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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Long-Liu Lin · Pei-Ru Chou · Yu-Wen Hua · Wen-Hwei Hsu

Overexpression, one-step purification, and biochemical characterization of a recombinant γ-glutamyltranspeptidase from *Bacillus licheniformis*

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Abstract A truncated gene from *Bacillus lichenifromis* ATCC 27811 encoding a recombinant γ-glutamyltranspeptidase (BLrGGT) was cloned into pQE-30 to generate pQE-BLGGT, and the overexpressed enzyme was purified from the crude extract of IPTG-induced *E. coli* M15 (pQE-BLGGT) to homogeneity by nickel-chelate chromatography. This protocol yielded over 25 mg of purified BLrGGT per liter of growth culture under optimum conditions. The molecular masses of the subunits of the purified enzyme were determined to be 41 and 22 kDa, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimum pH and temperature for the recombinant enzyme were 6–8 and 40 °C, respectively. The chloride salt of metal ions Mg^{2+}, K^{+}, and Na^{+} can activate BLrGGT, whereas that of Pb^{2+} dramatically inhibited it. The substrate specificity study showed that L-γ-glutamyl-*p*-nitroanilide (L-γ-Glu-*p*-NA) is a preference for the enzyme. Steady-state kinetic study revealed that BLrGGT has a k_{cat} of 105 s^{-1} and a K_m of 21 μM when using L-γ-Glu-*p*-NA as the substrate. With this overexpression and purification system, BLrGGT can now be obtained in quantities necessary for structural characterization and synthesis of commercially important γ-glutamyl compounds.

Introduction

γ-Glutamyltranspeptidase (GGT) (EC 2.3.2.2) catalyzes the transfer of the γ-glutamyl moiety to a large variety of amino acids and dipeptide acceptors (Orlowski and Meister 1965). In mammalian tissues, GGTs play a major role in glutathione metabolism (Tate and Meister 1981; Meister and Anderson 1983). These enzymes can use γ-glutamyl peptides as substrates in the reciprocal hydrolysis reaction, thus playing a role in the synthesis of glutathionine (Tate and Meister 1981). Additionally, GGT is clinically significant because elevation of its activity in obesity is associated with insulin resistance and the metabolic syndrome (Marchesini et al. 2005). Although GGTs from mammalian tissues have been extensively studied (Meister and Tate 1985; Coloma and Pitot 1986; Laperche et al. 1986; Goodspeed et al. 1989), only few bacterial GGTs have been characterized at the biochemical and the molecular levels (Suzuki et al. 1989; Ishiye et al. 1993; Xu and Strauch 1996; Chevalier et al. 1999), and very little is known regarding the physiological role of these enzymes in bacteria. It has been reported that bacterial GGTs might play a role in the transport of amino acid across cell membranes (Orlowski and Meister 1970; Meister 1973; Robins and Davies 1981); however, it remains unclear whether the amino acids are directly transported by transpeptidation via γ-glutamyl cycle or through the hydrolysis peptides used as substrates together with aminopeptideases (Suzuki et al. 1993).

GGTs belong to the structural superfamily of the N-terminal nucleophilic (Ntn) hydrolases. Members of this superfamily, despite lacking any discernible sequence similarity, share the same tertiary fold. A characteristic feature is the autoproteolytic activation of inactive precursors to release a catalytic serine, threonine, or cysteine at the N-terminal position (Brannigan et al. 1995). The core three-dimensional folding pattern shared by Ntn-hydrolases consists of a four-layer αββα-structure with two antiparallel β-sheets between α-helical layers (Oinonen and Rouvinen 2000). Smith and Meister (1995) demonstrated that the enzymatic reaction catalyzed by GGT proceeds via a γ-glutamyl-enzyme intermediate followed by nucleophilic substitution by water, amino acids, or peptides. The affinity-labeling study on several GGTs with ^{14}C-acivicin identified Thr-523 of rat kidney enzyme (Stole et al. 1990), Ser-405 of pig kidney enzyme, and Ser-406 of human kidney enzyme (Smith et al. 1995)

L.-L. Lin (✉) · P.-R. Chou · Y.-W. Hua
Department of Applied Chemistry, National Chiayi University,
300 University Road,
Chiayi, Taiwan, 60083, Republic of China
e-mail: llin@mail.ncyu.edu.tw
Fax: +886-5-2717901

