Application of recombinant Bacillus subtilis \( \gamma \)-glutamyltranspeptidase to the production of L-theanine

Xingyi Chen \(^{a,b} \), Lingqia Su \(^{a,b} \), Dan Wu \(^{a,b} \), Jing Wu \(^{a,b,\ast} \)

\(^{a}\) State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Ave., Wuxi 214122, People’s Republic of China

\(^{b}\) School of Biotechnology and Key Laboratory of Industrial Biotechnology Ministry of Education, Jiangnan University, 1800 Lihu Ave., Wuxi 214122, People’s Republic of China

A R T I C L E   I N F O

Article history:
Received 1 November 2013
Received in revised form 11 May 2014
Accepted 13 May 2014
Available online 7 June 2014

Keywords:
L-Theanine
\( \gamma \)-Glutamyltranspeptidase (GGT)
Bacillus subtilis 168
Industrial-scale production
Enzymatic synthesis

A B S T R A C T

L-Theanine, which has seen increasing use in the functional food industry, can be prepared via enzymatic synthesis using \( \gamma \)-glutamyltranspeptidase (GGT; EC 2.3.2.2). In this study, the GGT from Bacillus subtilis 168 was cloned and expressed as a secreted protein using Escherichia coli BL21(DE3). The enzymatic properties of the GGT and the optimal conditions for the enzymatic synthesis of L-theanine were investigated in detail. The activity of the enzyme was optimal at pH 10; the optimal temperature was 50 °C. Desirable pH stability was observed between pH 5 and pH 12, and adequate thermostability was seen at 50 °C. In 5 h at 37 °C, the enzyme converted 200 mM L-glutamine and 2.2 M ethylamine to L-theanine with a final yield of 78%. Yields of L-theanine decreased to 58% when using 500 mM Gln and 45% when using 1 M Gln. The yield of L-theanine obtained at high substrate concentration provides the basis for the industrial-scale production of L-theanine.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

L-Theanine (\( \gamma \)-glutamylethylamide; 5-\( \text{N} \)ethylglutamine) is a unique non-protein amino acid present in green tea that has been reported to impart an umami taste to the tea [1]. Several studies have shown or suggested that ingestion of L-theanine has physiological and pharmacological effects on humans. Ingestion of 200 mg of L-theanine has been shown to reduce psychological and physiological stress [2,3]. When combined with caffeine, as in tea, L-theanine has been shown to improve cognitive performance and learning ability [4–6]. Substantial doses have also been shown to promote weight loss in mice [7,8], reduce blood pressure [6,9], improve immune response (when combined with L-cysteine) [10,11], and inhibit the growth and survival of cancer cells [12,13]. Since these beneficial effects have been demonstrated at concentrations above the amounts available in tea, L-theanine is being increasingly used as a dietary supplement. In the United States, the Food and Drug Administration (FDA) has classified L-theanine as generally recognized as safe (GRAS).

L-Theanine can be extracted from tea, prepared by chemical synthesis, or produced by biological synthesis [14]. Extraction from natural sources produces the natural enantiomer, but requires considerable time and energy. It also produces substantial quantities of waste while producing a relatively small quantity of material. Chemical synthesis can produce larger quantities, but it also produces undesirable waste streams and generally results in a racemic product. Therefore, considering the environmental friendliness and overall cost-effectiveness of the process, as well as the production of a chiral product, the development of biological or enzymatic methods has been an increasing trend.

