Overexpression of a recombinant $\gamma$-glutamyltranspeptidase from 
*Escherichia coli* Novablue

Ya-Feng Yao¹, Yih-Ming Weng¹, Hui-Yu Hu² and Long-Liu Lin³*

¹Graduate Institute of Food Science, National Chiayi University, 300 University Road, 60083 Chiayi, Taiwan
²Department of Food and Nutrition, Hungkuang University, Taichung 433, Taiwan
³Department of Applied Chemistry, National Chiayi University, 300 University Road, 60083 Chiayi, Taiwan

Received 23 May 2006; revised 03 October 2006

A truncated *Escherichia coli* Novablue $\gamma$-glutamyltranspeptidase (EcGGT) gene, lacking the first 48-bp coding sequence for part of the signal sequence, was amplified by polymerase chain reaction (PCR) and cloned into expression vector pQE-30 to generate pQE-EcGGT. The maximum production of His6-tagged enzyme by *E. coli* M15 (pQE-EcGGT) was achieved with 0.1 mM IPTG induction for 12 h at 20°C. The overexpressed enzyme was purified to homogeneity by nickel-chelate chromatography to a specific transpeptidase activity of 4.25 U/mg protein and a final yield of 83%. The molecular masses of the subunits of the purified enzyme were determined to be 41 and 21 kDa respectively by SDS-PAGE, indicating the precursor EcGGT still undergoes the post-translational processing even in the truncation of signal sequence. His6-tagged EcGGT migrated relative to the molecular mass of approximately 120 kDa and its heterodimeric structure was confirmed by a native-PAGE gel.

**Keywords:** *Escherichia coli*, $\gamma$-Glutamyltranspeptidase, Signal sequence, Overexpression, Nickel-chelate chromatography

$\gamma$-Glutamyltranspeptidase (GGT; EC 2.3.2.2) catalyzes the cleavage of the $\gamma$-glutamyl bond of glutathione and other related $\gamma$-glutamyl compounds. It is widely distributed in living organisms and plays an important role in the regulation of the intracellular levels of glutathione. The open-reading-frame (ORF) of *Escherichia coli* GGT (EcGGT) codes a polypeptide precursor with N-terminal signal peptide of 25 amino-acid residues, the large subunit of 365 residues, and the small subunit of 190 residues. Besides the cleavage by signal peptidase I, the precursor EcGGT precedes the post-translational cleavage between Gln-390 and Thr-391 to yield a heterodimer. The enzyme utilizes many amino acids and peptides as $\gamma$-glutamyl acceptors and inexpensive L-glutamine as a good $\gamma$-glutamyl donor.

The synthesized $\gamma$-glutamyl-L-DOPA has been shown to be a possible pro-drug for Parkinson’s disease. Additionally, an EcGGT-mediated method has also been developed for synthesizing $\gamma$-glutamyl amino acids, such as $\gamma$-Glut-Phe and $\gamma$-Glut-Trp.

To obtain large amount of GGT for industrial utilization, the entire *E. coli* K-12 ggt gene was cloned into pUC18 and the recombinant plasmid was transformed into GGT-deficient mutants for protein expression. The specific GGT activity in the recombinant *E. coli* cells was 37-fold higher than in the wild-type cells and the expressed enzyme could be isolated from the periplasmic fraction of the recombinant cells by two-steps purification. A high-level expression system for *E. coli* HB101 ggt gene was also constructed; however, the separately purified subunits were needed to reconstitute GGT activity.

In this study, we attempted the removal of 5’ sequence region encoding the signal sequence of *E. coli* NovaBlue ggt gene and cloned the truncated gene into pQE-30 for the enzyme overexpression.

**Materials and Methods**

**Bacterial strains, plasmids, and culture conditions**

The *E. coli* NovaBlue (Novagen, Inc., Madison, WI) and *E. coli* M15 (Qiagen Inc., Valencia, CA)
strains were used in the study. Plasmids used were pGEM®-T Easy (Promega Co., Madison, WI) and pQE-30 (Qiagen). Bacterial strains were cultivated under conditions recommended by the supplier. Solid medium was prepared by the addition of bacteriological agar to a final concentration of 1.5% (w/v), and when required, ampicillin (100 μg/ml) and kanamycin (25 μg/ml) were also added.

