Enzymatic production of theanine, an “umami” component of tea, from glutamine and ethylamine with bacterial γ-glutamyltranspeptidase

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Abstract
Theanine (γ-glutamylethylamide) is the major “umami” (good taste) component of tea and its favorable physiological effects on mammals have been reported. An enzymatic method for the synthesis of theanine involving bacterial γ-glutamyltranspeptidase (GGT) was developed. The optimum reaction conditions were 200 mM Gln, 1.5 M ethylamine, and 0.4 units/ml GGT, pH 10. After 2 h incubation at 37 °C, 120 mM theanine was obtained, the conversion rate against Gln being 60%. Theanine was purified on Dowex 50 W × 8 and Dowex 1 × 8 columns, and then identified by 1H-NMR.

This is the first report that a GGT, regardless of its source, can utilize an alkyl amine as an acceptor of the γ-glutamyl moiety in its transpeptidation reaction.

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Keywords: Theanine; γ-Glutamyltranspeptidase; γ-Glutamyl amino acid; Enzymatic synthesis; Umami

1. Introduction
Theanine (γ-glutamylethylamide) (see the structural formula in Scheme 1), which was first identified by Sakato [1] in tea leaves, is not only the main free amino acid component of tea (more than half), but also the major umami component of tea. In general, a higher grade of Japanese green tea contains more theanine [2,3]. Green tea of the highest quality, “Maccha” or “Gyokuro,” contains on an average 1.5–2% of theanine per dry weight. Theanine is synthesized from Glu and ethylamine by theanine synthetase [l-glutamate: ethylamine ligase, EC 6.3.1.6] using ATP [4] in the roots of tea trees (Camellia sinensis), and the synthesized theanine is transported to the leaves and accumulated there [5,6].

It has been suggested that orally administered theanine is absorbed into the blood circulation through the intestinal tract [7,8]. Theanine is also known to be incorporated into the brain through the blood–brain barrier via a Leu-prefering transport system, and it may affect the metabolism and/or release of some neurotransmitters in the brain [9]. Intraperitoneal administration of theanine to spontaneously hypertensive rats results in a decrease in blood pressure [10]. Inhibitory effects of theanine on the convulsive action [11] and spontaneous activity [12] caused by caffeine administration have been reported. Later, Kakuda et al. confirmed its inhibitory effect on the excitation caused by caffeine at the concentration regularly associated with drinking of tea [13]. It has also been shown that the oral intake of theanine causes a feeling of relaxation in human volunteers [14]. Its prevention of ischemic neuronal damage has also been reported [15].

Because of its good taste and favorable physiological effects on mammals, theanine could be a new food additive and several investigators have studied its effective production. The production of theanine with cultured cells of C. sinensis has been performed, but it took 4-week cultivation [18,19]. Enzymatic production of theanine with glutaminase from Pseudomonas nitroreducens has been reported [20]; however, methods for the overproduction and simple purification of this enzyme have not yet been established. Abe and coworkers employed immobilized P. nitroreducens cells to overcome these defects [21,22].

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γ-glutamyl compounds, but also the transfer of their γ-glutamyl moieties to other amino acids or peptides (Scheme 1). We found that GGT from *Escherichia coli* K-12 can utilize a less expensive γ-glutamyl donor, Gln, and transfer its γ-glutamyl moiety to various compounds [23]. We also established a simple two-step purification method for a strain overproducing GGT [24]. Using bacterial GGT, we have developed an effective enzymatic method for synthesizing γ-glutamyl amino acids, such as γ-Glu-DOPA [25], γ-Glu–Phe [26], and γ-Glu-taurine [27]. However, whether or not GGT from any source can transfer the γ-glutamyl moiety to an alkyl amine was not known.

In this paper, we report an effective enzymatic method for synthesizing theanine from Gln and ethylamine involving bacterial GGT.

2. Materials and methods

2.1. Reagents and the enzyme

L-Theanine was purchased from Tokyo Kasei (Tokyo, Japan), and L-glutamine and ethylamine were from Nacalai Tesque (Kyoto, Japan). *Escherichia coli* K-12 strain SH642, which harbors pUC18 containing the *E. coli* GGT gene, was grown at 20 °C in LB broth containing 100 μg/ml ampicillin, and the over-produced GGT was purified as described previously [24] from its periplasmic fraction [28].