The enzymatic synthesis of L-theanine can be carried out using the enzyme glutaminase (EC 3.5.1.2) derived from a variety of bacterial sources. This reaction requires L-glutamine (Gln) and ethylamine as reactants, and produces L-theanine and ammonia. A process using the glutaminase from Pseudomonas nitroreducens IFO 12694 led to L-theanine yields ranging from 90% to 39% within 3–7 h, starting with concentrations of Gln varying from 100–700 mM [15]. The development of continuous reaction methods using immobilized \( P. \) nitroreducens cells further increased the yield of L-theanine [16,17]. Taiyokagaku Co. Ltd. reported using glutaminase derived from \( P. \) citronellolis GEA for L-theanine production. Their yields ranged from 20% to 78% within 48–72 h, starting with 200–400 mM Gln and various concentrations of ethylamine [18].
The production of L-theanine has also been studied using the enzyme glutamine synthetase (EC 6.3.1.2). This enzyme uses L-glutamic acid (Glu) and ethylamine as substrates, and requires the hydrolysis of cofactor ATP to drive the synthesis of L-theanine. Yamamoto et al. reported the production of L-theanine using the glutamine synthetase of *Pseudomonas taetrolens* Y-30. They achieved an 85% yield using 200 mM sodium glutamate and 1.2 M ethylamine as substrates, and sugar fermentation by baker’s yeast cells as an ATP-regeneration system [19]. However, due to the low reactivity of *P. taetrolens* Y-30 with ethylamine, its application to L-theanine production at high concentrations was limited. They subsequently reported a γ-glutamylmethylamide synthetase of *Methylovorax mays* No. 9 with high theanine-forming activity. Under conditions to those used with *P. taetrolens* Y-30, they achieved a 100% yield at 48 h using 600 mM sodium glutamate and 600 mM ethylamine as substrates [20]. The obvious inconvenience of using glutamine synthetases is that a specialized ATP regeneration system is required. Meanwhile, this process took tens of hours, which may decrease the intensity of production.

GGTs from bacterial resources have been used with increasing frequency to produce valuable new γ-glutamyl compounds [21]. GGT catalyzes transfer of the γ-glutamyl group of glutathione, or other compounds containing the γ-glutamyl moiety, to acceptors such as amino acids, dipeptides, or water, resulting in the corresponding transpeptidation product or hydrolysis [22]. GGTs are widely distributed in nature and involved in the metabolism of glutathione [23]. Suzuki et al. reported an L-theanine production method using the GGT from *Escherichia coli* K-12 in which a yield of 60% was achieved using 200 mM Gln and 1.5 M ethylamine as substrates [24]. Shuai et al. used the GGT from the newly isolated *Bacillus subtilis* SK11.004 strain, to obtain a 94% yield with 20 mM Gln and 50 mM ethylamine as substrates [25]. Notably, most bacterial GGTs exhibit maximal transferase activity at alkaline pH, which is consistent with the conditions under which L-theanine is produced. In addition, the enzyme reaction is quite efficient. The maximum yield is reached within several hours, providing another advantage in increasing the intensity of production. However, GGTs robust and active enough for industrial applications and able to reach high yields still need to be found.

Since the GGT from *B. subtilis* has been reported to be a very effective enzyme for the production of L-theanine [25], we chose to study the GGT from *B. subtilis* 168. *E. coli* BL21(DE3) was selected as a host strain for extracellular expression of this GGT, and the recombinant enzyme was characterized in detail. In this study, high production of recombinant enzyme could be achieved through extracellular expression. The ability of the GGT from *B. subtilis* 168 to produce L-theanine using different concentrations of substrate was also investigated. Compared with most other enzymes used in L-theanine production, this GGT has the advantages of ease of operation and higher yields of L-theanine at high substrate concentration.

### 2. Materials and methods

#### 2.1. Bacterial strains, plasmids, and materials

The *B. subtilis* 168 used in this study was a laboratory stock strain. *E. coli* JM109 cells for plasmid construction and *E. coli* BL21(DE3) cells for protein expression were purchased from Promega Co. The pMD18-T simple vector, EZ-10 Spin Column Plasmid Mini-Preps kit, agarose gel DNA purification kit, restriction enzymes, Primer STAR® DNA Polymerase and T4 DNA ligase were obtained from TakaRa (Dalian, China). The pET-20b(+) expression vector was obtained from Novagen. Other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

#### 2.2. Cloning of the ggt gene from *B. subtilis* 168

The ggt gene (GenBank accession no. 194373044) from *B. subtilis* 168 genomic DNA was amplified using PrimeSTAR® HS DNA Polymerase and a standard polymerase chain reaction method. The forward primer was ATCCATGGATAAAAAACCCAAAAGCTACGA (Ncol restriction site underlined), and the reverse primer was GCGCCGCGCACATTATTTACCTAATTATGC (XhoI restriction site underlined). The amplification product was isolated and ligated directly into the cloning vector pMD18T-simple, which was then transformed into chemically competent *E. coli* JM109 cells. We verified the presence of a plasmid containing the desired gene in several transformants using restriction analysis (using Ncol and XhoI, underlined above) and DNA sequencing. The resulting plasmid was digested with Ncol and Xhol, and the ggt-containing fragment was ligated into the similarly restricted expression vector pET-20b(+). The ligation mixture was used to transform *E. coli* JM109 cells. The plasmid isolated from several transformants was verified by restriction analysis and the desired plasmid, pET/20b(+), was transformed into competent *E. coli* BL21(DE3) cells for overexpression.

