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Production of Bioactive γ-Glutamyl Transpeptidase in *Escherichia coli* Using SUMO Fusion Partner and Application of the Recombinant Enzyme to L-Theanine Synthesis

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Abstract The amino acid L-theanine (γ-glutamylethylamide) has potential important applications in the food and pharmaceutical industries and increased demand for this compound is expected. It 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 L-theanine involving recombinant *Escherichia coli* γ-glutamyltranspeptidase (GGT) has been developed. We report here the application of small ubiquitin-related modifier (SUMO) fusion technology to the expression and purification of recombinant *Escherichia coli* γ-GGT. In order to obtain γ-GGT with high theanine-forming activity, safety, and low cost for food and pharmaceutics industry, M9 (consisting of glycerol and inorganic salts) and 0.1% (w/v) lactose were selected as culture medium and inducer, respectively. The fusion protein was expressed in soluble form in *E. coli*, and expression was verified by SDS-PAGE and western blot analysis. The fusion protein was purified to 90% purity by nickel–nitrilotriacetic acid (Ni–NTA) resin chromatography with a yield of 115 mg per liter fermentation culture. After the SUMO/γ-GGT fusion protein was cleaved by the SUMO protease, the cleaved sample was reapplied to a Ni–NTA column. Finally, about 62 mg recombinant γ-GGT was obtained from 1 l fermentation culture with no less than 95% purity. The recombinant γ-GGT showed great transpeptidase activity, with 1500 U of purified recombinant γ-GGT in a 1-l reaction system, a biosynthesis yield of 41 g of L-theanine was detected by paper chromatography or high pressure liquid chromatography (HPLC). Thus, the application of SUMO technology to the expression and purification of γ-GGT potentially could be employed for the industrial production of L-theanine.

Abbreviations

γ-GGT	γ-Glutamyltranspeptidase
SUMO	Small ubiquitin-related modifier
Gst	Glutathione S-transferase
Trx	Thioredoxin
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
Ni–NTA	Nickel–nitrilotriacetic acid
PVDF	Polyvinylidene difluoride
LB	Luria–Bertani
PTC-THEA	Phenylthiocarbamyl theanine
PITC	Phenyl isothiocyanate
RP-HPLC	Reverse-phase high pressure liquid chromatography

Introduction

L-Theanine (γ-glutamylethylamide), which was first identified by Sakato [8] 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 [4]. L-Theanine has several physiological functions. For example, it has been demonstrated that it has positive effects on immune system responsiveness to infection, blood-pressure reduction, relaxation, neuroprotection, and modulation of

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chemotherapy. It has been approved in Japan since 1964 for use in foods (except infant foods) [3].

To date, L-theanine has been used extensively in pharmaceuticals and functional foods and several investigators have studied its effective production. γ-Glutamyltranspeptidase (GGT, EC 2.3.2.2), which is widely distributed in living organisms, catalyzes not only the hydrolysis of the γ-glutamyl linkages of γ-glutamyl compounds, but also the transfer of their γ-glutamyl moieties to other amino acids or peptides. Some researchers also developed some new glutamine synthetase and γ-glutamyl transpeptidase as the biocatalyst to produce L-theanine, but the shortcomings are still obvious such as formation of byproducts and low activity of the enzyme in a neutral pH range where the yeast sugar fermentation system works [16, 17]. Therefore, the study about how to obtain γ-GGT enzyme with high theanine-forming activity and low cost is very significant.

Several major protein fusion technologies have been developed to improve expression and purification of heterologous recombinant proteins in bacterial cells. However, the effects of the affinity tag on different target proteins differ and no affinity tag appears to be generally superior for all recombinant proteins. Earlier attempts to express recombinant γ-GGT for L-theanine production in our laboratory using Trx [15] or Gst fusion technology [14] have some disadvantages such as low theanine-forming activity and high cost. Small ubiquitin-related modifier (SUMO) is an ubiquitin-related protein that functions by covalent attachment to other proteins. It is known that SUMO, fused at the N-terminus with other proteins, can fold and protect the protein by its chaperoning properties, making it a useful tag for heterologous expression. These advantages include the manner in which protein expression is enhanced, proteolytic degradation of the target protein is decreased, protein folding and solubility are increased, and purification and detection are simplified [2, 10]. Consequently, we subcloned the γ-GGT into pSUMO vector for production.

We have developed a novel SUMO fusion system that provides increased levels of soluble expression proteins in *E. coli* and allows rapid purification of proteins of interest. Here we provide the first report of the procedures for obtaining a recombinant *E. coli* γ-GGT with high theanine-forming activity by using SUMO fusion technology.

