Amino Acids (2010) 39:1177–1182
DOI 10.1007/s00726-010-0553-z

ORIGINAL ARTICLE

Enzymatic synthesis of theanine from glutamic acid γ-methyl ester and ethylamine by immobilized *Escherichia coli* cells with γ-glutamyltranspeptidase activity

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Received: 15 June 2009 / Accepted: 2 March 2010 / Published online: 19 March 2010

Abstract Theanine (γ-glutamylethylamide) is the main amino acid component in green tea. The demand for theanine in the food and pharmaceutical industries continues to increase because of its special flavour and multiple physiological effects. In this research, an improved method for enzymatic theanine synthesis is reported. An economical substrate, glutamic acid γ-methyl ester, was used in the synthesis catalyzed by immobilized *Escherichia coli* cells with γ-glutamyltranspeptidase (GGT) activity. The results show that GGT activity with glutamic acid γ-methyl ester as substrate was about 1.2-folds higher than that with glutamine as substrate. Reaction conditions were optimized by using 300 mmol/l glutamic acid γ-methyl ester, 3,000 mmol/l ethylamine, and 0.1 g/ml of immobilized GGT cells at pH 10 and 50°C. Under these conditions, the immobilized cells were continuously used ten times, yielding an average glutamic acid γ-methyl ester to theanine conversion rate of 69.3%. Bead activity did not change significantly the first six times they were used, and the average conversion rate during the first six instances was 87.2%. The immobilized cells exhibited favourable operational stability.

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Keywords Theanine · Enzymatic synthesis · Glutamic acid γ-methyl ester · γ-Glutamyltranspeptidase

Introduction

Theanine (γ-glutamylethylamide) is a characteristic, non-proteinous amino acid in tea plant (*Camellia sinensis*). It is the main amino acid component in green tea, accounting for 50% of the plant's total free amino acids and 1–2% of the dry weight of tea leaves (Graham 1992). It is also the major flavour component of green tea. Aside from the tea plant, theanine is also found in the mushroom *Xerocomus badius* and in other species belonging to the *Camellia* genus (*C. japonica* and *C. sasanqua*) (Casimir et al. 1960; Gregory and Burgunda 2006). It can cross the blood–brain barrier and penetrate the brain, so it exhibits psychoactive effects (Yokogoshi et al. 1998; Kimura et al. 2007). Recent studies suggest that theanine poses multiple physiological and pharmacological effects on relaxation (Juneja et al. 1999; Lu et al. 2004) and assists in blood pressure reduction (Yokogoshi et al. 1995; Rogers et al. 2008), anti-obesity (Zheng et al. 2004; Maeda et al. 2005), antitumor efficacy enhancement (Sadzuka et al. 1996, 2000), neuro-protection (Kakuda 2002; Nagasawa et al. 2004), and enhancement of defence against microbes (Bukowski and Percival 2007). Due to its special flavour and versatile physiological effects, an increasing demand for theanine is observed in the food and pharmaceutical industries.

Since the isolation and identification of theanine in 1949 (Sakato 1949), several methods have been developed for the production of this amino acid. One of the most effective methods is theanine extraction from dry tea or fresh tea plant leaves, but this procedure is laborious and time consuming. Other methods such as tea callus cultivation

(Orihara and Furuya 1990; Matsuura et al. 1994) and fermentation of the mushroom *Xerocomus badius* (Li et al. 2008) are also reported, but the production efficiency of these procedures is low. Another effective method is chemical synthesis (Lichtenstein 1942; Yan et al. 2003; Gu et al. 2004). The earliest report on chemical theanine synthesis is the preparation of γ-glutamylethylamide by an Israeli scientist in1942 in which he treated pyrrolidonecarboxylic acid with aqueous ethylamine solutions (Lichtenstein 1942). The chemical synthesis process is simpler than that presented in the previously mentioned method, and the main starting material, glutamic acid, is inexpensive. However, synthetic methods also have limitations including low yield, low purity, and a requirement for the protection and unblocking of reactive groups.