#### 2.3. Enzyme preparation and purification

A single colony of *E. coli* BL21(DE3) cells harboring plasmid pET/20b(+) was used to inoculate 10 ml Luria–Bertani medium containing 100 μg/ml ampicillin at 37 °C. This starter culture was shaken for 8–10 h. A 2.5 ml aliquot of the starter culture was diluted into 50 ml of terrific broth (TB) medium containing 100 μg/ml ampicillin and 0.75% (w/v) glycine in a 250 ml flask, and incubated on a rotary shaker (200 rpm) at 37 °C until it reached an optical density (OD) of 1.5 at 600 nm. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce expression. After induction, the culture was grown at 25 °C. Cell growth (OD<sub>600</sub>) and enzyme activity were measured at regular intervals. We also conducted an expression experiment using TB medium without glycine, to determine the effect of glycine on the production of GGT.

The culture supernatant was obtained by centrifugation at 12,000 × g for 10 min at 4 °C. Solid ammonium sulfate was slowly added to the culture supernatant, with stirring, to a final concentration of 70% (w/v). The solution was kept at 4 °C overnight to precipitate proteins. The precipitate was collected by centrifugation and dissolved in buffer A (50 mM Tris–HCl buffer, pH 8.0) and dialyzed against 2 L of buffer A at 4 °C overnight. The sample was filtered (0.22 μm) and loaded onto a DEAE-Sepharose column pre-equilibrated with buffer A. The column was eluted using a linear gradient from 0–1 M NaCl in buffer A at the flow rate of 1.0 ml/min. The active fractions were pooled and dialyzed against buffer A at 4 °C overnight. The dialyzed sample was applied to a Superdex 75 10/300 GL column equilibrated with buffer A at the flow rate of 0.5 ml/min and the fractions in the UV peak were collected and measured for enzyme activity.

#### 2.4. Electrophoresis

SDS-PAGE was performed under reducing conditions on a 12% polyacrylamide gel with a Mini-PROTEAN II electrophoresis unit (Bio-Rad) and visualized with 0.25% Coomassie Brilliant Blue G-250 stain.

#### 2.5. Enzyme assay

The activity of GGT was determined by the method of Orlowski and Meister with slight modification [26]. Each reaction mixture contained 5 mM L-γ-glutamyl-p-nitroanilide, 80 mM glycylglycine,
and 50 mM borate–NaOH buffer (pH 10.0) in a total volume of 1.0 mL. The mixture was pre-incubated at 37 °C for several minutes, then 20 μL of diluted enzyme solution was added. The reaction was performed at 37 °C for 5 min and then stopped by adding 0.4 mL of 4 M acetic acid to make the final pH around 3. One unit of GGT activity (also transpeptidation activity) was defined as the amount of the enzyme that transferred 1 μmol of p-nitroaniline per minute from L-γ-glutamyl-p-nitroanilide under the conditions described above. The hydrolysis activity was measured in the same reaction mixture without the addition of glycylglycine.

2.6. pH optimum and stability

The effect of pH on the transpeptidation and hydrolysis activities was measured between pH 3 and pH 11, in increments of 1 pH unit, using 50 mM sodium citrate (pH 3.0–5.0), potassium phosphate (pH 5.0–8.0), Britton–Robinson (pH 8.0–10.0) and borate–NaOH (pH 10.0–11.0) buffers. To determine the stability of the enzyme as a function of pH, the enzyme was incubated in various buffers between pH 3 and 11 at room temperature for 24 h, and then the residual activity was assayed.