W.-H. Hsu
Institute of Molecular Biology,
National Chung Hsing University,
Taichung, Taiwan, 402, Republic of China

as the labeled residue in the small subunit. However, site-directed mutagenesis of the human enzyme has shown that neither of these hydroxyl residues is essential for the catalysis or for the inactivation by acivicin (Smith et al. 1995). Another well-conserved hydroxyl residue in the small subunit is the N-terminal Thr (Thr-391 in *Escherichia coli* enzyme). Affinity labeling coupled with site-directed mutagenesis has proved that the N-terminal Thr-391 in small subunit of *E. coli* GGT (ECGGT) is the nucleophile (Inoue et al. 2000). Besides the cleavage of pro-ECGGT by signal peptidase I, this enzyme precedes the posttranslational cleavage between Gln-390 and Thr-391 to generate a heterodimer (Hashimoto et al. 1995). This notion gave impetus to identify the catalytic nucleophile not only involves in the catalytic reaction but also plays a role in the processing of GGTs.

Several γ-glutamyl peptides have been successfully synthesized through ECGGT. For example, γ-Glu-Trp (SCV-07) and γ-glutamyltaurine were synthesized with ECGGT using inexpensive L-glutamine as a γ-glutamyl donor (Suzuki et al. 2003; Suzuki et al. 2004). SCV-07 and γ-glutamyltaurine have been shown to have a broad spectrum of immunostimulatory activities against murine tuberculosis (Simbirtsey et al. 2003) and an antagonistic effect against excitatory amino acids (Jones et al. 1984), respectively. To conduct future industrial applications and structural studies, we anticipate the need for large quantities of purified GGT. This work describes our efforts to develop a reproducible and efficient overexpression and purification protocol for a recombinant GGT from the industrial bacterium *Bacillus licheniformis* ATCC 27811. We also demonstrate that the N-terminal truncation of *B. licheniformis* GGT (BLrGGT) did not have a detrimental effect on the production of active enzyme in the recombinant host cells. Also, the purified enzyme was characterized in the aspects of optimum reaction conditions, substrate specificity, effects of metal ions, and its in vitro maturation.

Materials and methods

Materials

Restriction and modification enzymes were purchased from Promega Life Sciences (Madison, WI, USA). Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resin was acquired from Qiagen (Valencia, CA, USA). Reduced and oxidized GSH, *S*-methylglutathione, L-Glu-γ-monohydroxamate, γ-glutamyl-L-tyrosine, L-Glu-γ-monomethyl ester, L-Glu-γ-monoethyl ester, L-Glutamine, D-Glutamine, L-Glu-γ-(3-carboxy-4-hydroxyanilide), and γ-glutamyl-L-leucine were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). All other chemicals were commercial products of analytical grade or molecular biological grade.

Bacterial strains, plasmid, and growth conditions

B. licheniformis ATCC 27811 obtained from the Bioresources Collection and Research Center in Food Industry Research & Development Institute (Hsinchu, Taiwan) was used as a source of chromosomal DNA for the *ggt* gene cloning. *E. coli* Novablue (Novagen, Maidson, WI, USA) was used for the routine preparation and construction of recombinant plasmid. *E. coli* M15 from Qiagen (Valencia, CA, USA) was employed for T5 RNA polymerase-mediated overexpression of BLrGGT. Plasmids used were pGEM-T Easy vector (Promega, Madison, WI, USA) and pQE-30 (Qiagen).

B. licheniformis was grown in nutrient broth at 37 °C for 20 h, while *E. coli* was grown in Luria–Bertani (LB) medium at 20 °C during isopropyl-β-D-thiogalactopyranoside (IPTG)-induced gene expression. The antibiotics, ampicillin, and kanamycin were used at 100 and 25 μg ml^{-1}, respectively.

Molecular techniques

Chromosomal DNA of *B. licheniformis* was prepared through the method described by Doi et al. (1983). Standard techniques for plasmid DNA preparation, restriction enzyme digestion, DNA ligation, and transformation were performed as described by Sambrook and Russell (2001). DNA fragments were recovered from agarose gel by a DNA extraction kit (Viogene, 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, USA). Nucleotide and amino acid sequences were analyzed with the programs BLASTX from the National Center for Biotechnology Information (National Library of Medicine, National Institute of Health, USA) and Alignment from the ExPAsy molecular server (Swiss Institute of Bioinformatics, Basel, Switzerland). The amino acid sequences from *B. licheniformis* ATCC 14580 GGT (Swiss-Prot Q62WE3), *Bacillus subtilis* GGT (Swiss-Prot P54422), *Thiobacillus denitrificans* GGT (Swiss-Prot Q3SJ07), *E. coli* GGT (Swiss-Prot P18956), *Helicobacter pyroli* GGT (Swiss-Prot O25743), *Pseudomonas aeruginosa* GGT (Swiss-Prot Q9I406), *Homo sapiens* GGT (Swiss-Prot P19440), *Sus scrofa* GGT (Swiss-Prot P20735), and *Mus musculus* GGT (Swiss-Prot Q60928) are included in this study.

