalanine. When 197 mM of Ala was used instead of ethylamine, 165 mM of γ-glutamylalanine was produced within 10 h (Fig. 6b). Production of 12.1 mM of theanine requires the same amount of ATP, 15 times as much as the amount of ATP added at the beginning of the reaction. These results indicate that theanine was produced by resting cells of *E. coli* overexpressing γGCS from Glu and ethylamine using the ATP-regeneration activity of the cell itself.

*γ-Glutamylamide synthesizing ability of* E. coli *γGCS*

The substrate specificity of *E. coli* γGCS for other amines was examined. The reaction mixtures of His-tagged γGCS, Glu, amines, and ATP were analyzed by HPLC. Significant peaks were detected when several kinds of amines were used as substrates (Fig. 4). These peaks were absent in the reactions that omitted γGCS, as exemplified in Fig. 4d, indicating that they were unique reaction products. *sec*-Butylamine, 2-heptylamine, and heptylamine did not yield new products. It should be noted that the practical insolubility of 2-heptylamine and heptylamine might have hampered the reaction.

The products from *n*-propylamine and allylamine were purified and analyzed by MS and NMR. On MS analysis, peaks at $m/z$ 189 ($[M+H]^+$), 211 ($[M+Na]^+$), 377 ($[2M+H]^+$), and 399 ($[2M+Na]^+$) were detected in the *n*-propylamine sample, and peaks at $m/z$ 187 ($[M+H]^+$), 209 ($[M+Na]^+$), 373 ($[2M+H]^+$), and 395 ($[2M+Na]^+$) were detected from the allylamine sample. The high-resolution ESI mass spectrum of the *n*-propylamine sample gave a $[M+H]^+$ at $m/z$ 189.1244, which corresponds to the molecular formula of γ-glutamylpropylamide, $C_8H_{16}N_2O_3$. The high-resolution ESI mass spectrum of the allylamine

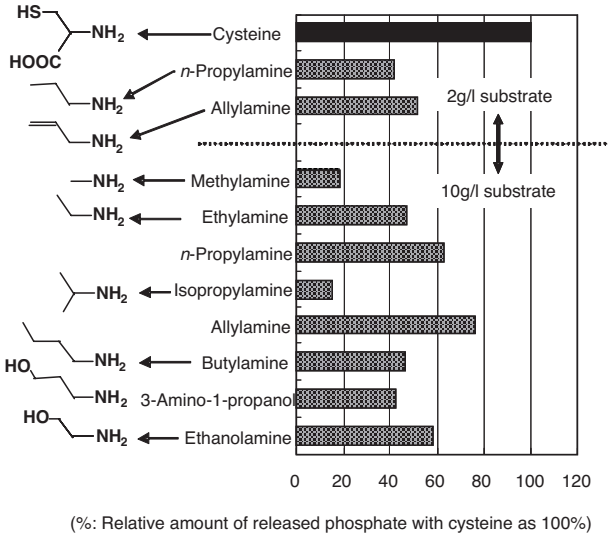


**Fig. 7.** Substrate Specificity of *E. coli* γGCS at pH 8.5.

Each bar indicates phosphate concentration released in the reaction mixtures with the substrate shown on the left. The phosphate concentration is expressed relative to that of the reaction, with cysteine as 100%. The substrate concentrations of cysteine, *n*-propylamine, and allylamine were 2 g/l, and of the others, 10 g/l.

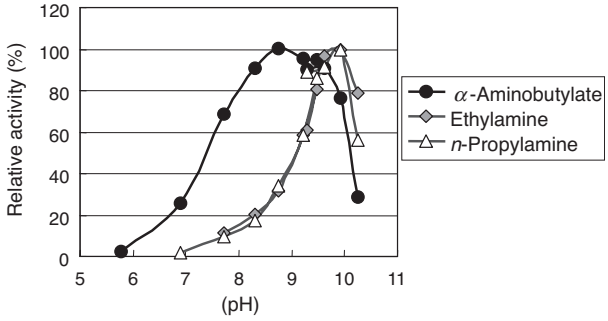


**Fig. 8.** Effects of pH on γ-Glutamylamide Forming Reaction by *E. coli* γGCS.

γ-Glutamyl compounds were determined by HPLC peak area, and activity was expressed relative to the various maximum activities, as 100%. One hundred mg/l of purified His-tagged γGCS protein was incubated with 15 mM of ATP, 100 mM of $MgSO_4$, 5 g/l of MSG, and 2 g/l of amines in 0.1 M Tris–HCl buffer (pH 7 to 9.5) or 0.1 M CAPS buffer (pH 9.2 to 10.3). The reaction was carried out at 37 °C for 60 min, and was stopped by boiling (100 °C for 5 min). The reaction mixture was analyzed by HPLC. Symbols: ●, α-aminobutylate; ◆, ethylamine; △, propylamine.

sample gave $[M + H]^+$ at $m/z$ 187.1086, which corresponds to the molecular formula of γ-glutamylallylamide, $C_8H_{14}N_2O_3$. The NMR spectra ($^1H$, $^{13}C$, $^1H$–$^1H$ COSY, NOESY, HSQC, and HMBC) revealed that the amines were connected to the γ-carboxyl unit of Glu in both samples (Fig. 5b, c, Table 1). Based on these results, the products from the reaction of *n*-propylamine and allylamine were concluded to be γ-glutamylpropylamide and γ-glutamylallylamide respectively, indicating that γGCS of *E. coli* can synthesize various γ-glutamylamide compounds from amines and Glu.