Materials and Methods

Bacterial Strains, Vectors, and Enzymes

Escherichia coli DH5α (maintained in our laboratory) was used for subcloning and plasmid amplification. *Escherichia coli* BL21 (DE3) (Novagen, USA) was used as the expression host. The linearized pSUMO vector with Bsa I and Xho I restriction sites and T7 promoter and kanamycin resistance and 6× His sequence was purchased from LifeSensors (LifeSensors, Malvern, PA, USA). SUMO protease containing a histidine-tag was also the product of Lifesensors (Malvern, PA, USA). All the restriction enzymes and T4 DNA ligase were purchased from Takara Biotech Co. Ltd (Dalian, China).

Construction of Expression Vectors

The *E. coli* γ-GGT gene of interest was amplified from plasmid pET32a/γ-GGT which was constructed previously in Yin's laboratory [15] according to sequences reported in EMBL data bank under accession NO. M28722. The PCR fragments were separated by 1.0% gel electrophoresis, purified with a DNA gel extraction kit (Takara, China). The resulting PCR product was digested with Bsa I and Xho I, and ligated into the pSUMO plasmid at the corresponding restriction sites [9]. The ligation mixture was transformed into *E. coli* DH5α cells for verification by sequencing (Shanghai Invitrogen Bio. Co. Ltd).

Expression and Characterization of SUMO Fusion Protein

The pSUMO/γ-GGT plasmid that had been constructed was transformed into competent *E. coli* BL21 (DE3). Three colonies were picked and cultured in 4 ml sterilized Luria–Bertani (LB) medium with vigorous shaking (220 rpm) at 37°C to an optical density (A_{600}) of 0.6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was then added to induce the expression of the recombinant protein at 24°C for 8 h. M9 medium (1% glycerol, 1% NH_4Cl, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 0.5% NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) at pH 7.0 and lactose (1 g/l) were also selected to optimize the recombinant γ-GGT expression and enzyme activity.

SDS-PAGE and Western Blot

SDS-PAGE analysis was performed according to Laemmli [6] using 12% polyacrylamide gels. The total expression protein samples from cell lysates after induction were mixed with equivalent sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 10% 2-mercaptoethanol). Gels were stained with Coomassie brilliant blue R-250.

For western blotting, the same protein sample was separated on a 12% polyacrylamide gel under reducing conditions and then transferred to a polyvinylidene difluoride

(PVDF) membrane (Roche Applied Science). The immunoblotting was performed as described [12]. The IRdye 800 conjugated mouse IgG secondary antibody was used against His-tag primary antibody (Novagen). The proteins were visualized using the Odyssey infrared imaging system (LI-COR).

Purification of SUMO Fusion Protein

BL21(DE3)-pSUMO/γ-GGT strains were cultured in 200 ml sterilized LB medium with vigorous shaking (220 rpm) at 37°C to an optical density (A_{600}) of 0.6. Lactose (1 g/l) was then added to induce the expression of the recombinant protein at 24°C for 8 h. Cultures were collected by centrifugation at 12,000×*g*, at 4°C for 10 min and the cell pellet frozen at −80°C. The pellet from 200 ml culture was then resuspended in 20 ml binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, and 10 mM phenylmethylsulfonyl fluoride, pH 8.0) and lysed on ice by sonication at 400 W for 100 cycles (4 s working, 8 s free). The supernatant of the cell lysate resulting from centrifugation at 12,000×*g* at 4°C for 20 min was applied to a nickel–nitrilotriacetic acid (Ni^{2+}–NTA, Novagen) affinity chromatography column according to the manufacturer's instruction. After extensive washing with binding buffer (10 column volumes), the fusion protein was eluted with five column volumes of elution buffer (20 mM Tris, 500 mM NaCl, and 250 mM imidazole, pH 8.0). The peak fractions with high-UV values at A_{280}, which were detected by LPDataView (BIORAD), and containing the fusion protein were pooled and dialyzed overnight at 4°C against phosphate buffered saline (PBS, pH 8.0).