Recently, methods for enzymatic theanine synthesis have also been developed and intensively investigated by several groups. In general, three enzymes, namely, glutaminase (EC 3.5.1.2) (Aberlian et al. 1993; Tachiki et al. 1998), glutamine synthetase (GS, EC 6.3.1.2) (Tachiki et al. 1986; Yamamoto et al. 2005, 2008), and γ-glutamyltranspeptidase (GGT, EC 2.3.2.2) (Suzuki et al. 1986, 2002), can generate theanine (Fig. 1a, b). The reaction catalyzed by GS can utilize glutamic acid, a cheap chemical, as a substrate, but this reaction requires a continuous supply of ATP. In a recent study (Yamamoto et al. 2008), 110 mg/ml of theanine was produced in 48 h, and a 100% conversion rate was achieved from coupled fermentation with energy transfer. On the other hand, the reactions catalyzed by glutaminase or GGT require no ATP input, but a high concentration of ethylamine is required to drive glutamine conversion. In a previous report (Suzuki et al. 2002), a glutamine to theanine conversion rate of 60% was obtained using a glutamine to ethylamine mole ratio of 2:15. In addition, L-amidohydrolase (EC3.5.1.14) was also reported to be suitable for use in DL-theanine resolution (Li et al. 2007). At any rate, enzymatic synthesis provides another possibility for rapidly producing theanine on an industrial scale.

We find the study of Suzuki et al. (2002) relevant as it is our view that better conversion rates at reduced substrate costs are still possible and will be necessary for cost-effective industrial scale processes. In addition, an immobilized cell system can increase cell concentration, enhance bead stability, and allow easier separation for reuse. Therefore, the immobilized cell system is a desirable technique in enzymatic theanine production. In the present research, we optimized the conditions for theanine synthesis from a novel substrate, glutamic acid γ-methyl ester (L-Glu-γ-methyl ester), catalyzed by GGT (Fig. 1c). Key factors such as substrate specificity, pH, temperature, and substrate mole ratio were investigated.

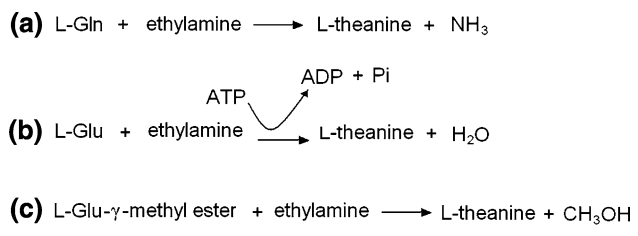


Fig. 1 Enzymatic theanine synthesis. **a** Catalyzed by glutaminase or GGT; **b** catalyzed by GS; **c** catalyzed by GGT

Materials and methods

Chemicals

Theanine was purchased from Taiyo Kagaku Co., Ltd. Glutamine, ethylamine (65%), sodium alginate, peptone, and yeast extract were purchased from China Medicine (Group) Shanghai Chemical Reagent Corporation. Isopropyl-β-D-thiogalactopyranoside (IPTG) (purity ≥ 98%) was purchased from Nanjing Jitian Biotechnology Co., Ltd., while L-Glu-γ-methyl ester and γ-L-glutamylhydrazide were prepared as described in a previous paper (Chen et al. 1994). All other chemicals were of analytical grade.

Microorganisms

The gene encoding γ-glutamyltranspeptidase, *ggt*, was cloned from *Escherichia coli* k-12 MG1655. The *E. coli* strain BL21 (DE3) carrying the recombinant plasmid pET32a-ggt was constructed in our laboratory. The strain was cultured in LB broth containing 50 μg/ml ampicillin and induced with 0.4 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C.