2.2. Measurement of GGT activity

GGT activity was measured as described previously [23]. One unit of enzyme was defined as the amount of enzyme that released 1 μmol p-nitroaniline per minute from γ-glutamyl-p-nitroanilide through the transpeptidation reaction.

2.3. Measurement of theanine, Gln, and γ-Glu–Gln

The concentrations of theanine, Gln, and γ-Glu–Gln were measured with a high-performance liquid chromatograph (HPLC) equipped with a Shim-pack Amino-Na column (Shimadzu, Kyoto, Japan), with a gradient elution at 60 °C at a flow rate of 0.6 ml/min. Gradient of the mobile phase was formed with buffer A (66.6 mM citrate, 1% perchloric acid, and 7% ethanol, pH 2.8) and buffer B (200 mM citrate, 200 mM boric acid, and 0.12 N NaOH, pH 10). Concentration of buffer B was kept 0% until 9 min. It was linearly increased to 7% from 9 to 13 min, to 8% from 13 to 17.2 min, and then to 11%. o-Phthalaldehyde was used as the detection reagent and the fluorescence was detected with a fluorescence detector (model LC-9A; Shimadzu, Kyoto, Japan) as the absorbance at 450 nm, with excitation at 348 nm. Typically, γ-Glu–Gln, Gln, and theanine were eluted at 6.1, 13.6, 14.5 min, respectively.

2.4. NMR analysis

Theanine (5 mg) was dissolved in 0.5 ml D$_2$O and then analyzed with a Bruker 500 MHz spectrometer, and the spectrum was compared with that obtained with theanine purchased from a commercial source.

3. Results

3.1. Enzymatic synthesis of theanine with GGT

Gln and ethylamine were dissolved in water (final = 20 mM each), and the pH was adjusted to 10 with NaOH. GGT was added to this solution (final = 0.04 units/ml), and
Fig. 1. Effect of the ethylamine concentration on theanine synthesis. The reaction was carried out with 20 mM Gln and 0.04 units/ml GGT at pH 10 and 37 °C for 5 h, the concentration of ethylamine being varied as indicated in the figure. Theanine (shaded boxes) and γ-Glu-Gln (open boxes) were assayed by HPLC as described under Section 2.

then the reaction mixture was incubated at 37 °C for 5 h. As controls, the reaction mixtures without GGT were incubated similarly. The reaction was terminated by the addition of trichloroacetic acid to 10%. The solution was passed through Cosmowipe Filter W (pore size = 0.45 μm, Millipore) and then subjected to HPLC analysis. About 4 mM theanine was synthesized, while the major product was γ-Glu-Gln.

3.2. Optimization of the reaction conditions for enzymatic synthesis of theanine

The reaction conditions for the synthesis of theanine were investigated. First, the concentration of Gln was fixed at 20 mM and the concentration of ethylamine was varied. As shown in Fig. 1, 150 mM of ethylamine was the optimum. The pH was varied and its effect was examined (Fig. 2). After 5-h incubation, the best yield was obtained at pH 10. Similarly, the optimum concentration of GGT was determined to be 0.04 units/ml (data not shown).

When 20 mM Gln was used, 150 mM ethylamine, 0.04 units/ml GGT, pH 10, and incubation at 37 °C for 5 h were the optimum conditions. The concentrations of the substrates and GGT were increased 2-, 3-, 4-, 6-, 8-, and 10-fold, and the effects were examined (Fig. 3). The yield of theanine increased as the concentrations increased, and 120 mM theanine was synthesized on 5-h incubation with 200 mM Gln, 1.5 M ethylamine, and 0.4 units/ml GGT, the conversion rate of Gln as to theanine being 60%.

Fig. 3. Effects of increasing concentrations of the substrates and GGT on theanine synthesis. The one-fold reaction mixture contained 20 mM Gln, 150 mM ethylamine, and 0.04 units/ml GGT. The two-fold one 40 mM Gln, 300 mM ethylamine, and 0.08 units/ml GGT, and so forth. The reaction was carried out at 37 °C for 5 h. The concentration of theanine is shown as shaded boxes and that of γ-Glu-Gln as open boxes.