2.7. Temperature optimum and thermostability

The temperature dependence of enzyme activity was determined by measuring the enzyme activity between 20 °C and 60 °C at pH 10, using L-γ-glutamyl-p-nitroanilide and glycylglycine as the substrates. The thermostability of the enzyme was determined by incubating the enzyme in 50 mM Tris–HCl buffer (pH 8.0) at 50 °C, 60 °C and 70 °C. At different time intervals, samples were taken and assayed for residual activity.

2.8. Kinetic studies

The kinetic parameters for the transpeptidation activity of GGT were studied using both L-γ-glutamyl-p-nitroanilide and Gln as γ-glutamyl donors. Enzyme assays using both substrates were performed in borate–NaOH buffer (pH 10.0) at 37 °C. For studies using L-γ-glutamyl-p-nitroanilide, the enzyme activity was determined using 10–4500 μM concentrations of L-γ-glutamyl-p-nitroanilide and 80 mM glycylglycine as the acceptor. For studies using Gln, the enzyme activity was determined using 1–30 mM concentrations of Gln and 100 mM ethylamine as the acceptor. The amounts of l-theanine formed were determined by HPLC. The K_m and V_max were calculated by nonlinear regression using GraphPad Prism version 5.0 software.

2.9. Optimization of the reaction conditions for l-theanine production

In order to observe the yield of L-theanine from Gln and ethylamine under various conditions, Gln and ethylamine were dissolved in water to a final volume of 10 mL and the reaction was carried out in a constant temperature shaker incubator with continuous agitation (150 rpm). An equal volume of 10% trichloroacetic acid was added to terminate the reaction as well as maintain an acidic pH to prevent the decomposition of the products. The reaction products were then separated and quantified by HPLC.

2.10. HPLC analysis

The quantities of l-theanine, Gln, and Glu were measured using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Eclipse XDB-C18 5 μm (4.6 mm × 150 mm) column (Agilent Technologies). Analyses were conducted with gradient elution at 40 °C and a flow rate of 0.8 mL/min. The gradient of mobile phase was formed with buffer A (4.52 g/L sodium acetate anhydrous, 200 μg/L triethylamine, 5 mL/L tetrahydrofuran, pH 7.2 ± 0.05) and buffer B (22.6 g/L sodium acetate anhydrous, pH 7.2 ± 0.05/acetonitrile/methanol = 1:2:2, by volume). The elution profile consisted of (i) a linear change from 8% B to 26% B over 12 min, (ii) a linear change from 26% B to 100% B over 2 min, (iii) a hold at 100% B for 1.5 min, and (iv) a linear return to 8% B over 3 min. α-Phthalaldehyde was used as the precolumn derivatization reagent and the sample was detected with a UV detector (G1314B VWD, Agilent Technologies) at 338 nm. Typically, Glu, Gln and l-theanine were eluted at 2.8, 5.9 and 11.0 min, respectively (see Supplementary Material).

Supplementary Fig. 1 can be found, in the online version, at doi:10.1016/j.procbio.2014.05.019.

2.11. UPLC-Q-TOF MS analysis

The reaction product of L-theanine synthesis was diluted with H_2O to 0.1–0.2 g/L l-theanine then analyzed with UPLC-Q-TOF MS system, and the spectrum was compared with that of the L-theanine standard. UPLC was performed on a Waters ACQUITY UPLC™ system equipped with a binary solvent-delivery system and an autosampler. Mass spectrometry was performed with a Waters Synapt Q-TOF system (Waters MS Technologies, Manchester, UK) [25].

3. Results and discussion

3.1. Cloning and expression of ggt in E. coli BL21(DE3)

A 1680 bp fragment containing the ggt gene was amplified by PCR using chromosomal DNA from B. subtilis 168 as the template. The PCR product was cloned into pMD18-T and sequenced. This 1680 bp fragment showed 100% sequence identity with the sequence reported for the ggt gene of B. subtilis 168 (GenBank accession no. 194373044).

The recombinant plasmid ggt/pET-20b(+) was constructed and transformed into E. coli BL21(DE3). Since it has been reported that glycine increases the permeability of the outer membrane of E. coli by modifying the peptidoglycan structure in the cell wall [27], 0.75% (w/v) glycine was added to the TB medium to enhance extracellular secretion of the recombinant GGT. The maximum secreted enzyme activity (80 U/mL) was reached after cultivation for 40 h. When glycine was omitted from the culture medium, a similar maximum secreted enzyme activity could be obtained, but only if the cultivation period was extended by approximately 10 h (Fig. 1).