Molecular techniques

Restriction enzymes and T4 DNA ligase were used according to the manufacturer’s instructions. Standard protocols were used for manipulation of DNA and transformation of E. coli strains. Chromosomal DNA of E. coli NovaBlue was isolated with a genomic DNA extraction kit according to the manufacturer’s instructions (Protech Technology, Taipei, Taiwan). Oligonucleotide primers were synthesized by Mission Biotechnology (Taipei, Taiwan). DNA sequencing was done by the chain-termination method with an automated 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Cloning, expression and purification of His<sub>6</sub>-tagged EcGGT

The putative ggt gene of E. coli NovaBlue was obtained by PCR (Thermocycler PE 2400, Perkin-Elmer, Boston, MA) using the genomic DNA as a template. The PCR mixture contained (total volume 50 μl) 10 ng of genomic DNA, 10 pmol of primer Ecggt-f (5′-GAGCTCTGAGGATTTTAGC-3′; a SacI site is underlined), 10 pmol of Ecggt-r (5′-GGTACCATAGTACCCCCCGTTAA-3′; a KpnI site is underlined), 1 × PCR buffer, each deoxynucleotide triphosphate at a concentration of 1 mM, and 2.5 U of Taq DNA polymerase (Promega). After an initial denaturation at 94°C, gene fragments were amplified by 30 cycles consisting of 2 min of denaturation at 94°C, 1.5 min of annealing at 55°C, and 2 min of extension at 74°C, followed by a final extension at 74°C for 10 min. The PCR product was then cloned into the pGEM®-T Easy vector and transformed into E. coli NovaBlue cells. Transformants containing the recombinant vector were selected on LB medium, supplemented with ampicillin and 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plasmid DNA with the insert was digested with SacI and KpnI and inserted into the corresponding sites of the E. coli expression vector pQE-30 to create pQE-EcGGT.

For high-level expression of the recombinant enzyme, E. coli M15 cultures harboring pQE-EcGGT were incubated in 100 ml of LB medium, supplemented with ampicillin and kanamycin at 37°C until the optical density (OD) at 600 nm of the culture was 1.0. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The cultivation continued at 20°C for 12 h. Cells were harvested by centrifugation (4,000 × g for 10 min at 4°C) and the cell pellets were stored at 20°C until required.

To purify His<sub>6</sub>-tagged EcGGT, cell pellets were resuspended in the binding buffer (5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl; pH 7.9) and lysed by sonication (30 s bursts for 5 min). The cell extract was obtained by centrifugation, and the soluble His<sub>6</sub>-tagged EcGGT was bound to 2 ml of Ni<sup>2+</sup>-NTA resin by gentle mixing at 4°C for 30 min. Subsequently, the resin was loaded on to a column and washed with 3 vols of 50 mM phosphate buffer (pH 7.9) containing 0.3 M NaCl and 20 mM imidazole, and the bound protein was eluted with 5 ml of 250 mM imidazole added to the washing buffer.

Protein methods

Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical mini-gel system (Mini-Protean III; Bio-Rad Laboratories, Inc., Hercules, CA). GGT activity in the gel was detected according to the method described previously. Briefly, a 10% non-denaturing polyacrylamide gel was run at 4°C and at a constant voltage of 100 V for 2 h. To detect the transpeptidase activity, the gel was immersed into 1 mM 5-L-glutamyl-2-naphthylamide and 50 mM Gly-Gly in 100 mM Tris-HCl buffer (pH 10), and incubated at 37°C for 30 min. The gel was then transferred into a solution containing 0.05% Fast garnet GBC (2-methyl-4-((2-methylphenyl)azo)-benzenediazonium salt; Sigma-Aldrich catalog no. F8761) in 8% acetic acid until the blue dark GGT band appeared.

Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed using the Laemmli buffer system. Prior to electrophoresis, the samples were heated at 100°C for 5 min in dissociating buffer containing 2% SDS and
5% 2-mercaptoethanol. Protein markers used were phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa).

Protein concentration was measured using a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as the standard. For protein sequencing, the purified His-tagged EcGGT was run on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane using a Mini Trans-Blot cell (Bio-Rad Lab). The membrane was then rinsed several times in distilled water and stained with 0.2% Coomassie brilliant blue in 50% methanol for 10 min. The target bands were cut off from the membrane and destained with 50% methanol. After being air-dried, the target bands were wrapped in plastic, and stored at 20°C. The N-terminal sequence was determined by the Edman degradation method using gas phase sequencer (Applied Biosystems 475A).

Size-exclusive chromatography was performed with the procedure as described previously16.

Enzyme activity assay
GGT activity was assayed spectrophotometrically by determining the p-nitroaniline released from L-γ-glutamyl-p-nitroanilide (L-γ-Glu-p-NA) as described elsewhere17, with slight modifications. Unless otherwise indicated, the standard reaction mixture contained 50 mM Tris-HCl buffer (pH 9.0 at room temperature), 1.25 mM L-γ-Glu-p-NA, 30 mM Gly-Gly, 1 mM MgCl2, 20 μl of enzyme solution at a suitable dilution, and enough distilled water to bring the final volume to 1 ml. The reaction mixtures were incubated at 37°C for 10 min. The reaction was stopped by adding 100 μl of 3.5 N acetic acid and the absorbance was measured at 410 nm. One unit of GGT activity was defined as the amount of enzyme that produced 1 μmol of p-nitroaniline per min under the assay conditions.