Immobilization of cells

A modified method from Birgisson et al. (2007) was used for cell immobilization. To make a final concentration of 3% alginate (w/v), 5 g cells (wet wt.) were mixed with 20 ml sodium alginate water solution. After stirring the components together, the mixture was added into 500 ml of 2% (w/v) $CaCl_2$ solution by using a syringe to drip the mixture from a 10 cm height. The average diameter of the resulting beads was about 3 mm. Prior to use, the beads were allowed to harden for 2 h at 4°C without agitation.

GGT activity assays

By measuring the *p*-nitroaniline released from γ-glutamyl-*p*-nitroanilide as described by Nakayama et al. (1984), GGT activity was determined spectrophotometrically.

The reaction mixture (final volume, 2 ml) containing 2.5 mM γ-L-glutamyl-*p*-nitroanilide, 50 mM Tris–HC1 (pH 8.5), 100 mM glycylglycine, and enzymes (i.e. free or immobilized cells) was incubated at 37°C for 5 min. The reaction was discontinued by adding 1.5 mol/l acetic acid (4 ml), and the amount of liberated *p*-nitroaniline was determined from the optical density at 410 nm. One enzyme unit (U) was defined as the amount of free or immobilized cells that released 1 μmol of *p*-nitroaniline per minute from γ-glutamyl-*p*-nitroanilide at 37°C. The specific activity is defined as units/g of free or immobilized cells.

Theanine assay

The concentrations of theanine and glutamic acid γ-methyl ester were determined by high-performance liquid chromatograph (HPLC) as previous described (Suzuki et al. 2002). The IR spectra of theanine in KBr was recorded on a Perkin–Elmer 297 spectrometer calibrated with polystyrene. ^{1}HNMR spectra was recorded on a Bruker 400 (400 MHz).

Results

GGT substrate specificity

To investigate GGT substrate specificity, five different γ-glutamyl compounds (γ-glutamyl-*p*-nitroanilide, L-Gln, GSH (glutathione), L-Glu-γ-methyl ester, and γ-L-glutamylhydrazide) were used as substrates in the enzymatic synthesis of theanine catalyzed by GGT. As shown in Table 1, all of these compounds could serve as γ-glutamyl donors in theanine synthesis. The GGT activity with L-Glu-γ-methyl ester as substrate was about 1.2-folds higher than that with glutamine as substrate.

The reaction was carried out in a 10 ml mixture containing 1,000 mmol/l ethylamine and 100 mmol/l γ-glutamyl donors, and 1 g immobilized GGT cells at pH 9 and 37°C. A gram of immobilized cells contains 550 units of enzymes. Theanine concentrations were measured by HPLC after 20 min.

Table 1 GGT substrate specificity

Substrate	Theanine (mmol/l)	Relative activity (%)
γ-Glutamyl-*p*-nitroanilide	15.9	100
L-Gln	11.5	72.5
GSH (glutathione)	14.3	89.9
L-Glu-γ-methyl ester	13.5	85.4
γ-L-Glutamylhydrazide	7.0	44.2

pH and temperature effect

Enzyme activity can be affected by environmental factors, such as pH and temperature. In this study, using L-Glu-γ-methyl ester and ethylamine as the substrates, a pH range from 7 to 11 was used to determine the optimal initial pH. As shown in Fig. 2, with the initial pH increasing from 7 to 10, GGT activity increased significantly and reached its maximum at pH10. An activity decrease was observed at pH 11. The optimal temperature for the reaction was subsequently optimized. When assayed at pH 10, the optimal temperature for the reaction was about 50°C (Fig. 3). These results indicate that the optimal reaction conditions, with glutamic acid γ-methyl ester as γ-glutamyl donor, were similar to those with glutamine as γ-glutamyl donor (Suzuki et al. 2002).

Effect of substrate mole ratio

In enzymatic synthesis, a frequently used means to increase production is in excess of one substrate. In this study, the mole ratios of L-Glu-γ-methyl ester to ethylamine varied from 1:4 to 1:12. The relationship between the conversion rate of L-Glu-γ-methyl ester and the mole ratios is shown in Fig. 4. Ethylamine excess could significantly promote theanine synthesis. The highest conversion rates of 96.1 and 96.3% occurred at mole ratios of 1:10 and 1:12.

