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Enzymatic synthesis of L-theanine from L-glutamine and ethylamine by *Bacillus licheniformis* γ-glutamyltranspeptidase and its mutants specialized in transpeptidase activity

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ABSTRACT

In this investigation, *Bacillus licheniformis* γ-glutamyltranspeptidase (*Bl*GGT) together with its two transpeptidase-specialized mutants (N450D and N450Q) were employed for the biocatalytic synthesis of L-theanine. A high-pressure liquid chromatographic method coupled with ultraviolet detection (HPLC/UV) was used to quantify L-theanine content in the reaction mixture. Through a series of experiments, the perfect conditions for the biocatalytic synthesis were found to be an operational pH of 10.5, a reaction time of 4 h, L-glutamine and ethanolamine in a 1:2.4 M ratio, and enzymes at a working concentration of 25 μg/mL. In a batch process for N450D-mediated synthesis of L-theanine at 37 °C, a conversion rate of ~94% was achieved from 250 mM L-glutamine and 600 mM ethylamine. Successful synthesis of the desired product was further verified by mass spectrometry analysis. Conclusively, the experimental results suggest a high potential application in the production of L-theanine by *Bl*GGT-mediated biocatalysis.

1. Introduction

L-Theanine (γ-glutamyl-L-ethylamide) is an abundant non-protein derived amino acid that was first isolated from green tea leaves by water extraction (Sakato, 1949). With the exception of being identified in the mycelia of the basidiomycete mushroom *Xerocomus badius* (Li et al., 2008), its occurrence appears to be limited to the *Camellia* genus, mostly the tea plants *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*, and two closely related species *C. japonica* and *C. nitidissima* (Tsushida and Takeo, 1984; Vuong et al., 2011; Liu et al., 2016; Cheng et al., 2017). In the leaves of the tea plants, L-theanine accounts for about 500 g/kg of the free amino acids and has been intimately linked with the distinctive umani taste of Japanese green tea (Balentine et al., 1998).

Several observational and experimental epidemiological studies have suggested that daily intake of L-theanine at doses of greater than 50 mg can improve health and well-being through the influence of a variety of psychological and physiological factors, such as stress levels, improvements in learning ability, prevention of cancers and vascular diseases, enhancement of immune response and promotion of weight loss (Juneja et al., 1999; Huber, 2003; Zheng et al., 2004; Bryan, 2007; Kimura et al., 2007; Kurihara et al., 2007; Rogers et al., 2007; Miyagawa et al., 2008; Owen et al., 2008; Liu et al., 2009). Reviews of previous literature have stated that a bolus ingestion of L-theanine facilitates the generation of alpha brain waves, which are closely associated with a relaxed but alert mental state (Bryan, 2007; Cooper and Morré, 2005). Moreover, L-theanine was reported to promote the release of the principle inhibitory neurotransmitter γ-aminobutyric acid that in turn regulated dopamine and serotonin levels in the brain (Mason, 2001). An imbalance in the levels of dopamine and serotonin could get serious effects on mental health, digestion, and sleep cycle (Monti and Jantos, 2008; Camilleri, 2010; Narvaes and Martins de Almeida, 2014). Definitely, the health benefits have provided valuable support for L-theanine to serve as a key bioactive ingredient for food and beverage applications (Williams et al., 2016).

Bioactive phytochemicals are extensively used in medicinal and commercial industries for cosmetics, food aids and additives (Dillard

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and German, 2000; Joshi and Pawar, 2015). L-Theanine, a good example of bioactive phytochemicals, can be obtained by direct extraction of tea leaves with water; however, the practical isolation of this non-proteinaceous amino acid in high purity presents a considerable challenge due to the presence of other soluble substances, such as caffeine and polyphenols, in a certain amount (Vuong et al., 2011). Chemical synthesis has been shown to provide an alternative to prepare this γ-glutamyl compound, but one of the main limitations for the production of synthetic L-theanine is that the synthesis process normally produces a racemic mixture of L- and D-enantiomers (Vuong et al., 2011), leading to considerable uncertainties regarding its safety and efficacy. In this regard, biocatalysis becomes a more persuasive approach for the production of L-theanine over the past few decades (Suzuki et al., 2007; Mu et al., 2015). Four different kinds of bacterial enzymes, including L-glutamine synthetase, L-glutaminase, γ-glutamylmethylamide synthetase, and γ-glutamyltranspeptidase (GGT), were being employed to biocatalytically synthesize L-theanine (Mu et al., 2015). Among them, the biocatalytic synthesis of L-theanine from a combination of different γ-glutamyl donors and various γ-glutamyl acceptors through the transpeptidation activity of bacterial GGT enzymes has been studied extensively (Suzuki et al., 2002; Shuai et al., 2010; Zhang et al., 2010; Wang et al., 2012; Bindal and Gupta, 2014; Chen et al., 2014).

Previously, the gene encoding a *Bacillus licheniformis* GGT (*Bl*GGT) has been cloned by our laboratory and overexpressed in *Escherichia coli* M15 cells (Lin et al., 2006). Low-resolution structures of the mature form and a precursor mimic (T399A-*Bl*GGT) of *Bl*GGT are also readily available (Lin et al., 2014; Pica et al., 2016). Three recent investigations have further revealed that the recombinant *Bl*GGT confers the ability to catalyze the synthesis of γ-L-glutamyl-*S*-allyl-L-cysteine, γ-glutamyl-L-phenylalanine and γ-glutamyl-L-leucine (Chen et al., 2015; Chi et al., 2017; Lee et al., 2018). However, the intrinsic ability of GGT enzymes to carry out the hydrolysis reaction is always a serious drawback for their practical use in the field of biocatalysis. Recently, a significant increase in the ratio of transpeptidation to hydrolysis, with up to 3.6-fold enhancement, has been observed in two mutant versions (N450D and N450Q) of *Bl*GGT (Lin et al., 2016). The peculiar characteristics offered by N450D and N450Q may be useful for the enzymatic synthesis of L-theanine. The objective of this study was therefore aimed to produce L-theanine by performing a descriptive research of *Bl*GGT-, N450D-, and N450Q-catalyzed reactions.