Results and Discussion
The sequence analysis of the E. coli strain K-12 genome18 allowed the identification of an ORF (b3447) coding for a GGT. The derived amino acid sequence predicted a polypeptide of 580 residues with a calculated molecular mass of 61,768 Da. All the non-redundant databases were screened for entries showing similarity to this ORF with the BLASTP program, available at the site www.ncbi.nlm.nih.gov/Blast/. The gene product deduced from the 1743-bp DNA sequence exhibited high similarity to several GGTs, cephalosporin acylases, and capsule biosynthesis proteins.

For high-level expression of truncated E. coli Novablae ggt gene, the PCR-amplified DNA fragment was digested with SacI and KpnI and inserted into the expression vector pQE-30 to yield pQE-EcGGT. The nucleotide sequence of this construct was confirmed by sequence analysis. The sequencing data from two independent clones revealed one codon difference (GCC→GTC), compared with E. coli K-12 gene sequence4 at position 1307 of the ggt gene, indicating the replacement of Ala-436 with Val. By the above construction, the expressed protein contained 10 additional amino acid residues at its N-terminus, which facilitated one-step purification of the recombinant protein by metal-affinity chromatography. Moreover, we also changed the termination codon (TAA) of the ggt gene into TAT, resulting into the presence of 12 extra amino acid residues at the C-terminus of EcGGT. As the previous constructions for leucine aminopeptidase-amylace fusion proteins16,19, elimination of stop codon from the ggt genes help to introduce the starch-binding domain of Bacillus sp. TS-23 α-amylace into EcGGT. Such construction would offer an inexpensive strategy to purify the fusion protein by adsorption-elution on a starchy column.

E. coli M15 cells transformed with pQE-EcGGT were grown in liquid medium at different temperatures, inducer concentrations and induction times to test their ability as a good source of the recombinant enzyme. After 1, 3, 6, 12 and 24 h induction, 1 ml of bacterial culture was centrifuged and disrupted by sonication, and the crude extracts were analyzed for specific transpeptidase activity. Results demonstrated that the synthesis of His6-tagged EcGGT reached a maximum after a 12 h induction. Two bands corresponding to the molecular masses of 41 and 21 kDa, respectively were observed in the crude extracts of IPTG-induced E. coli M15 (pQE-EcGGT), when the incubation temperature was set at 20 or 28°C (Fig. 1a). These bands were absent in the control crude extract of E. coli M15 harboring pQE-30. It is worthwhile to note that only small amount of the recombinant enzyme was produced by E. coli M15 (pQE-EcGGT) cultivated at 10 or 37°C. Apparently, the optimum IPTG concentration for production of active His6-tagged EcGGT was 0.1 mM (Fig. 1b). Thus, the best conditions for the maximum
production of His\textsubscript{6}-tagged EcGGT by \textit{E. coli} M15 (pQE-EcGGT) were: incubation of the recombinant cells at 20°C in the presence of 0.1 mM IPTG and 12 h induction.

**Purification, N-terminal sequencing, and molecular mass determination**

The recombinant enzyme in the crude extract was purified by nickel column chromatography. As shown in Fig. 2, molecular masses of subunits of the purified enzyme were 41 and 21 kDa, respectively. The purification scheme for the recombinant enzyme is summarized in Table 1. The specific transpeptidase activity for the purified enzyme was 4.25 U/mg protein, indicating the protein was purified approx 32.7-fold by one-step chromatography. The purified enzyme was further subjected to N-terminal amino acid sequencing and the sequences of the large and small subunits were determined to be RGSHHHHH and TTHYSVV, respectively.

The enzymes belonging to the N-terminal nucleophile (Ntn)-hydrolase superfamily are synthesized as inactive polypeptides and undergo intramolecular autocatalytic activation\textsuperscript{20}. A range of autocatalytic events proceeds via protein splicing\textsuperscript{21}, where the initial steps are thought to involve an N→O or N→S acyl rearrangement to generate a branched intermediate\textsuperscript{22}. In Ntn-hydrolases, catalytic nucleophile also acts as the autocatalytic nucleophile\textsuperscript{23-26}.