Cleavage of SUMO Fusion Protein and Purification of γ-GGT

The dialyzed fusion protein was reacted with 1 U SUMO protease per 50 μg fusion protein at 30°C for 1 h. Since both SUMO and SUMO protease had 6× His tags, but γ-GGT did not, the cleaved SUMO fusion samples could be re-applied to the nickel column to obtain the purified γ-GGT by subtracting the 6× His-tagged proteins. Briefly, after the SUMO fusions were cleaved by the SUMO protease, the sample was loaded onto a nickel column with Ni–NTA resin. Most of the γ-GGT without 6× His tags was eluted (five column volumes) in the flow-through (unbound) fractions, and the rest was recovered by washing the resin with binding buffer (10 column volumes). The eluted and washed proteins appearing in fractions with high-UV values at A_{280} were pooled as the final purified sample. The purified proteins were checked on SDS-PAGE and the samples were stored at −80°C for activity assay.

Assay of Transpeptidase Activity of γ-GGT and Synthesis of L-Theanine Products

The γ-GGT activities of crude and purified recombinant γ-GGT were determined spectrophotometrically at 530 nm, 37°C following the instruction of γ-GGT kit from Jiancheng Bioengineering Institute of Nanjing, China (glutamyl-α-naphthylamine as substrate; 1 U = 1 μmol α-naphthylamine 15 min^{-1}; U = Unit).

The 1 l reaction system (267 mM glutamine, 2 M ethylamine, pH 10.5 at 37°C with the shaking speed of 150 rpm for 24 h, 1500 U crude γ-GGT or purified γ-GGT) was used to test L-theanine production at 37°C.

Paper Chromatography and HPLC Assay of L-Theanine

Paper chromatography assay was performed according to normal procedure [11]. Enzyme reaction mixture (containing glutamine and L-theanine) yielded good chromatograms for qualitative or quantitative assay after placing the sample on paper.

High pressure liquid chromatography (HPLC, Agilent 1100 series, USA) equipped with a Pico-TagTM Amino Acid column (300 × 3.9 mm, 5 μm, Waters, USA) was used for determining the L-theanine synthesized by recombinant γ-GGT. The separation of L-theanine from the other free amino acids of reaction mixture was achieved on a commonly used reverse phase column using gradient elution and diode array ultraviolet detection. The phenyl isothiocyanate (PITC) precolumn derivatization procedure and reverse-phase high pressure liquid chromatography (RP-HPLC) reported by Bindlingmeyer et al. [1] was also used to quantitate L-theanine. The samples from reaction mixture were treated according to the procedure by Agilent. The phenylthiocarbamyl theanine (PTC-THEA) derivatized product was detected by a Diode Array Detector (Agilent G1362A series, USA) as the absorbance at 254 nm.

Results

Plasmid Construction and the Expression of SUMO Fusion Protein

The construct for γ-GGT expression, containing a His-tag for affinity purification, is depicted in Fig. 1. Recombinant plasmid pSUMO/γ-GGT sequence was verified by DNA sequencing (Shanghai Invitrogen Bio. Co. Ltd). The correct construct was transformed into the expression host *E. coli* BL21 (DE3), and subjected to a pilot expression test. As shown in Fig. 2, there was an obvious protein band after IPTG induction that could be detected by anti-His_6

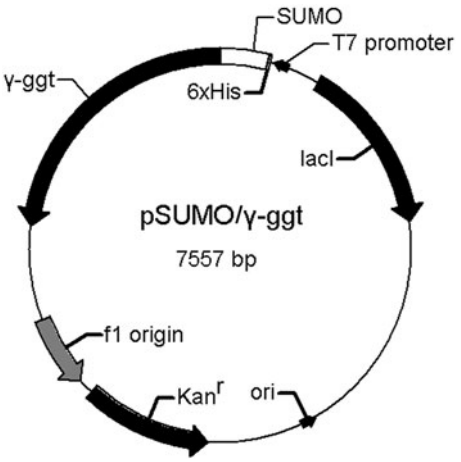

Fig. 1 Schematic representation of the expression vector pSUMO/γ-GGT. γ-GGT was expressed as a fusion protein with the SUMO

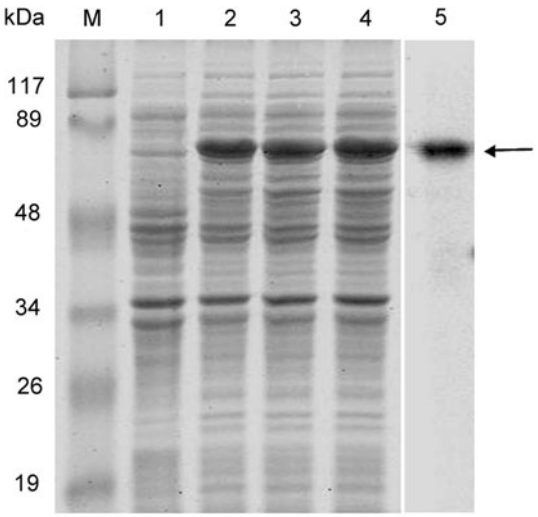

Fig. 2 Analysis of expressed fusion protein by SDS-PAGE. *Lane M*: molecular mass marker; *Lane 1*: negative control; *Lanes 2, 3, 4*: colony 1, 2, 3 induced by 0.5 mM IPTG; *Lane 5*: western blot of total protein from colony. The *arrow* indicated the location of the recombinant fusion protein