2. Materials and methods

2.1. Materials and enzyme preparation

Essential reagents for enzyme assay and biocatalytic synthesis, including L-γ-glutamyl-*p*-nitroanilide (L-γ-Glu-*p*-NA), *p*-nitroaniline (*p*-NA), glycylglycine (Gly-Gly), L-glutamine (L-Gln), L-glutamate (L-Glu), and ethanolamine, were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). The commercially available L-theanine was a product of Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nickel-nitrilotriacetic acid (Ni-NTA) resin for affinity purification of His$_6$-tagged enzymes was purchased from Qiagen Inc. (Valencia, CA, USA). The ready-to-use protein assay dye reagent concentrate (5 ×) was acquired from Bio-Rad Laboratories, Taiwan Branch (Taipei, Taiwan). All other chemicals were of either chemical or biological grade supplied by different vendors.

The cultivations of *Escherichia coli* M15 cells harboring pQE-*Bl*GGT, pQE-*Bl*GGT/N450D, or pQE-*Bl*GGT/N450Q, and IPTG-induced expression of His$_6$-tagged enzymes were carried out as described previously (Pica et al., 2016; Lin et al., 2016). The overexpressed His$_6$-tagged enzymes were purified to near homogeneity from the culture-free extract using the Ni-NTA Superflow Cartridge (Qiagen). Protein concentration was determined by reference to a standard curve established with bovine serum albumin. The purity of affinity-purified enzymes was checked by SDS-12% polyacrylamide gel. The transpeptidation activity of *Bl*GGT, N450D, and N450Q was assayed in triplicate with L-γ-Glu-*p*-NA and Gly-Gly as the substrates according to details outlined elsewhere (Yang et al., 2011). One unit of GGT activity is defined as the amount of enzymes that release 1 μmol of *p*-NA from the chromogenic substrate per minute through the transpeptidation reaction.

2.2. Analysis of the reaction products by high performance liquid chromatography (HPLC)

Amino acids, ethanolamine, and L-theanine were analyzed by an HPLC instrument (model L-2000; Hitachi, Tokyo, Japan) equipped with a MIGHTYSIL RP-18 GP column (4.6 by 250 mm) (Kanto Chemical Co., Inc., Tokyo, Japan). A gradient elution was performed at ambient temperature and at a flow rate of 1.0 mL/min. The gradient of the mobile phase was formed with buffer A (8 g/L $CH_3COONa{\cdot}3H_2O$, 0.16 mg/L trimethylamine, and 4.4 mg/L tetrahydrofuran; pH 7.2) and buffer B (20 g/L $CH_3COONa{\cdot}3H_2O$, pH 7.2/acetonitrile/methanol; 1:2:2 by volume). Initially, the concentration of buffer B was kept at 8% until 20 min. It was linearly increased to 38% from 20 to 24 min, to 100% from 24 to 25.5 min, and then to 8% from 25.5 to 28.5 min. Fluoraldehyde™ *o*-phthaldialdehyde (OPA) reagent solution (ThermoFisher Scientific, Taiwan Co., Ltd.) was used as the pre-column derivatization reagent, and the fluorescence was detected with a UV detector (model L-2420; Hitachi) as the absorbance at 338 nm, with excitation at 262 nm. Typically, the retention time for L-Glu, L-Gln, L-theanine, and ethylamine is 4.3, 9.9, 15.9 and 26.9 min, respectively.

2.3. Biocatalytic synthesis of L-theanine

To investigate the effect of reaction time on the biocatalytic synthesis of L-theanine, a reaction mixture (5 mL) containing 250 mM L-Gln, 600 mM ethylamine, 50 mM borate buffer (pH 10.5), and the individual enzyme at a working concentration of 25 μg/mL was prepared. After incubating each reaction mixture at 37 °C for up to 6 h, 25-μL aliquots were taken at regular intervals to measure the amount of L-theanine by HPLC. The percentage yield of L-theanine was calculated as follows:

$$Yield\ \ (\%) = \frac{The\ \ amount\ \ of\ \ L-theanine(mM)}{The\ \ initial\ \ amount\ \ of\ \ L-Gln\ \ (mM)} \times 100\%$$

The effect of enzyme concentration on the biocatalytic synthesis of L-theanine was carried out in a reaction mixture (1 mL) consisting of 250 mM L-Gln, 600 mM ethylamine, 50 mM borate buffer (pH 10.5), and the individual enzyme at the working concentrations of 2.5–30 μg/mL. After incubating the reaction mixtures at 37 °C for 4 h, 25 μL of each mixture was taken to measure the amount of L-theanine by HPLC.

The effect of pH on the biocatalytic synthesis of L-theanine was evaluated with the following two buffer systems: 50 mM Tris-HCl buffer, pH 7.2–9.0 and 50 mM borate buffer, pH 9.5–11. The reaction mixture (1 mL) contained each of the aforementioned buffers, 250 mM L-Gln, 600 mM ethylamine, and the individual enzyme at a working concentration of 25 μg/mL. After incubating the reaction mixtures at 37 °C for 4 h, the amount of L-theanine was determined by HPLC as afore-stated.

To study the effect of donor/acceptor ratio on the biocatalytic synthesis of L-theanine, the individual enzyme at a final concentration of 25 μg/mL was added to 50 mM borate buffer (pH 10.5) containing 250 mM L-Gln and different amounts of ethylamine (200–800 mM). Reversely, the same enzyme-buffer mixture contained the acceptor substrate (L-Gln) in the concentration range of 50–300 mM and a fixed amount (600 mM) of the acceptor substrate (ethylamine). All these mixtures were then incubated at 37 °C for 4 h, followed by concentration determination of L-theanine by HPLC. Quantitative estimation of the conversion efficiency was done in accordance with an evaluation of the molar ratio between L-theanine/L-Gln.