To quantify the reaction rates, the amounts of phosphate released from ATP through the γGCS catalyzed reactions with Glu and amino acids and with amines were monitored (Fig. 7). The amount of phosphate was highest when Cys was the substrate. The amount of phosphate from allylamine and from *n*-propylamine was 40–50% of Cys. The amount of phosphate released was elevated by increasing the concentrations of the amines (Fig. 7). The amount of phosphate obtained from the reactions with the amines depended on the length of the carbon chain of the amines: *n*-propylamine (carbon chain length, 3) > butylamine (4) > ethylamine (2) > methylamine (1). The influence of pH on the activity was examined using α-aminobutyrate, ethylamine, and propylamine as substrates. It was determined that the optimum pH varied with the substrate (Fig. 8). While the optimum pH for α-aminobutyrate was broad and around neutral, those for ethylamine and propylamine were narrower and shifted to alkaline conditions (pH 9.5–10).

## Discussion

More than 40 years ago, Rathbun[27] tested 71 compounds, including some amines, as substrates for γGCS from bovine lens, and reported no detectable activities for ethylamine or isopropylamine. In this study, however, we found that γGCS from *E. coli* had the ability to synthesize γ-glutamylamide compounds from Glu and amines. The reason our results differ from Rathbun *et al.* is thought to be differences in the origin of γGCS or the reaction conditions. This is the first report showing that γGCS has γ-amidation activity with amines. Our finding should expand the possibility of producing useful compounds using γGCS and should provide new clues towards elucidating the substrate recognition mechanism of γGCS.

Tokutake *et al.*[28] concluded that the carboxy group in Cys is the major recognition element of *E. coli* γGCS in the transition state. On the other hand, Hiratake *et al.*[29] suggested the importance of the length of the methylene chain of the substrate amino acid, which has a large influence on the hydrophobic interactions between the substrate and the enzyme. Kelly *et al.*[25] reported that γGCS from *E. coli* took 17 amino acids instead of Cys, and showed that the relative activity ($V_{max}/K_m$ values) for amino acids was α-aminobutylate (carbon chain length, 3) > alanine (2) > glycine (1) > norvaline (4). Based on our experiments, the relative activities of the amines depended on the length of the carbon chain as follows (Fig. 7): *n*-propylamine (3) > butylamine (4) > ethylamine (2) ≫ methylamine (1). The order of amine reactivities was similar to the order of amino acid reactivity except for the position of the C4 compounds. In both amino acids and amines, C3 compounds (α-aminobutyrate and *n*-propylamine) were the most highly reactive compounds. This appears to confirm the importance of the hydrophobic interaction. Amines with longer side chains are correspondingly more reactive than the comparable amino acids. This suggests that the carboxyl group of the amino acids contributes to substrate recognition at the Cys binding site of the enzyme. The reason the optimum pH for the reaction of the amines was narrower and shifted to alkaline pHs is difficult to determine but it might be related to the difference in acid dissociation constants between the amino acid and the amine.

In this study, we also found that theanine can be synthesized from Glu and ethylamine by a resting cell reaction of *E. coli* overexpressing γGCS. Despite the

fact that the optimal pH shifted to alkali conditions, it is remarkable that theanine was synthesized in gram quantities with coupling to *E. coli*'s own ATP regeneration system (Fig. 3). The amount of ATP initially added was 0.8 mM, whereas 12.1 mM of theanine and 11.5 mM of γ-glutamylalanine were formed, indicating that most of the ATP for the synthesis reactions was regenerated by the glycolytic activity of the cells. Yamamoto *et al.*[17] reported that theanine could be synthesized from GS coupled with ATP regeneration through the glycolytic activity of dry yeast. It would be interesting to determine whether the theanine synthesis found here can be achieved only in *E. coli* cells, because we found that *E. coli* cells contained sufficient ATP regeneration activity to drive the reaction.

The formation of γ-glutamylalanine is a drawback of our theanine production system. The substrate Ala is formed from pyruvate, the glycolytic product. As Fig. 3 suggests, the reactivity of γGCS for Ala is higher than that for ethylamine. Thus to reduce the γ-glutamylalanine byproduct, alternating the host sugar metabolic pathway or changing the substrate specificity of γGCS is necessary. The structure of γGCS from *E. coli* was recently published and the Cys binding sites were determined.[30] Perhaps by gaining additional insight from the crystal structure into ways to alter the substrate specificity of γGCS, opportunities might be revealed to engineer the system to avoid unwanted byproducts further.

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