antibody according to western blot. The apparent molecular weight of the SUMO fusion protein was about 80 kDa (*E. coli* γ-GGT native enzyme has subunits of 39.2 and 22 kDa, which encoded a protein of 250 amino acids; the theoretical molecular weight of SUMO is about 10 kDa, but it runs as an 18–20 kDa band with SDS-PAGE [7]). The fusion protein of colony 1 was produced more efficiently in a soluble form induced by 0.5 mM IPTG at 24°C, and it was chosen for the purification experiment soon after. Considering the high cost of LB and the toxicity of IPTG, finally M9 (consisting of glycerol and inorganic salts) and lactose were chosen as the culture medium and inducer according to the results of SDS-PAGE expression assay and enzyme activity test of recombinant γ-GGT. The expression yield (65% of total cellular protein, estimated by SDS gel scanning using Scion Image Analysis Software analysis) of recombinant γ-GGT strain in M9 induced by lactose were almost the same as those in LB, but the crude γ-GGT activity were obviously higher than those cultured in LB and induced by IPTG (data not shown).

Purification of SUMO/γ-GGT Fusion Protein

As described above, Ni–NTA resin was used for fusion protein purification. Most of the proteins without 6× His tags were removed from the Ni–NTA resin using washing buffer containing 20 mM imidazole, and the 6× His-tagged SUMO/γ-GGT was eluted with more than 90% purity using elution buffer containing 250 mM imidazole (Fig. 3). The purity was estimated by SDS gels stained by Coomassie Blue. About 115 mg fusion protein determined using a Bradford assay can be obtained per liter of fermentation culture.

Purification of γ-GGT

The SUMO/γ-GGT protein (50 μg) was competently cleaved after incubation with SUMO protease (1 U) at 30°C for 1 h, as confirmed by checking the proteins on a 12% gel in the presence of SDS. After the cleaved sample was re-applied to a Ni–NTA column to remove His_6-tagged SUMO and SUMO protease, finally purified γ-GGT was obtained, and 12% gel results indicated that γ-GGT had been purified successfully to more than 95% purity (Fig. 3). Table 1 shows the comparison of the SUMO fusion system with Gst or Trx fusion systems in production of recombinant γ-GGT. The purified γ-GGT was filtered through a 0.22 μm filter membrane and stored at −80°C for activity assay. Finally, the purified recombinant γ-GGT was produced at a yield of 62 mg/l culture.

Transpeptidase Activity and Synthesis of L-Theanine Products

The specific transpeptidase reaction activities of the crude His_6–SUMO–γ-GGT, purified γ-GGT are 39, 51.41 U/ml. The data showed that the affinity purification could be used to obtain good yield of active enzyme and addition of a N-terminal hexahistidine and SUMO tag did not significantly affect the activity of the enzyme.

To investigate whether future industrial production of L-theanine was feasible using the recombinant γ-GGT, the 1 l reaction system (details are described in the legend) was tested. Ethylamine was added every 2 h. L-Theanine content was about 41 g/l after 24 h reaction, and the conversion of glutamine was about 80% (Fig. 4). As shown in Fig. 5, the purity of L-theanine crystal products from