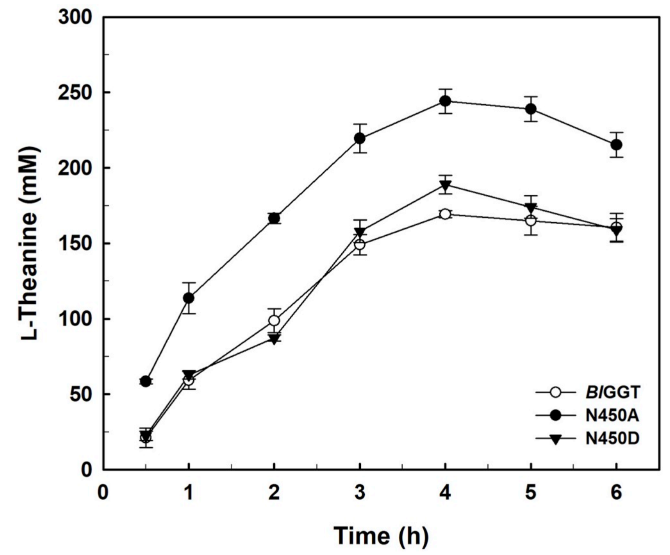

Fig. 1. Effect of reaction time on the biocatalytic synthesis of L-theanine by *Bl*GGT, N450D, and N450Q. The data were expressed as mean ± SD from three independent experiments.

2.4. Preparative-scale synthesis and subsequent isolation of L-theanine

A reaction mixture (20 mL) consisting of 250 mM L-Gln, 600 mM ethanolamine, 50 mM borate buffer (pH 10.5), and N450D at a working concentration of 25 μg/mL was shaken by means of an orbital shaker at 37 °C for 4 h. The reaction solution was then loaded onto a preparative column (1.9 by 20 cm) packed with Dowex® 1 × 8 ion exchange resin (200–400 mesh; Sigma-Aldrich) in its acetate (CH_3COO^-) form. The loaded column was firstly washed with one column volume of distilled water and eluted stepwisely with a scalar gradient of acetic acid solutions (0.5, 1.0, 2.0, and 5.0 M). Afterwards, the appropriate fractions were pooled together and evaporated to dryness to give 0.29–0.41 g white solid.

2.5. Verification of the desired product

Structural analysis of the reaction products was performed by using a QSTAR XL Q-Tof mass spectrometer (AB Sciex, MA, USA) combined with an electrospray ionization (ESI) probe. Recovery of the reaction products was accomplished by means of solid-phase extraction (SPE) and the analytes were eluted from the SPE cartridge by methanol. The resultant solution was subsequently infused into the ESI probe through a syringe pump at a flow rate of 5 μL/min. Positive mode electrospray ionization was used for the structural characterization of the reaction products. The full ESI-MS spectrum of the prepared sample was firstly carried out, and then followed by the collision-induced dissociation (CID) spectra of the selected ions. Typical settings were a spray voltage of +3.2 kV, a capillary temperature of 195 °C, a sheath gas flow rate of 10 units, and a normalized ionization energy of 36%.

3. Results and discussion

3.1. Condition optimization for the biocatalytic synthesis of L-theanine

Our previous studies on *Bl*GGT, N450D, and N450Q have shown that these enzymes are active at a temperature of 40 °C (Lin et al., 2016; Yang et al., 2011), which provides a solid base for the biocatalytic synthesis of γ-glutamyl compounds at around this temperature. By keeping in mind the lower boiling point of ethylamine (bp = 20 °C), the biocatalytic synthesis of L-theanine by several other researchers has been performed at 37 °C (Wang et al., 2012; Chen et al., 2014; Lin et al., 2006). Hence, the reaction temperature for the biocatalytic synthesis of L-theanine by these three enzymes was set at 37 °C throughout the study.