3.2. Purification of GGT

The recombinant GGT was purified by ammonium sulfate precipitation, DEAE-Sepharose anion exchange chromatography, and Superdex 75 gel filtration chromatography (Table 1). The purified enzyme was determined to be homogeneous by SDS-PAGE analysis (Fig. 2) and exhibited a specific activity of 136.0 U/mg using L-γ-glutamyl-p-nitroanilide with glycylglycine as the substrates. Besides, the hydrolysis activity (without glycylglycine added) of the purified GGT was 25.7 U/mL, only 18.9% compared with the GGT activity (transpeptidation activity). It should be noted that, as a number of reports suggest that, in the presence of a strong acceptor and at millimolar levels of the donor, the hydrolysis activity of GGT may be negligible [28,29]. Thus, the actual extent of hydrolysis under transpeptidation reaction conditions was probably lower.

SDS-PAGE analysis (Fig. 2) showed that the purified enzyme was composed of two subunits with molecular masses of approximately 43 kDa and 21 kDa respectively, which is consistent with the
from 55 °C to 60 °C (Fig. 3A). The thermostability of GGT was determined at pH 8.0 and temperatures of 50 °C, 60 °C and 70 °C (Fig. 3B). The enzyme showed 50% residual activity when incubated for 130 h at 50 °C. Upon incubation at 60 °C, the enzyme lost activity rapidly and the half-life of the enzyme decreased to about 30 min. At 70 °C, the enzyme was immediately inactivated. However, the enzyme showed more than 90% residual activity for seven days at temperatures between 30 °C and 40 °C (data not shown). This thermostability is desirable for its application to the enzymatic production of L-theanine, which is commonly performed between 30 °C and 40 °C.

3.4. pH optimum and stability of GGT

Recombinant GGT exhibited optimal transpeptidation and hydrolysis activities at pH 10.0 (Fig. 4A). The enzyme isolated directly from B. subtilis 168 has been reported to display optimal transpeptidation activity between pH 9 and 9.5 and optimal hydrolyase activity between pH 8.5 and 11 [30]. This modest difference, which may be caused by slight variations in the measurement method, is not likely to affect the recombinant enzyme’s suitability for L-theanine production. The pH stability of the recombinant enzyme was investigated by incubating the enzyme in buffers of different pH, at room temperature, and assaying samples at varying incubation times. The enzyme retained more than 90% of its maximal activity between pH 5.0 and 8.0 and 80% between pH 9.0 and 12.0 after incubation at room temperature for 24 h (Fig. 4B). These results suggest that the enzyme exhibits broad pH stability and that it retains stability at the extremely alkaline pH required for premium enzyme performance.

To compare these results with those of other enzymes that have been used in L-theanine production, the optimal pH and pH stability of the GGTs, glutaminase and the glutamine synthetases are presented in Table 2. The GGTs and glutaminase exhibited optimal transpeptidation activity at alkaline pH, and the GGTs from B. subtilis and glutaminase from P. nitroreducens showed great pH stability at alkaline pH (pH 10–11) [11,30,32,33]. Addition of different divalent cations (Mg²⁺/Mn²⁺) to the glutamine synthetase from P. taetorens Y-30 caused variation in the optimal pH [34]. Because the synthetase reaction was carried out at pH 7 [19,20], the Mn²⁺-dependent reaction (optimal pH 8.5), coupled with sugar fermentation by the yeast, was preferred for L-theanine production.

Since an alkaline environment is usually more conducive to the transfer reaction that forms L-theanine, the pH optimum and stability of the GGT used in this study are consistent with its application for the efficient production of L-theanine.