![Fig. 1](image)

**Table 1 — Purification scheme for His\textsubscript{6}-tagged EcGGT**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>524 ± 17</td>
<td>4032 ± 58</td>
<td>0.13</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Ni\textsuperscript{2+}-NTA</td>
<td>434 ± 12</td>
<td>102 ± 7</td>
<td>4.25</td>
<td>83</td>
<td>32.7</td>
</tr>
</tbody>
</table>

Fig. 1—(a): Analyses of total proteins and specific activity of \textit{E. coli} M15 (pQE-EcGGT) under specific conditions [Crude extracts were separated on 10% polyacrylamide-SDS gels and visualized by Coomassie brilliant blue staining. Lane M, protein size markers; lane 1, growth without IPTG induction; and lanes 2-7, growth with 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1 mM and 10 mM IPTG induction respectively; and (b): Effect of temperature and IPTG concentrations on production of active EcGGT [The amount of active enzyme was determined by measuring the specific transpeptidase activity of the soluble extract. Data represent three independent measurements]

![Fig. 2](image)
stereochemistry at the active site structure for the catalytic domain and equivalent cysteine, Ntn-hydrolases share a common tertiary Whether the nucleophile is threonine, serine or 6-tagged EcGGT confirmed that removal of part of the signal peptide of the enzyme did not influence its autocatalytic cleavage of the truncated precursor.

Fig. 3—(a): Gel filtration and native-PAGE of purified EcGGT [Purified enzyme at a concentration of approx 2 mg/ml was subjected to gel filtration chromatography. Chromatographic analysis of EcGGT was carried out on a TSK gel SW guard column (4 cm × 8 mm) and a TSK G-3000 SW (30 cm × 8 mm) (TosoHaas, Japan) loaded on a Hitachi D-7000 HPLC system (Hitachi Ltd., Tokyo, Japan). The mobile phase used was 0.05% (w/v) sodium azide in 50 mM Tris-HCl buffer (pH 8.0). A typical analysis was completed in 40 min with a flow rate of 0.5 ml/min. The standard compounds included albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran 2000 (2000 kDa); and (b): 10% Native-PAGE (lane 1) and activity staining (lane 2) analyses of purified enzyme were as described in the ‘Materials and Methods’. Standards included albumin (67 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa) and ferritin (440 kDa).

Whether the nucleophile is threonine, serine or cysteine, Ntn-hydrolases share a common tertiary structure for the catalytic domain and equivalent stereochemistry at the active site. In EcGGT, the oxygen atom of the side chain of the N-terminal amino acid residue (Thr-371) of small subunit is the nucleophilic atom and the processing also takes place autocatalytically. In the present study, the N-terminal sequencing of the small and large subunits of His6-tagged EcGGT confirmed that removal of part of the signal peptide of the enzyme did not influence its autocatalytic processing. However, the reason for the autocatalytic cleavage of the truncated precursor remains to be investigated.

The molecular mass of His6-tagged EcGGT was determined by chromatographic analysis. To make the molecular mass determination more accurate, calibration curves with correlative coefficients of 0.97 and 0.99 for high and low molecular mass, respectively were drawn using protein standards. The enzyme was eluted at 16.8 min (Fig. 3a), whose molecular mass was determined to be around 120 kDa. The data of Fig. 2 and Fig. 3a indicated that this enzyme existed as a heterodimeric structure. Non-denaturing PAGE analysis of the purified enzyme confirmed the chromatographic data, in which the molecular mass was determined to be around 120 kDa (Fig. 3b). Zymogram analysis also revealed that the native enzyme had the GGT activity.

Conclusion
We used the post-genomics technique, coupled with the E. coli complete DNA sequence to rapidly clone the industrially important genes. The truncated coding region of the ggt gene was subcloned downstream of the T5 promoter of pQE-30 and the production of active protein was improved by optimizing cultivation conditions. One-step purification of His6-tagged EcGGT overexpressed in recombinant E. coli M15 cells allowed us to easily obtain mg quantity of fully active enzyme. Also, the purified His6-tagged EcGGT was active towards a range of substrates (data not shown), indicating that the recombinant enzyme was folded correctly and retained a quaternary structure, allowing a normal substrate recognition and catalytic reaction.

Interestingly, we found that part of the signal peptide of E. coli NovaBlue GGT was not essential for the production and post-translational modification of the precursor enzyme in the recombinant host cells. Moreover, the overexpressed enzyme was easily purified from cell-free extract by nickel-chelate chromatography. Although the detailed mechanism for the active production of His6-tagged EcGGT remains to be elucidated, the overproduced enzyme may prove beneficial for the synthesis of commercially important γ-glutamyl compounds.

Acknowledgement
This work was supported by a grant (NSC-93-2313-B-241-005) from National Science Council of the Republic of China.
17 Orlowski M & Meister A (1963) *Biochim Biophys Acta* 73, 679-681