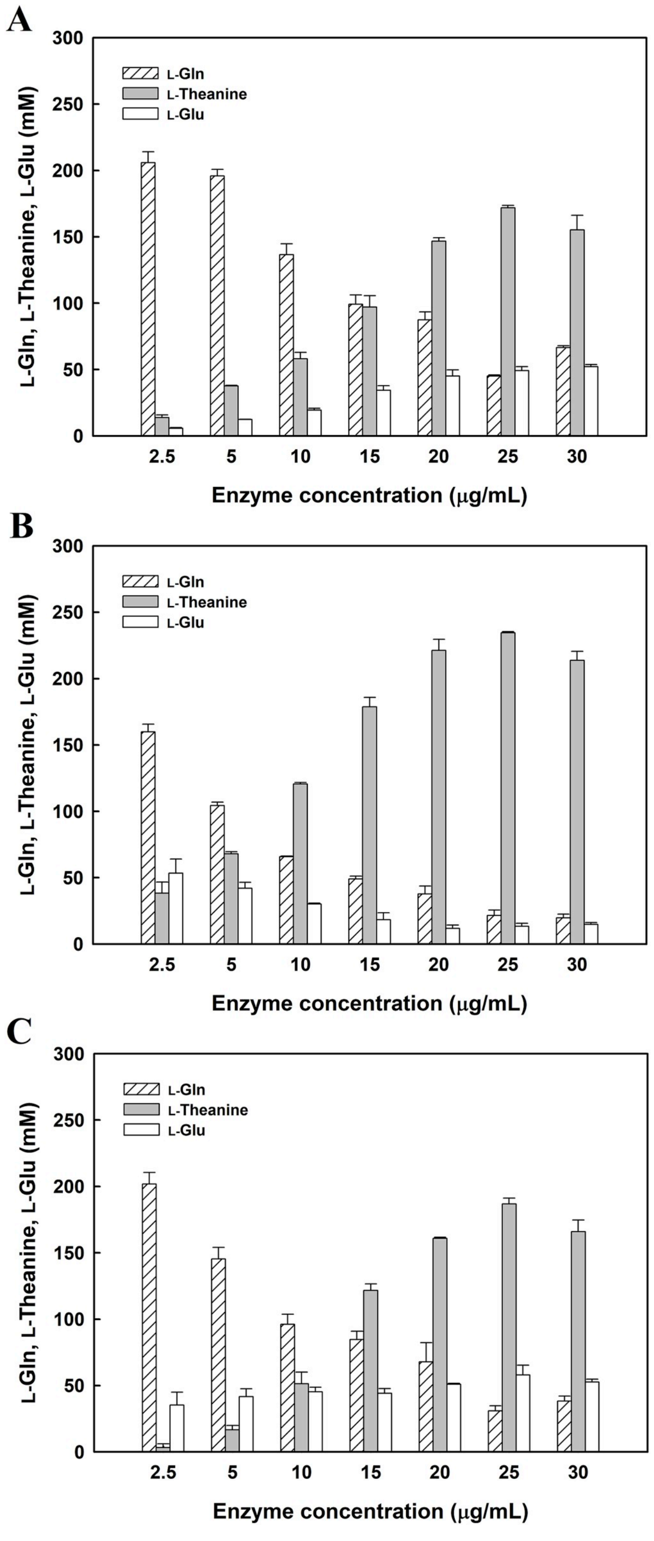

Fig. 2. Effect of enzyme concentration on the biocatalytic synthesis of L-theanine by *Bl*GGT (A), N450D (B), and N450Q (C). The data were expressed as mean ± SD from three independent experiments.

The effect of reaction time on the biocatalytic synthesis of L-theanine was studied at an enzyme concentration of 25 μg/mL and 37 °C. As shown in Fig. 1, the biocatalytic synthesis of L-theanine by *Bl*GGT, N450D, and N450Q was increased rapidly within the first 2 h and

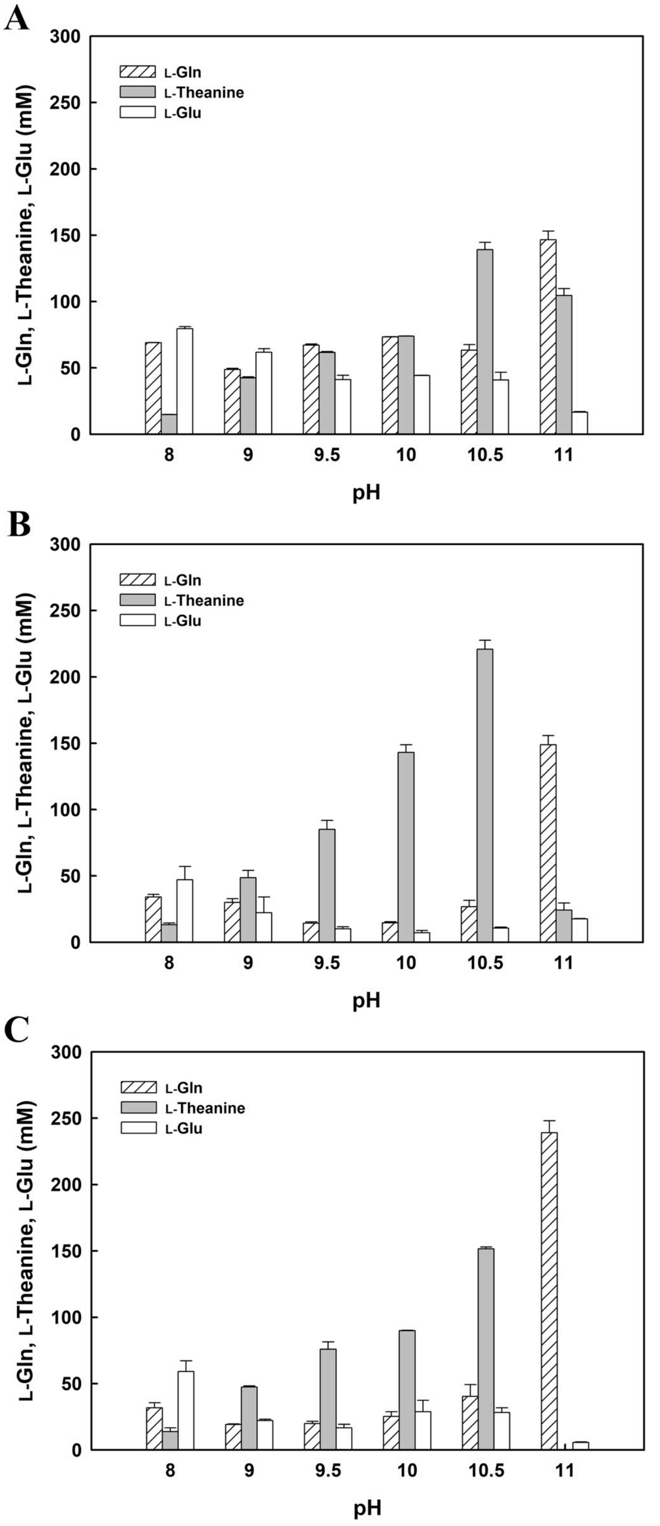

Fig. 3. Effect of pH on the biocatalytic synthesis of L-theanine by *Bl*GGT (A), N450D (B), and N450Q (C). The data were expressed as mean ± SD from three independent experiments.

reached a maximum after 4 h of reaction. The highest amount (235 mM) of L-theanine that corresponds to a conversion rate of 94% was achieved by N450D-mediated biocatalysis, while both N450Q and *Bl*GGT exhibited a less ability to catalyze the conversion. Consequently, further parameters of the biocatalytic reaction were studied for 4 h with one single variable at a time approach.