3.5. Kinetic studies

The kinetic parameters for the transpeptidation activity of GGT were obtained using L-γ-glutamyl-p-nitroanilide as the donor and 80 mM glycglycine as the acceptor. Separate experiments were performed using L-Gln as donor and 100 mM ethylamine as acceptor. The data were fit using the Michaelis–Menten equation to determine $K_m$ and $V_{max}$ values. The $K_m$ was 2.15 mM for L-γ-glutamyl-p-nitroanilide, and 0.93 mM for Gln. The $V_{max}$ was 191.30 pmol/min/mg using L-γ-glutamyl-p-nitroanilide and...
Fig. 3. Effect of temperature on activity and stability of GGT. (A) The temperature optimum was determined in borate-NaOH buffer (pH 10.0); (B) thermostability of the enzyme in Tris–HCl (pH 8.0) at 50 °C (■), 60 °C (▲), and 70 °C (●). The error bars correspond to the standard deviations from three independent measurements.

Table 2
Comparison of optimal pH and pH stability with other enzymes used in l-theanine production.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme source</th>
<th>Optimal pH for transpetidation activity</th>
<th>Optimal pH for hydrolysis activity</th>
<th>pH stability</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGT (Escherichia coli K-12)</td>
<td></td>
<td>8.73 (γ-GpNA and Gly-Gly)</td>
<td>9.25 (γ-GpNA only)</td>
<td>Not shown</td>
<td>[32]</td>
</tr>
<tr>
<td>GGT (Bacillus subtilis SK11.004)</td>
<td></td>
<td>10 (γ-GpNA and Gly-Gly), 10 (Gln and ethylamine)</td>
<td>8 (γ-GpNA only)</td>
<td>80% activity after 24 h at pH 4–12 and 4 °C</td>
<td>[33]</td>
</tr>
<tr>
<td>GGT (Bacillus subtilis 168)</td>
<td></td>
<td>9.0–9.5 (γ-GpNA and Gly-Gly)</td>
<td>8.5–11 (γ-GpNA only)</td>
<td>Not shown</td>
<td>[30]</td>
</tr>
<tr>
<td>GGT (recombinant enzyme)</td>
<td>Bacillus subtilis 168</td>
<td>10 (γ-GpNA and Gly-Gly), 10 (Gln and ethylamine)</td>
<td>10 (γ-GpNA only)</td>
<td>80% activity after 24 h at pH 5–12 and 25 °C</td>
<td>This study</td>
</tr>
<tr>
<td>Glutaminase (Pseudomonas nitroreducens IFO 12694)</td>
<td></td>
<td>10–11 (Gln and ethylamine)</td>
<td>9 (Gln only)</td>
<td>Glutaminase activity can be assayed after 24 h at pH 11</td>
<td>[15]</td>
</tr>
<tr>
<td>Glutamine synthetase (Pseudomonas taetrolens Y-30)</td>
<td></td>
<td>11 (ethylamine and Mg²⁺)</td>
<td>Stable at pH 6.0</td>
<td></td>
<td>[34]</td>
</tr>
<tr>
<td>γ-Glutamyl-methylamide synthetase (Methylovorus mays No. 5)</td>
<td></td>
<td>7.5–8.0 (ethylamine and Mg²⁺)</td>
<td>Stable at pH 6.0</td>
<td></td>
<td>[36]</td>
</tr>
</tbody>
</table>

95.42 μmol/min/mg using Gln. Interestingly, the $K_m$ and $V_{\text{max}}$ values measured using Gln as the donor were larger than those reported for the enzyme isolated directly from *B. subtilis* 168 (102 μM and 7.01 μmol/min/mg) [30]. This difference may be caused by differences in the methods used for the determinations. In addition, the $K_m$ values for l-γ-glutamyl-p-nitroanilide and Gln were similar to those of the GGT of unknown sequence isolated from *B. subtilis* SK11.004 [33], which were 1.74 mM for l-γ-glutamyl-p-nitroanilide using 20 mM glycylglycine acceptor and 0.83 mM for Gln using 50 mM ethylamine acceptor, respectively.