Substrate concentration effect

The substrate concentration effect on the conversion rate from L-Glu-γ-methyl ester to theanine was studied. The conversion rate time course under different substrate concentrations is shown in Fig. 5. The results indicate that with a substrate concentration increase, the conversion rate

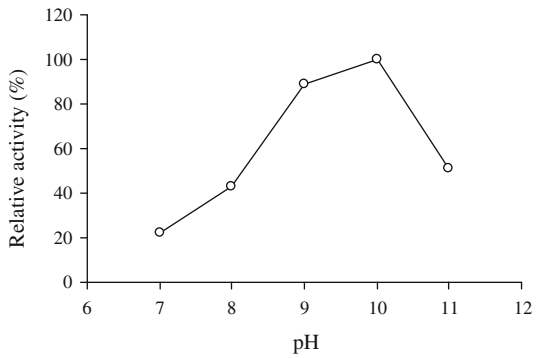


Fig. 2 Effect of pH on GGT activity. The reaction was carried out in a 10 ml mixture containing 1,000 mmol/l ethylamine, 100 mmol/l L-Glu-γ-methyl ester, and 1 g immobilized GGT cells. The mixture was kept in a plastic tube at 37°C as it was being shaken. Theanine concentrations were measured after 20 min. The highest relative activity of 100% denoted 15.4 mmol/l of theanine

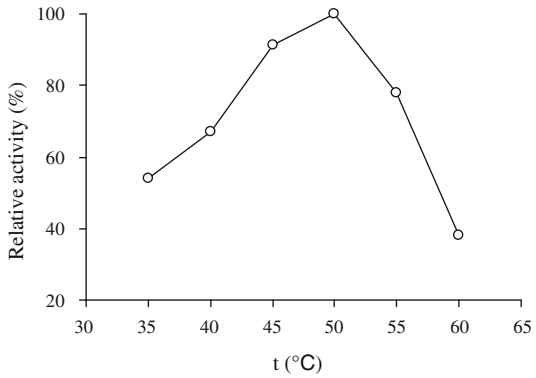


Fig. 3 Effect of temperature on GGT activity. The mixture components were the same as those mentioned in Fig. 2. The mixture was kept in a plastic tube at pH 10 as it was being shaken. Theanine concentrations were measured after 20 min. The highest relative activity of 100% denoted 27.9 mmol/l of theanine

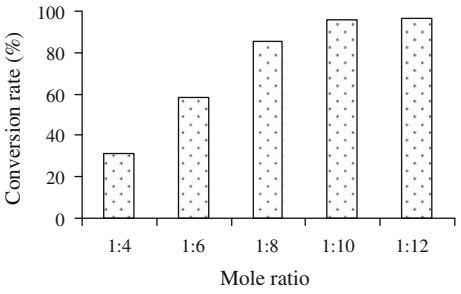


Fig. 4 Effect of the initial mole ratios of L-Glu-γ-methyl ester to ethylamine on the conversion rate of L-Glu-γ-methyl ester. The reaction was carried out in a 10 ml mixture containing two substrates and 1 g immobilized GGT cells at pH10. The L-Glu-γ-methyl ester concentration fixed at 100 mmol/l and ethylamine concentrations were 400, 600, 800, 1,000, and 1,200 mmol/l, respectively. The mixture was kept at 50°C as it was being shaken. Theanine concentrations were measured after 8 h. The highest conversion rate of 100% denoted 100 mmol/l of theanine