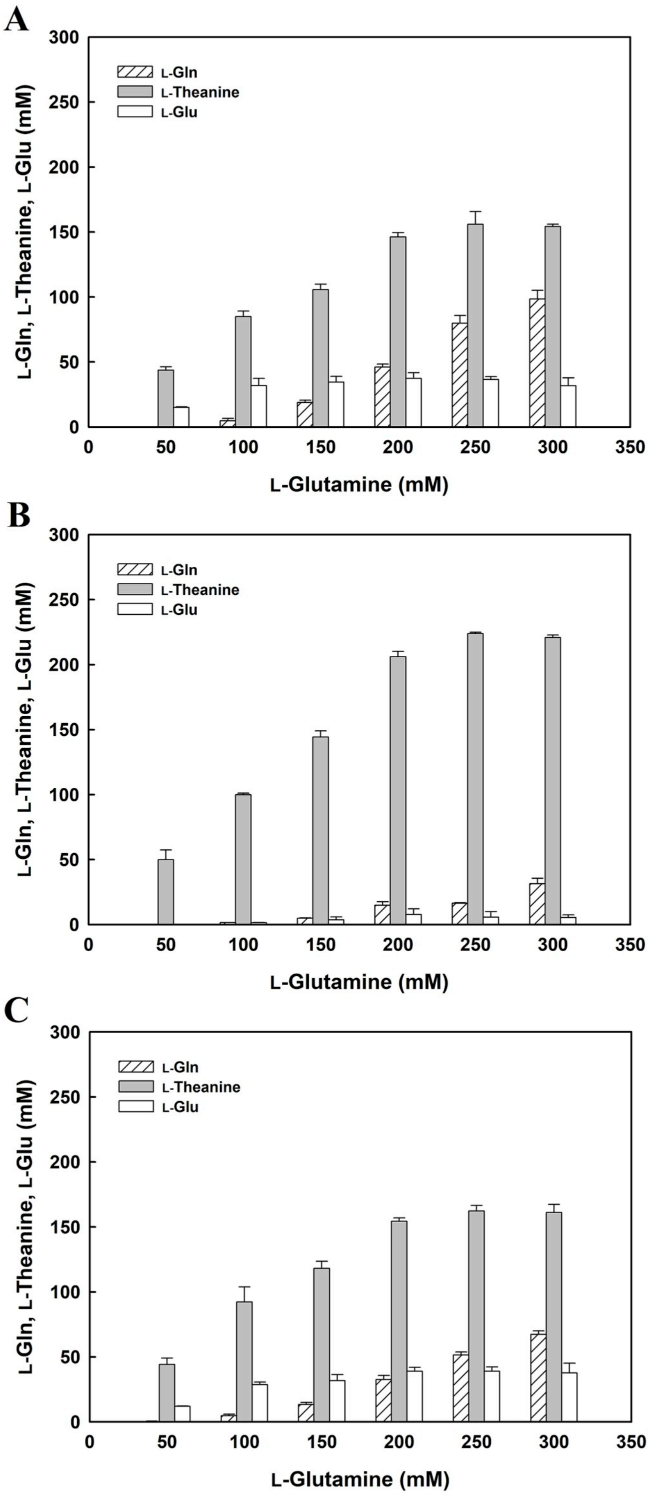

Fig. 4. Effect of donor substrate concentration on the biocatalytic synthesis of L-theanine by *Bl*GGT (A), N450D (B), and N450Q (C). The data were expressed as mean ± SD from three independent experiments.

The effect of different enzyme concentrations on the biocatalytic synthesis of L-theanine was subsequently investigated. At a working concentration of 2.5 μg/mL, the percent yield of L-theanine was found to be less than 16% for all enzyme samples (Fig. 2). Gradual increases in the product yield could be seen when the enzyme concentrations were increased from 5 to 20 μg/mL. The maximum yield for *Bl*GGT, N450D, and N450Q was 69, 94, and 75%, respectively, at a working