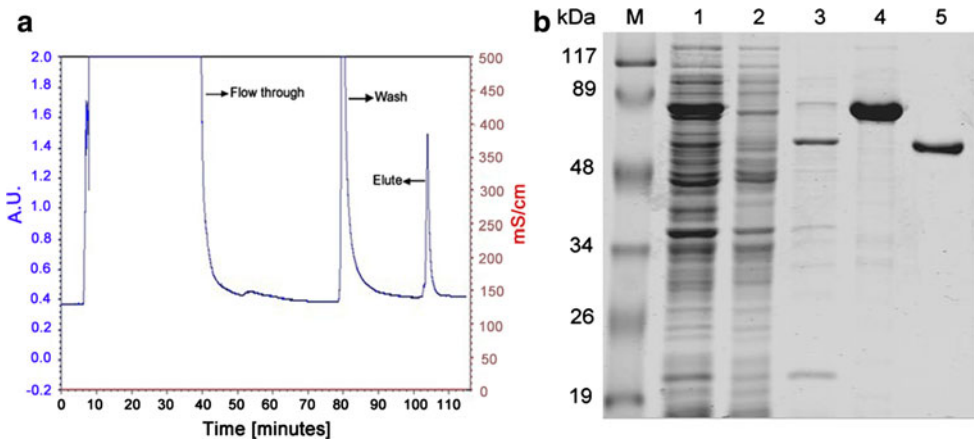

Fig. 3 Purification of recombinant soluble γ-GGT protein. **a** Ni–NTA affinity chromatography of fusion protein by using LPDataView (BIORAD). **b** SDS-PAGE analysis of lactose-induced expression and purification of recombinant soluble γ-GGT. *Lane M*: protein molecular weight marker; *Lane 1*: supernatant of cell lysate; *Lane 2*: flow-through; *Lane 3*: wash; *Lane 4*: elution; *Lane 5*: recombinant γ-GGT following purification of the SUMO protease cleavage reaction mixture and Ni–NTA resin chromatography

Table 1 Comparison of the SUMO fusion partner with Trx or Gst fusion partners

Fusion protein	Time cost (days)	Molecular weight of fusion tag (kDa)	Yield (mg/l)	Purified enzyme activity[a] (U/ml)
Trx/γ-GGT	4–5	18	1.2–12	4.7 ± 0.4
Gst/γ-GGT	3	25	28.2	33.05 ± 1.2
Sumo/γ-GGT	2	10	62	51.41 ± 2

[a] Purified enzyme activity was determined spectrophotometrically at 530 nm, 37°C following the instruction of γ-GGT kit (glutamyl-α-naphthylamine as substrate; 1 U = 1 μmol α-naphthylamine 15 min^{-1}; U = Unit). Experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD)

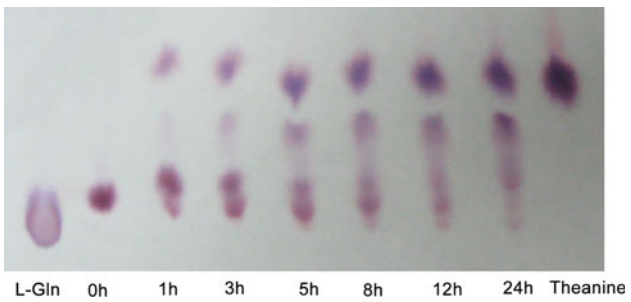

Fig. 4 Paper chromatography analysis of L-theanine synthesis by crude γ-GGT from M9. The reaction system (1 l) containing 267 mM glutamine, 2 M ethylamine, pH 10.5, 1500 U purified γ-GGT from BL21-pSUMO/γ-GGT grown in M9 was carried out at 37°C, with shaking (150 rpm) for 24 h. 0, 1, 3, 5, 8, 12, 24 h, reaction time lasted 0–24 h, respectively; L-Gln (glutamine), 0.5 M; L-theanine, 0.4 M

enzyme-catalyzed synthesis determined by HPLC reached about 98% finally, which almost matched the standard samples of L-theanine (Sigma, USA). This result demonstrated that the recombinant γ-GGT was applicable for large scale L-theanine production.

Discussion

Expressing large recombinant enzyme proteins, especially proteins with disulfide bridges, is quite an arduous task because they often fold incorrectly and aggregate, leading to either rapid degradation or to the accumulation of inclusion bodies when they are expressed in *E. coli*. Fortunately, these problems are somewhat alleviated when the desired protein is expressed with a fusion partner. SUMO is superior to commonly used fusion tags in enhancing expression and solubility with the distinction of generating recombinant protein with native sequences [2, 13]. We hypothesized that the attachment of a highly stable and compact SUMO structure to the N-terminus of the γ-GGT would facilitate correct protein folding and enhance solubility and expression.