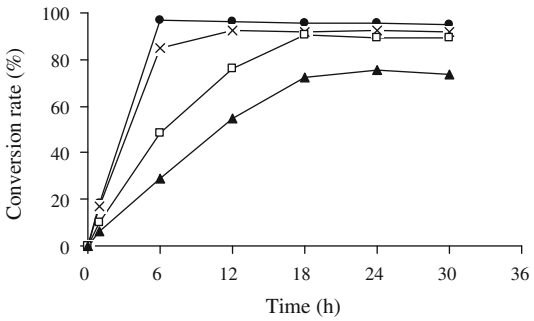


Fig. 5 Conversion rate time course of L-Glu-γ-methyl ester. The reaction was carried out in a 10 ml mixture containing 1 g immobilized GGT cells at pH10. The L-Glu-γ-methyl ester concentrations were 100 mmol/l (*filled circle*), 200 mmol/l (*cross*), 300 mmol/l (*empty square*), and 400 mmol/l (*filled triangle*), respectively. The ethylamine concentrations were tenfolds higher than those of L-Glu-γ-methyl ester, correspondingly. The mixture was kept at 50°C as it was being shaken. The highest conversion rate of 100% denoted 100 mmol/l of theanine

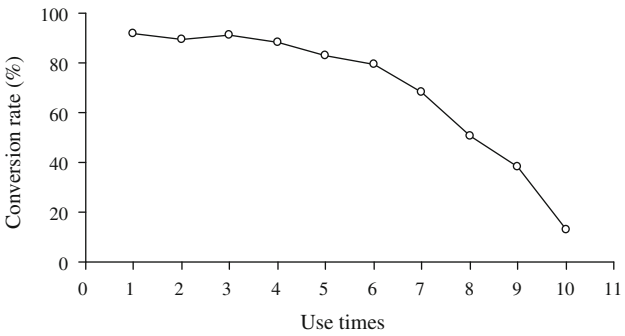


Fig. 6 Operational stability of the immobilized cells. The reaction was carried out in a 10 ml mixture containing 300 mmol/l glutamic acid γ-methyl ester, 3,000 mmol/l ethylamine, and 1 g immobilized cells at pH10. The mixture was kept at 50°C as it was being shaken. Theanine concentrations were measured after 18 h

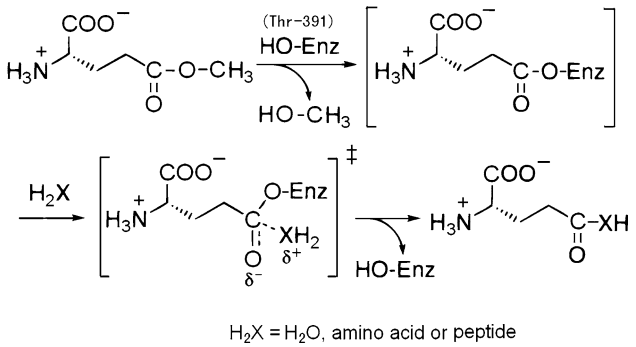
from L-Glu-γ-methyl ester to theanine decreased. Reaction inhibition was observed at a high substrate concentration. Taking production efficiency into consideration, the optimal substrate concentration would be 300 mmol/l. The optimal reaction time was prolonged with substrate concentration increase. When the L-Glu-γ-methyl ester concentration was 300 mmol/l, the optimal reaction time was 18 h.

Operational stability of immobilized cells

In evaluating immobilized cell stability, the beads were continuously used ten times. As shown in Fig. 6, bead activity did not change significantly during the first six times the beads were used, and the average conversion rate from L-Glu-γ-methyl ester to theanine during these times was 87.2%. When the beads were used more than six times, a significant enzyme activity degradation was observed. Upon tenth usage, the conversion rate reduced to 12.8%. The average conversion rate during the ten-time usage was 69.3%. The immobilized cells exhibited favourable operational stability.