3.3. Isolation and identification of theanine

The pH of the reaction mixture (30 ml) comprising 20 mM Gln, 150 mM ethylamine, and 0.04 units/ml GGT was adjusted to 10, followed by incubation at 37 °C for 5 h. After the reaction has been terminated, the reaction mixture was applied to a column (50 ml) of Dowex 50 W × 8, which had been prepared as the Ca²⁺ form. Theanine was eluted with water, and the fractions containing theanine were collected and lyophilized. It was dissolved with water and applied to a column (30 ml) of Dowex 1 × 8, which had been prepared as the Cl⁻ form. Theanine was eluted with water, and the fractions containing only theanine were collected and lyophilized.

The reverse phase HPLC chromatograms of synthesized and commercial theanine were compared, their retention times being found to match well (data not shown). The 1H NMR spectra of synthesized and commercial theanine were identical (Fig. 4; the spectrum of commercial theanine is not shown).

Fig. 2. Effect of the pH of the reaction mixture on theanine synthesis. The reaction was carried out with 20 mM Gln, 150 mM ethylamine, and 0.04 units/ml GGT at 37 °C for 5 h, the pH of the reaction mixture being varied as indicated in the figure.

Fig. 4. The reverse phase HPLC chromatograms of synthesized and commercial theanine were compared, their retention times being found to match well (data not shown).
Fig. 4. $^1$H NMR spectrum of the isolated sample. The $^1$H NMR spectrum was measured in D$_2$O with a Bruker 300 MHz spectrometer.
4. Discussion

GGTs could transfer γ-glutamyl moiety of γ-glutamyl compounds to various amino acids and peptides and we have developed the enzymatic method to synthesize some γ-glutamyl amino acids using bacterial GGT [25–27]. Theanine is a very interesting amino acid and potential demand in food industry is expected. However, there was no report that a GGT, regardless of its source, could transfer γ-glutamyl moiety to an alkyl amine. Therefore, whether or not theanine could be synthesized from Gln and ethylamine through the transpeptidation reaction of bacterial GGT as a catalyst was examined using 20 mM Gln and ethylamine. Because amino acids have a buffering effect, the pH of the initial reaction solution was adjusted instead of adding a conventional buffer solution, such as Tris–HCl or phosphate buffer. Although theanine was synthesized, its yield was low and more γ-Glu-Gln was synthesized than theanine. This indicates that the GGT prefers Gln than ethylamine as an acceptor of γ-glutamyl moiety. To improve the yield, the reaction conditions for the synthesis of theanine were investigated. The concentration of Gln was fixed at 20 mM and an excess amount of ethylamine was used to improve the yield of theanine (Fig. 1). The optimum pH (Fig. 2) and GGT concentration were also examined. When 20 mM Gln was used, 150 mM ethylamine, 0.04 units/ml GGT, pH 10, and incubation at 37 °C for 5 h were the optimum conditions. The concentrations of the substrates and ethylamine were increased in this proportion, 200 mM Gln, 1.5 M ethylamine, and 0.4 units/ml GGT being the optimum (Fig. 3). Because 200 mM is almost the saturating concentration for Gln at this pH, we did not examine higher concentrations. This experiment showed that the GGT could tolerate up to 1.5 M ethylamine. After 2-h incubation, there was no increase in theanine throughout the concentration range tested because almost all Gln was converted to theanine and γ-Glu-Gln. The fact that only a trace amount of Gln was found in the reaction mixture (data not shown) indicates that under these reaction conditions, the GGT only catalyzed transpeptidation reaction. The yield might be improved by feeding of the substrates, especially Gln. Synthesized theanine was purified and identified as theanine with $^1$H NMR.

In conclusion, we developed an effective enzymatic method for the synthesis of theanine with bacterial GGT. This is the first report that a GGT, regardless of its source, can utilize an alkyl amine as an acceptor of the γ-glutamyl moiety in its transpeptidation reaction.

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References


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