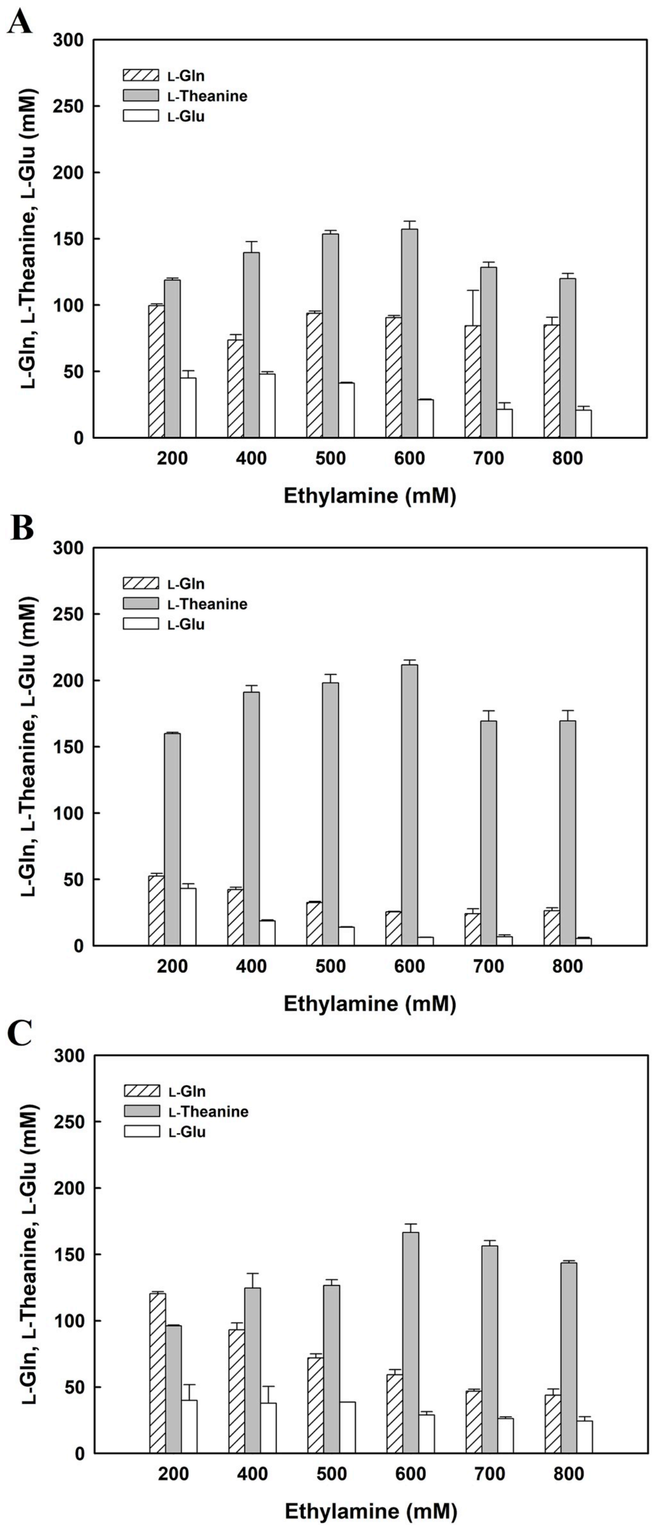

Fig. 5. Effect of acceptor substrate concentration on the biocatalytic synthesis of L-theanine by *Bl*GGT (A), N450D (B), and N450Q (C). The data were expressed as mean ± SD from three independent experiments.

concentration of 25 μg/mL. With a further increase in the enzyme concentrations to 30 μg/mL, there was a statistically significant decrease in the yield of the desired product. The decrease in the product yield is probably caused by the predominant use of L-theanine as one kind of γ-glutamyl donor for the transpeptidation action under the enzyme concentrations of more than 30 μg/mL.

Since the transpeptidation activity of *Bl*GGT could be reduced considerably under acidic conditions (Pica et al., 2016), the optimal level of L-theanine production was therefore studied at different alkaline conditions. As shown in Fig. 3, the optimal pH for L-theanine production by *Bl*GGT, N450D, and N450Q was all at 10.5, resulting in the individual yields of 56, 88, and 61%. The ideal pH for several other GGT-mediated productions of γ-glutamyl compounds is in the consistent range of 9.0–10.0 (Zhang et al., 2010; Wang et al., 2012; Bindal and Gupta, 2014; Lin et al., 2006; Chen et al., 2015; Speranza and Morelli, 2012). It is worthy of note that the product yield of *Bl*GGT-catalyzed synthesis operated at pH 11.0 was 42%. However, under the same pH condition, the product yield was sharply reduced from 88% to about 10% for N450D-catalyzed synthesis and much worse in N450Q-mediated reaction (Fig. 3B and C). The reason for these observations is still unknown and remains to be elucidated by determining the crystal structures of N450D and N450Q.

In the biocatalytic synthesis of L-theanine, the most frequently-used and accepted approach to increase an output of the production process is an excess of one substrate (Zhang et al., 2010; Wang et al., 2011, 2012; Bindal and Gupta, 2014; Lin et al., 2006, 2014; Tachiki et al., 1998; Yamamoto et al., 2005). So the molar ratios of L-Gln to ethylamine in the reaction mixture was accordingly varied from 0.08 to 0.5. The correlation between the conversion efficiency of L-theanine and the molar ratios was shown in Figs. 4 and 5. At the L-Gln concentration of 50 mM, the donor substrate was completely exhausted by each enzyme and the product yield for *Bl*GGT-, N450D-, and N450Q-catalyzed syntheses was very similar (Fig. 4). The product yield was proportionally increased with the L-Gln concentration and reached a maximum at a donor substrate concentration of 250 mM. It should be noted that the product yields were significantly reduced for all the biocatalytic syntheses if the donor concentration was larger than 250 mM (Fig. 4). The most likely explanation for this observation is that, when the concentration of L-theanine in the reaction mixture reaches a certain level, it becomes a new γ-glutamyl donor for the biocatalytic syntheses and acts as a competitive substrate with respect to L-Gln. Moreover, both L-Gln and the newly synthesized L-theanine can also be used as the substrates by theses enzymes to carry out the auto-transpeptidation reaction. Under these situations, the composition of reaction products becomes complicated due to the occurrence of adverse reactions and the formation of γ-glutamyl-L-theanine and other undesirable substances (see the HPLC chromatograms and ESI/MS spectrum shown below), and eventually leads to a reduction in the overall percent yield of L-theanine.

When the concentration of donor substrate (L-Gln) was fixed at 250 mM, the optimal amount of acceptor substrate (ethylamine) was found to be 600 mM for all the biocatalytic syntheses (Fig. 5). This result is consistent with the use of L-Gln as the donor substrate for the enzymatic synthesis of L-theanine by several other bacterial GGTs, in which excess ethylamine is preferred for producing this substance (Wang et al., 2011, 2012; Bindal and Gupta, 2014; Lin et al., 2006, 2014). Beyond that, the above observations are assumed to be interpretable from the aspect of catalytic mechanism. Excellent literature reviews have documented that the catalytic cycle of GGT enzymes follows a ping-pong mechanism (Castellano and Merlino, 2012; Allison, 1985). In the first step of GGT-catalyzed reaction, the nucleophilic oxygen atom of the catalytic Thr residue can attack the carbonyl of the donor substrate to form a γ-glutamyl-enzyme intermediate. The resulting intermediate in turn reacts with water to release glutamate in the hydrolysis reaction or with amino acids or peptides to produce new γ-glutamyl compounds in the transpeptidation reaction. It has been reported that the rate-determining step in the GGT-catalyzed reaction is the transfer of the γ-glutamyl moiety from the intermediate acyl-enzyme to the acceptor molecule (Allison, 1985; Castonguay et al., 2003). Consequently, it can be argued that higher yields observed in the ethylamine range of 400–600 mM are probably achieved by the molar excess of the acceptor. It is also worthy of note that the concentrations of L-Gln for the *Bl*GGT-catalyzed synthesis were remained at greater than 134 mM in the