Expression and purification of BLrGGT

Amplification of the truncated *ggt* gene from *B. licheniformis* ATCC 27811 was carried out by polymerase chain reaction (PCR) using the gene-specific primers designed from the published *ggt* gene (Rey et al. 2004). The cloning sites of *Bam*HI and *Kpn*I were introduced into the forward primer Bl*ggt*-f (5′-*GGATCC*GTCGGGAAAGACGGTA TGGTG-3′) and the reverse primer Bl*ggt*-r (5′-*GGTACC*

CAATTTAGCCGATGTTAATG-3′), respectively. A 1,644-bp DNA fragment was obtained through 30 cycles of PCR with denaturation at 94 °C for 1.5 min, annealing at 50 °C for 1.5 min, extension at 72 °C for 2 min, and a final incubation at 72 °C for 10 min. The amplified DNA was then cloned into the pGEM-T Easy vector and transformed into *E. coli* Novablue cells. Transformants carrying the recombinant plasmid were selected on LB medium supplemented with ampicillin and 0.01 % 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Plasmid DNA with the insert was digested with *Bam*HI and *Kpn*I and inserted into the corresponding sites of pQE-30 to yield pQE-BLGGT.

For high-level expression of the recombinant enzyme, *E. coli* M15 (pQE-BLGGT) was grown at 37 °C in LB broth containing ampicillin (100 μg ml^{-1}) and kanamycin (25 μg ml^{-1}) until the optical density at 600 nm of the culture was 0.6. IPTG was then added to a final concentration of 0.025 mM. Protein induction was carried out at a lower temperature of 20 °C, and the induction was continued for 48 h. Cells were harvested by centrifugation (4,000×*g* for 20 min at 4 °C). To purify BLrGGT, 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 BLrGGT was bound to 2 ml of Ni^{2+}-NTA resin by gentle mixing at 4 °C for 30 min. Subsequently, the resin was loaded onto a column and washed with 3 vol 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

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12 % acrylamide slabs using the buffer system of Laemmli (1970). Before electrophoresis, the samples were heated to 100 °C for 5 min in dissociating buffer containing 2 % SDS and 5 % 2-mercaptoethanol. Protein size markers were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Protein concentration was measured with a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as the standard.

Enzyme activity assay

GGT activity was assayed spectrophotometrically by monitoring the *p*-nitroaniline released from L-γ-glutamyl-*p*-nitroanilide (L-γ-Glu-*p*-NA) as described elsewhere (Orlowski and Meister 1963), with slight modifications. The standard reaction mixture contained 50 mM Tris–HCl buffer (pH 8.0 at room temperature), 1.25 mM L-γ-Glu-*p*-NA, 30 mM Gly-Gly, 1 mM $MgCl_2$, 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 40 °C for 10 min. The reaction was stopped by the addition of 100 μl of 3.5 N acetic acid. Absorbance was measured at 410 nm with a spectrophotometer and the boiled samples (5 min at 100 °C) taken at time zero were subtracted from the readings. One unit of GGT activity is defined as the amount of enzyme that produced 1 μmol of *p*-nitroaniline per min under the assay conditions.

Biochemical studies

The effects of pH and temperature on the GGT activity were determined using L-γ-Glu-*p*-NA as the substrate. The optimum pH for enzyme activity was determined at 40 °C from pH 3 to pH 13 in various buffers including 50 mM acetate/Na-acetate buffer (pH 3–6), 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 5–7), 50 mM Tris–HCl buffer (pH 7–10), and 50 mM glycine/NaOH buffer (pH 10–13). The optimum temperature for GGT activity was determined by measuring the rate of reaction at temperatures ranging from 30 to 60 °C under the standard assay conditions.