3.6. Optimization of reaction conditions for l-theanine production

3.6.1. Effect of pH on l-theanine production

The effect of reaction pH on l-theanine production was investigated first. Because the enzyme activity was substantially inhibited under acidic conditions, the optimum pH for l-theanine biotransformation was measured between pH 8 and 12. The reaction conditions for this experiment were: 200 mM Gln, 1.4M ethylamine, 2 U/mL enzyme, 37 °C, 150 rpm and 5 h. As shown in Fig. 5, the optimal pH for l-theanine production was pH 10, resulting in a 71% yield (Gln to l-theanine). This result was appreciably better than the 60% yield previously reported for GGT from *E. coli* K-12 under similar reaction conditions [24]. As the protein homology between sequences from *E. coli* K-12 and *B. subtilis* 168 is very low as 37%, this big difference may lead to different structures and functions of the two enzymes, which then determine their different performances on theanine-forming reaction. At pH 9 and 11, the yield declined to 40% and 31%, respectively. No l-theanine was formed at pH 8 or 12. These results suggest that GGT requires a relatively narrow range of alkaline pH to efficiently produce l-theanine. Lower pHs, such as pH 8 and 9, enhance the extent of the hydrolytic reaction, forming Glu as a main byproduct, while extreme...
alkaline pHs, like pH 12, limit the enzyme activity. In addition, only 70–80% of the total material can be detected at the conclusion of the reaction, indicating other by-products are produced. γ-Glu-Gln, for example, could be produced through an auto-transpeptidation reaction.

3.6.2. Effect of the amount of Gln to ethylamine on l-theanine production

To test the effect of varying the mole ratio of Gln to ethylamine, we tested ratios between 1:1 and 1:15. As shown in Fig. 6, when the ratio increased from 1:1 to 1:11, the yield of l-theanine exhibited a corresponding increase from 30% to 78%. The yield decreased appreciably when the mole ratio was increased to 1:13 or 1:15. These results demonstrate that the optimum yield (74–78%) was achieved when the Gln to ethylamine ratio was between 1:9 and 1:11. In addition, when the ratio exceeded 1:5, there was a minimal increase in the yield caused by the additional ethylamine. On the contrary, higher concentrations of ethylamine (above 1:11) obviously inhibited l-theanine formation. This inhibitory effect of high acceptor concentrations has been described in other reports [24,25]. We used the optimized mole ratio 1:10 when conducting subsequent studies.

3.6.3. Effect of temperature on l-theanine production

The temperature dependence of l-theanine production was studied using temperatures of 30 °C, 37 °C, 50 °C and 60 °C, pH 10, and a 1:10 ratio of Gln to ethylamine. A slightly larger amount of enzyme (3 U/mL) was added to reduce the time required to reach the final conversion. As shown in Fig. 7, the reaction reached its final conversion at the 2 h time point. The yields at 30 °C, 37 °C and 50 °C remained essentially constant between 2 and 5 h, while the yield at 60 °C decreased continuously after 2 h. These results showed that temperatures between 30 °C and 50 °C had little effect on the final yield, and the amount of l-theanine remained roughly stable during the reaction process. However, at 60 °C the process produced a lower yield, perhaps due to poor enzyme stability, but perhaps also due to an increasing rate of product decomposition. Subsequent experiments were performed at 37 °C to maintain consistency with other reports [24,25].

3.6.4. Effect of enzyme dosage on l-theanine production

According to previous studies (data not shown), increasing the enzyme dosage reduced the time required to reach the maximum yield but had little effect on enhancing the maximum l-theanine yield when analyzed as a single variable. Adding too much enzyme with respect to the concentration of certain substrates will increase the rate of by-product synthesis, sharply decreasing the yield of l-theanine with prolonged reaction time and making it difficult

Table 3

Comparison of l-theanine production at various concentrations of Gln.

<table>
<thead>
<tr>
<th>Gln (mM)</th>
<th>Ethylamine (M)</th>
<th>Enzyme dosage (U/mL)</th>
<th>Reaction time (h)</th>
<th>L-Theanine yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.5</td>
<td>0.2</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>200</td>
<td>2.2</td>
<td>2.0</td>
<td>5</td>
<td>78</td>
</tr>
<tr>
<td>500</td>
<td>3.0</td>
<td>4.0</td>
<td>5</td>
<td>58</td>
</tr>
<tr>
<td>1000</td>
<td>5.0</td>
<td>9.0</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Strain for enzyme source</td>
<td>Substrates</td>
<td>Yields (%) based on Gln</td>
<td>Reaction time</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------</td>
<td>------------</td>
<td>-------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>GGT</td>
<td>Escherichia coli K-12</td>
<td>0.2</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Bacillus subtilis SK11.004</td>
<td>0.02</td>
<td>94</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Bacillus subtilis 168</td>
<td>0.02</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas nitroreducens IFO 12694</td>
<td>0.2</td>
<td>73</td>
<td>5</td>
</tr>
<tr>
<td>Glutaminase</td>
<td>Pseudomonas citronellosis GEA</td>
<td>0.3</td>
<td>71</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>One or more of Bacillus, mold and yeast*</td>
<td>0.5</td>
<td>47</td>
<td>7</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>Pseudomonas taetrolens Y-30</td>
<td>0.2 (sodium glutamate)</td>
<td>85 (based on sodium glutamate)</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Methylovorus mays No. 9</td>
<td>0.6 (sodium glutamate)</td>
<td>100 (based on sodium glutamate)</td>
<td>48</td>
</tr>
</tbody>
</table>