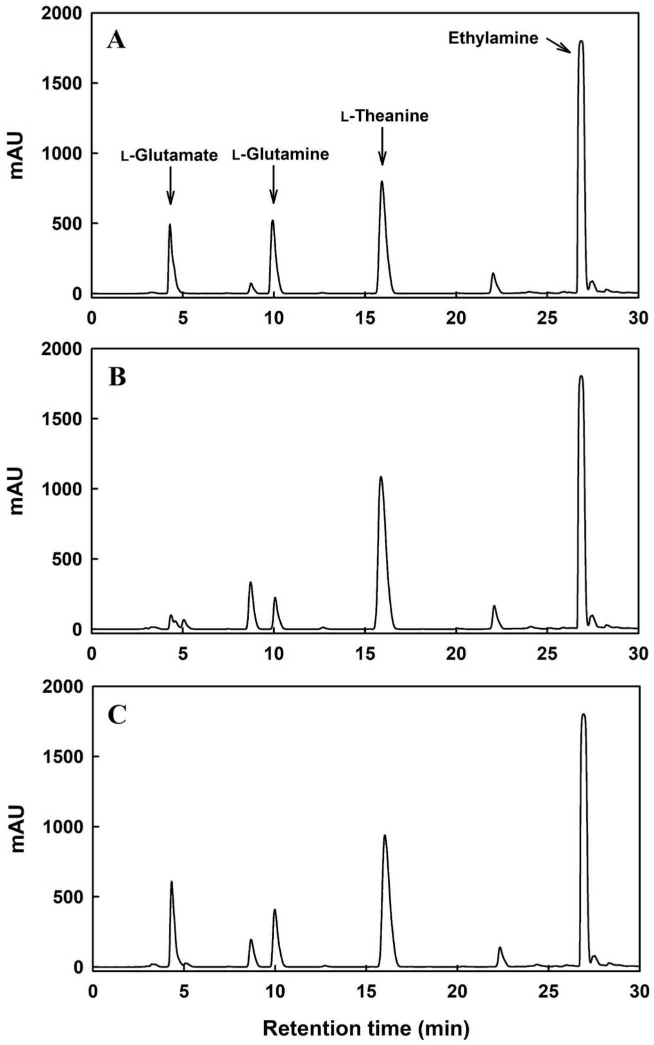

Fig. 6. HPLC analyses of the reaction products from *Bl*GGT (A)-, N450D (B)-, and N450Q (C)-mediated biocatalysis. The reactions were carried out at 37 °C for 4 h with a reaction mixture (20 mL) consisting of 50 mM borate buffer (pH 10.5), 250 mM L-Gln, 600 mM ethylamine, and each enzyme at a working concentration of 25 μg/mL.

reaction mixture with either 700 or 800 mM ethylamine (Fig. 5A). But this was not the case for N450D- and N450Q-associated syntheses where the donor substrate was more efficiently used by the enzymes (Fig. 5B and C). Such a result is mostly resulted from the intrinsic nature of the enzyme molecules in which transpeptidation is the predominant mode of action.

3.2. *Tentative synthesis of L-theanine*

The biocatalytic synthesis of L-theanine by *Bl*GGT, N450D, and N450Q was repeated at a preparative level with a reaction solution of 20 mL. After incubating the reaction mixtures at 37 °C for 4 h, the OPA-derived samples were subjected to HPLC analysis. As shown in Fig. 6A, four major peaks devoted to L-Glu (4.3 min), L-Gln (9.9 min), L-theanine (15.9 min), and ethylamine (26.9 min) were observed in the HPLC chromatogram of *Bl*GGT-catalyzed synthesis. It is worthy of note that some unknown peaks can also be seen in the chromatogram. The HPLC profile of N450Q-mediated synthesis was fairly similar to that of the wild-type enzyme; however, the amount of L-Glu in the chromatogram of N450D-catalyzed reaction was very low and the intensity of an unknown peak (8.2 min) was conversely increased (Fig. 6B and C). By comparison to the standard curve of L-thenaine, the conversion efficiency for *Bl*GGT-, N450D-, and N450Q-mediated synthesis was 62, 94, and 75%, respectively. Following an ion-exchange chromatographic process, L-theanine was eluted by a 5 M solution of acetic acid. The preparative-scale syntheses resulted in an overall yield of 33.1–47.1% after the subsequent purification.