At first, expression of the recombinant *E. coli* γ-GGT was successfully achieved using Trx or Gst fusion technology in our laboratory. However, the disadvantages of using these two technologies are that the enzyme protein was expressed mostly in the form of inclusion bodies and had lower theanine-forming activity, the production yield

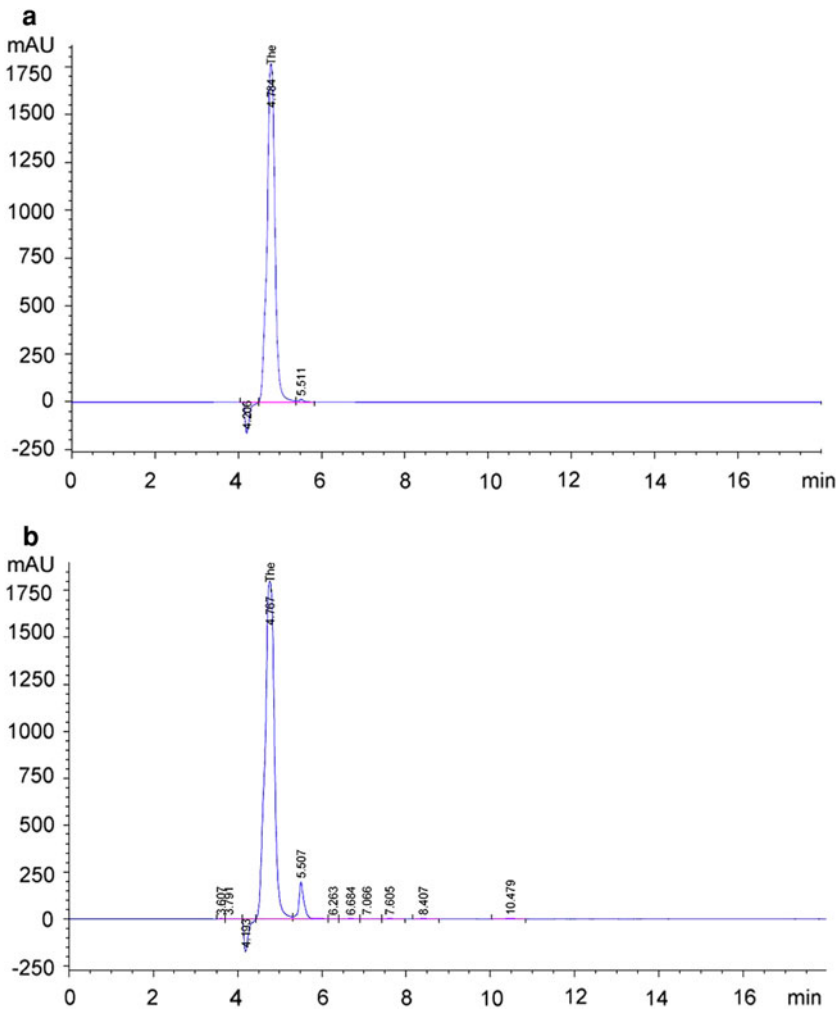

Fig. 5 The HPLC analysis of the standards and crystal samples of L-theanine. **a** Standards of L-theanine; **b** Crystal samples of L-theanine

of L-theanine by bioconversion with recombinant γ-GGT was 26.9 g/l, the rate of conversion from L-Gln to L-theanine was only 57.8% [5]. The control of pH and temperature was important to the activity of the protein, therefore, avoiding γ-GGT deactivation in vitro and retaining bioactivities were very important. So having fewer steps or days in the purification minimizes the possibility of deactivation. This method has these major advantages over the Gst or Trx fusion systems developed earlier as shown in Table 1.

In this study, we expressed γ-GGT as SUMO fusions in *E. coli* to evaluate its role in the production of L-theanine. The SUMO fusion protein was successfully expressed in *E. coli.* Because of the toxicity and the high cost of IPTG, lactose is a promising inducer in the lac-promotor based expression system. We selected economic M9 medium and lactose to induce the recombinant γ-GGT expression, so that high expression levels of soluble fusion protein and high activity of the crude enzyme or purified enzyme were achieved. The SUMO/γ-GGT fusion protein can be completely cleaved by SUMO protease as shown in Fig. 3. γ-GGT, which is about 62 kDa, was recovered with 95% purity by purification using nickel affinity chromatography again, and a final yield of 62 mg γ-GGT was obtained per liter of fermentation. The recombinant γ-GGT showed great transpeptidase activity, the yield of L-theanine was 41 g/l with the purity of about 98% detected by paper chromatography or HPLC in 1 l reaction system. Thus, the application of SUMO technology to the expression and purification of γ-GGT is potentially feasible for the industrial production of L-theanine.

In summary, the SUMO fusion system and customized expression and purification protocol described here have further improved the efficiency and lowered the costs of L-theanine production from enzyme-catalyzed synthesis. The SUMO fusion technology could also be widely applied to the industrial production of a variety of enzyme proteins in *E. coli.*

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