Isolation and identification of theanine

The reaction mixture (60 ml) containing 261.6 mmol/l theanine was concentrated, crystallized from 80% ethanol and dried in vacuum for 2 h to give 1.9 g (69%) of white crystals. Above crystals were 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 theanine were collected and lyophilized to give 1.1 g (57%) of white crystals, IR (KBr): 3,443, 3,300, 2,969, 1,647, 1,582 cm^{-1}. ^{1}HNMR



Scheme 1 GGT reaction mechanism

(D_2O): δ 3.75 (t, 1H), 3.20 (q, 2H), 2.14-2.38 (m, 4H), 1.11 (t, 3H).

Discussion

Animals, plants, and microorganism have been found to extensively contain γ-glutamyltranspeptidases (GGT) (Taniguchi and Ikeda 1998). The GGT catalyzes the hydrolysis of γ-glutamyl compounds and the transfer of their γ-glutamyl components to various primary amine acceptors including amino acids, peptides, or other amino compounds. As shown in Scheme 1, the catalytic mechanism involves the activated oxygen atom of the side chain of the N-terminal Thr-391 of the small sub-unit attacks the carbonyl carbon of γ-glutamyl compounds to form a γ-glutamyl-enzyme intermediate (Inoue et al. 2000; Okada et al. 2006; Suzuki et al. 2007). When this intermediate is exposed to a nucleophile, a γ-glutamyl acceptor, a transpeptidation reaction takes place, and a new γ-glutamyl compound is formed. A series of γ-glutamyl compounds including γ-L-glutamyl-L-DOPA, theanine, γ-D-glutamyl-L-tryptophan (SCV-07), and γ-L-glutamyltaurine can be produced in this manner (Suzuki et al. 2007).

In γ-glutamyl compound synthesis by GGT, the optional γ-glutamyl donors could be glutamine, glutathione (GSH), γ-L-glutamylmethylamide, and glutamic acid γ-methyl ester (Suzuki et al. 1986; Bridge and Zarka 2006). Among all these donors, glutamic acid γ-methyl ester may be the cheapest because it can be easily prepared from glutamic acid and methanol through esterification reaction. In previous literature, GGT activity with glutamine as the γ-glutamyl donor was about 4.4-folds higher than that that with glutamic acid γ-methyl ester as substrate (Lin et al. 2006). In contrast, our result displayed a 1.2-folds higher GGT activity with glutamic acid γ-methyl ester as γ-glutamyl donor as compared to that using glutamine as substrate. The inconsistency might be caused by the difference in measurement methods. In our method, the γ-glutamyl acceptor was exceedingly more excessive over γ-glutamyl donors. Side reactions were avoided this way.

The speed of transpeptidation reaction, a nucleophilic substitution, catalyzed by GGT is significantly affected by pH. Generally, a high pH inhibits nucleophile ionization, and the attack of the nucleophile, the γ-glutamyl acceptor, will be more efficient at a high pH. In our study's result, an acceleration of transpeptidation reaction was observed with pH increase. The GGT activity reached its maximum at pH10, which agreed with the results of a previous report (Suzuki et al. 2002).

In previous literature, glutamine as the γ-glutamyl donor was used in the enzymatic synthesis of theanine catalyzed by GGT, and the glutamine to theanine conversion rates ranged from 60% (Suzuki et al. 2002) to 27% (Hung et al. 2008). The low conversion rate in previous studies could have resulted from the low glutamine to ethylamine mole ratio, which could be verified indirectly by our present results. In our research, a glutamic acid γ-methyl ester to ethylamine substrate mole ratio of 1:10 enabled an enhanced conversion of 96.1%.

In our study, glutamic acid γ-methyl ester instead of glutamine was used in theanine synthesis, and immobilized *Escherichia coli* cells with GGT activity were used as catalysts. The results demonstrate an improvement in enzymatic theanine synthesis and display vast potential in industrialization. Our results also suggest an improvement in γ-glutamyl compound preparation.

Acknowledgments We thank Ji-Bing Xiong for synthesizing L-Glu-γ-methyl ester. This work was supported by the National Technology-Innovation Fund of P R China (02CJ-13-01-16).

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