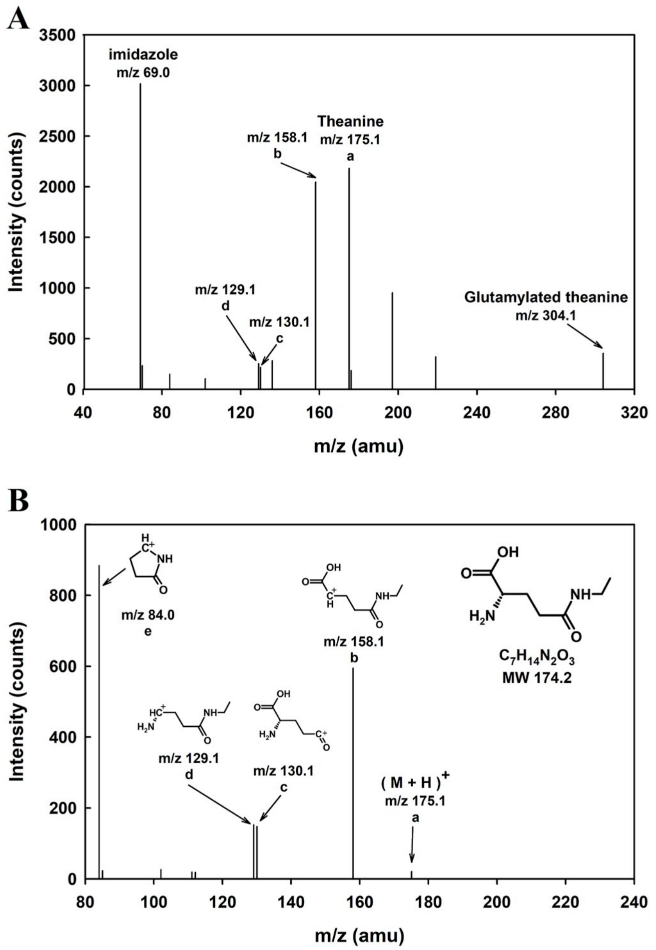

Fig. 7. Mass spectra of $[M + M^{+}]$ ions of the reaction products recorded on an ion trap. A, Mass spectrum of the protonated L-theanine (*m/z* 175.1) and the identified compounds. B, MS^2 mass spectrum of *m/z* 175.1 with the subsequent isolation of the fragments *m/z* 158.1, 130.1, and 129.1.

3.3. *Instrumental verification of the desired product*

The afore-prepared L-theanine was subjected to instrumental analysis by ESI-MS/MS. As shown in Fig. 7A, a strong signal with an *m/z* value of 175.1 corresponding to the ionized L-theanine in the positive mode could be seen. The protonated molecule at an *m/z* value of 175.1 lost either its amino group (17 u) to yield the fragment at *m/z* 158.1, or individually vanished the ethyl amine and carboxyl group to produce the fragments at the *m/z* values of 130.1 and 129.1 (Fig. 7B). The ESI-MS/MS spectra of the authentic L-theanine were fundamentally coincided with those of the synthesized product in the reaction mixtures (data not shown). These results clearly reveal that L-theanine has been successfully synthesized by the biocatalytic process using L-Gln and ethylamine as the substrates.

3.4. *A comparison on the production of L-theanine by bacterial enzymes*

Since the favorable contribution of L-theanine to umami taste and a wide variety of health benefits, there is a perceived increasing demand for its use as nutraceuticals, functional food ingredients and dietary

Table 1
Comparison of operational conditions and yields among bacterial enzymes employed for the biocatalytic synthesis of L-theanine.

Enzyme	L-Glutamine concentration (mM)	Ethylamine concentration (mM)	Reaction buffer	Temperature (°C)	Reaction time (h)	Conversion rate (%)[a]	References
*Bl*GGT	250	600	Borate buffer (pH 10.5)	37	4	62	This study
			Borate buffer (pH 10.5)				
N450A	250	600	Borate buffer (pH 10.5) pH 10[b]	37	4	94	This study
N450D	250	600		37	4	75	This study
			pH 10.5[b]				
E. coli K-12 GGT	200	1500		37	2	60	Zhang et al. (2010)
			Tris-HCl buffer, pH 10.5				
E. coli K-12 GGT	267	2000	pH 10[b]	37	24	80	Wang et al. (2011)
E. coli NovaBlue GGT	10	40	Tris-HCl buffer, pH 9	37	NA[c]	45	Yao et al. (2006)
			Borate buffer, pH 11				
B. subtilis SK 11.004 GGT	20	50		37	4	94	Wang et al. (2012)
			Borate buffer, pH 10				
B. licheniformis ER-15 GGT	80	600		37	4	>84	Lin et al. (2006)
Pseudomonas nitroreducens IFO 12694 L-glutaminase	700	1500		30	7	39	Tachiki et al. (1998)
P. nitroreducens DSM 14399 L-glutaminase	300	1500		37	5	40	Pu et al. (2013)

[a] The conversion rate is expressed as the amount of synthesized L-theanine against the initial concentration of L-Gln.
[b] The buffer system used is not indicated by the authors.
[c] NA, not available.

supplements in recent years (Williams et al., 2016; Eschenauer and Sweet, 2006; Adhikary and Mandal, 2017). In this regard, many efforts have been made to synthesize and produce L-theanine to meet this demand (Vuong et al., 2011; Shuai et al., 2010). Biocatalysis has several advantages over other production techniques due to its intrinsic features of stereo-selectiveness, high specificity, shorter reaction time, higher yield and recyclability (Sharma et al., 2018). Over the last two decades, two types of bacterial enzymes, L-glutaminase and GGT, together with two cost-effective substrates, L-Gln and ethylamine, have been mostly used in the biocatalytic synthesis of L-theanine (Vuong et al., 2011; Shuai et al., 2010; Sharma et al., 2018). As shown in Table 1, an L-Gln to L-theanine conversion rate between 39 - 94% can be obtained after the biocatalytic process. It is worthy of mention that an excess amount of ethylamine in the reaction mixture is a generally accepted principle for the biocatalytic synthesis of L-theanine. Through the use of 250 mM L-Gln and 600 mM ethylamine as the substrates, L-theanine could be produced with individual conversion rates of 62, 94, and 75%, respectively, by *Bl*GGT, N450D and N450Q (Table 1). A very high conversion rate observed in N450D-catalyzed synthesis renders this enzyme applicable to the industrial conversion of L-Gln and ethylamine to L-theanine.

4. Conclusion

In this study, *Bl*GGT and its Asn450 mutants with high transpeptidation activity are employed for the biocatalytic synthesis of L-theanine. Under the optimal conditions for L-theanine synthesis, a satisfactory yield of ~94% can be obtained from the cost-effective substrates. These experimental results are of considerable importance in the feasibility of industrial-scale production of L-theanine to meet the increasing health needs of consumers.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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