* Bacillus subtilis (75%), Bacillus amyloliquefaciens (74%), Bacillus coagulans (70%), Aspergillus oryzae (72%), Aspergillus niger (73%), Saccharomyces cerevisiae (75%), Saccharomyces rouxii (70%), Candida utilis (71%) and Candida Antarctica (72%).
Fig. 8. Identification of the reaction product by UPLC-Q-TOF MS. (A) The UPLC profile and Q-TOF MS spectrum of the L-theanine standard. (B) The UPLC profile and Q-TOF MS spectrum of the compound in the reaction mixture.
to control the reaction process. Therefore, in the following study of substrate concentration, the enzyme dosage at each substrate concentration was roughly controlled to ensure maximal product yields and a stable reaction process within the specified reaction time.

3.6.5. Effect of substrate concentration on l-theanine production

In our hands, the recombinant GGT from B. subtilis consistently exhibited a better yield (78%), with the same substrate concentration, when compared with a previously reported bacterial GGT (60%) (Table 4) [24]. To get further information about the ability of GGT to produce l-theanine, enzymatic reactions were performed using different concentrations of Gln.

As shown in Table 3, the yields of l-theanine declined as the concentration of Gln increased, dropping from 90% at 20 mM Gln to 45% at 1 M Gln. As the concentration of Gln increased, the ratio of ethylamine to Gln decreased from 25 to 5 because the concentration of ethylamine approached its solubility limit. However, it was also found that at higher concentrations of Gln (i.e. 500 mM or 1 M), increasing the concentration of ethylamine had little effect on product formation.

In comparison with other enzymes used in l-theanine production, as shown in Table 4, Glutaminases from P. nitroreducens [15–17] have also been used to produce l-theanine with good results. For instance, using the enzyme from P. nitroreducens with 1.5 M ethylamine led to 90% yield at 100 mM Gln, a 73% yield at 200 mM Gln, a 71% yield at 300 mM Gln, a 47% yield at 500 mM Gln and a 39% yield at 700 mM Gln, within 3–7 h. Although yields were not ideal when using higher substrate concentrations, increasing the ethylamine concentration may raise the yields further. In addition, enzymes from some Bacillus and Aspergillus species [35] exhibited even better results, with yields of 72–75% achieved using 500 mM Gln and various concentrations of ethylamine, but the properties of these enzymes have not been studied.

3.6.6. Identification of l-theanine by UPLC-Q-TOF MS

The elution profiles of ultra performance liquid chromatography (UPLC) together with the identification of the reaction product by a quadrupole time-of-flight (Q-TOF) mass spectrometry were shown in Fig. 8. The UPLC retention time of the compound in the reaction mixture (Fig. 8B) was consistent with that of the l-theanine standard (Fig. 8A). In Q-TOF mass spectra, a strong signal with an m/z value of 175, which corresponds to the ionized theanine standard in positive ion mode, was observed in both figures. The loss of ammonia from m/z 175 yields m/z 158. The subsequent loss of an ethylamine group may lead to the formation of m/z 129 [25]. This verifies that the compound in the product was exactly the same as the standard, so the product obtained through enzymatic reaction were undoubtedly l-theanine.

In conclusion, the GGT from B. subtilis 168, which has desirable enzyme activity and stability under typical process conditions, gives a satisfactory yield of l-theanine even when starting with high substrate concentrations. The approach described in this study indicates a great potential for industrial-scale applications.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (31100048) and the 111 Project (111-2-06).

References