The effect of pH on BLrGGT stability was determined by incubating the purified enzyme (25 μg ml^{-1}) in the buffers with different pH values for 30 min at 4 °C, and the residual activity was assayed under the standard assay conditions. To determine the influence of temperature on BLrGGT stability, the purified enzyme (25 μg ml^{-1}) was preincubated for 30 min at temperature range of 30–60 °C in 50 mM Tris–HCl buffer, pH 8.0. Subsequently, the residual activity was measured under the standard assay conditions.

The effects of metal ions on the enzyme activity were determined by incubating aliquots of purified BLrGGT with the chloride salt of the metal ions such as Na^+, K^+, Mg^{2+}, Ca^{2+}, or Co^{2+} in 50 mM Tris–HCl buffer (pH 8.0) at 30 °C for 10 min. The residual activity was measured under standard conditions. GGT activity assayed in the absence of metal ions was taken as 100 %.

A steady-state kinetics study of the purified BLrGGT was performed at 40 °C in 50 mM Tris–HCl buffer (pH 8.0) with L-γ-Glu-*p*-NA concentrations in the range of 10–100 μM. K_m and k_{cat} values were determined by least-squared fitting of the kinetic data to the Michaelis–Menten equation.

The transpeptidase activity of the purified enzyme against several γ-glutamyl derivatives was also determined. The reaction mixture contained 50 μl of purified enzyme (30 μg ml^{-1}), 100 μl of 25 mM substrate, 100 μl of 10 mM ethylamine, 20 μl of 50 mM $MgCl_2$, and 730 μl of 50 mM Tris–HCl buffer (pH 8.0). Transpeptidation activity of BLrGGT was measured by high performance liquid chromatography equipped with a RP18-GP250 column (Kanto Chemical, Kanto, Japan) and with a gradient elution at a flow rate of 1 ml min^{-1}. Gradient of the mobile phase was formed with 100 % acetonitrile and 1 % formic acid, pH 2.1. Concentration of acetonitrile was kept at 25 % until 15 min. It was then increased to 70 % from 15 to 20 min,

and to 90 % from 20 to 25 min. *O*-Phthalaldehyde was used as the detection reagent and the adsorption was detected with a UV detector as the absorbance at 335 nm (Hill et al. 1979).

Results

Comparison of BLrGGT sequence with bacterial and eukaryotic homologues

The PCR-amplified DNA fragment lacking the coding sequence for the first ten amino acids of *B. licheniformis* ATCC 27811 *ggt* gene was digested with *Bam*HI and *Kpn*I, and inserted into the expression vector under the control of T5 promoter. *E. coli* M15 competent cells were transformed with the recombinant plasmid, pQE-BLGGT, and the cloned gene was confirmed by restriction analysis and DNA sequencing. The sequencing data revealed that six codon differences, compared with the published gene sequence of *B. licheniformis* ATCC 14580 GGT (BLIGGT) (Rey et al. 2004), occurred at positions 205, 211, 242, 566, 568, and 853 of the *ggt* gene, resulting in the substitution of Asp-69, Glu-71, Arg-81, Val-189, Gln-190, Ser-285 with His-69, Lys-71, Met-81, Ala-189, Glu-190, and Pro-285, respectively. The sequence alignment of the unprocessed pro-GGT from a variety of organisms is shown in Fig. 1. The multiple alignments of the GGTs illustrate that BLIGGT has an overall primary structure similar to that of the well-described mammalian GGTs. The gene encodes a protein consisting of 557 amino acids with a calculated molecular mass of 60,974 Da (Swiss-Prot Q62WE3). Bacterial GGTs are usually translated as a precursor protein followed by two proteolytic cleavages to remove the signal peptide and to process the pro-GGT into the large and small subunits (Suzuki et al. 1989; Ishiye et al. 1993; Xu and Strauch 1996). The cleavage site between amino acids 370 and 371, which results in the processing of

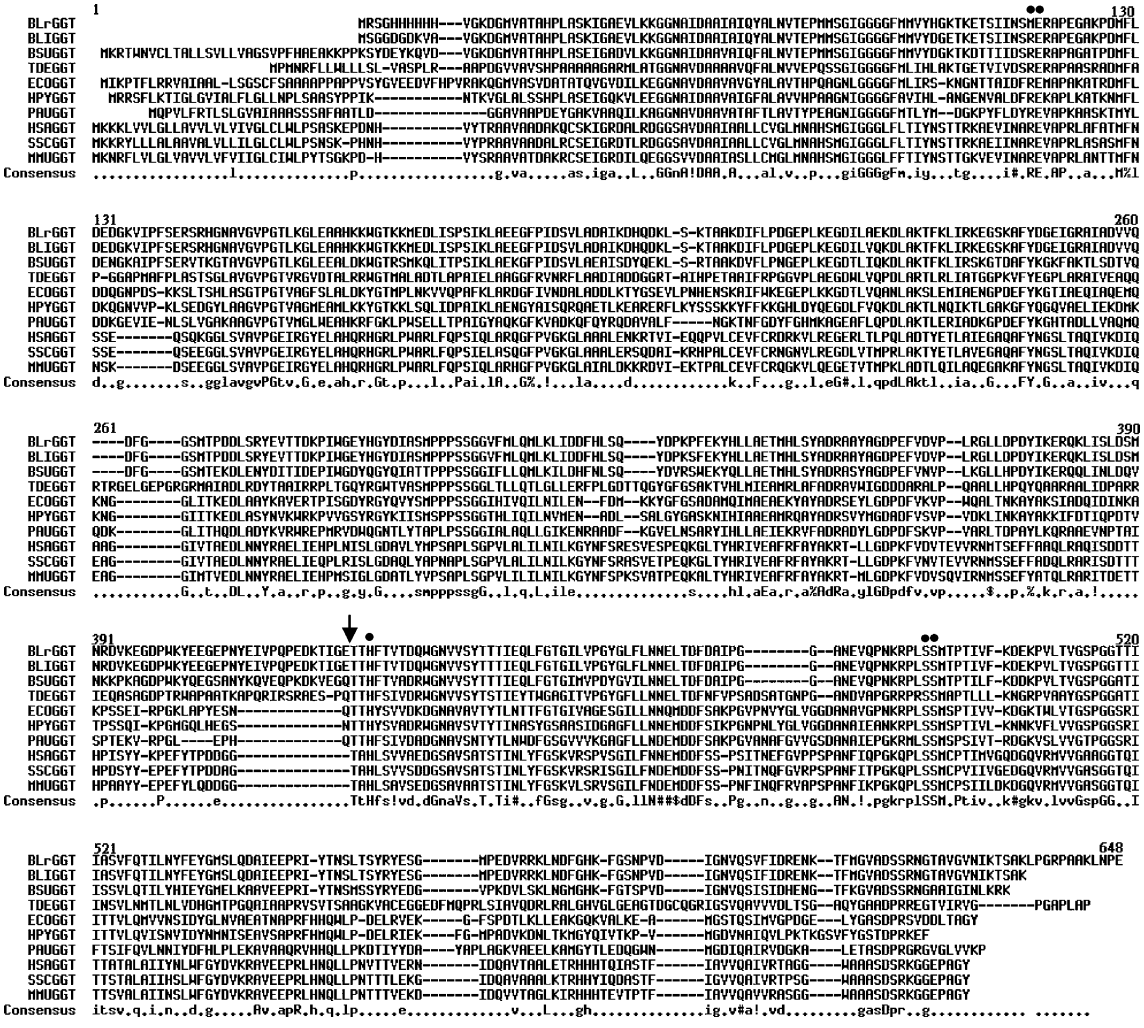

Fig. 1 Sequence alignment of microbial and animal GGTs. The deduced amino acid sequence for the recombinant *B. licheniformis* ATCC 27811 GGT (BLrGGT), *B. licheniformis* ATCC 14580 GGT (BLIGGT), *B. subtilis* GGT (BSUGGT), *T. denitricans* (TDEGGT), *E. coli* GGT (ECOGGT), *P. aeruginosa* GGT (PAEGGT), *H. pylori* GGT (HPYGGT), *H. sapiens* (HSAGGT), *S. scrofa* GGT (SSCGGT), and *M. musculus* (MMUGGT) are shown. Sequence accessions are listed in the "Materials and methods" section. Gaps in aligned sequences (*dashes*) were introduced to maximize similarities. The *vertical arrow* shows the putative proteolytic cleavage site. *Solid circles* indicate the residues assigned to the catalytic activity of human GGT

the pro-GGT into a large and a small subunit, with calculated molecular masses of 40,475 Da and 20,517 Da, was deduced for BLIGGT by comparison with other enzymes (Fig. 1). BLIGGT shares 76, 42, 40, 38, and 37 % identity with the amino acid sequences of *B. subtilis*, *T. denitrificans*, *H. pyroli*, *E. coli*, and *P. aeruginosa* GGTs, respectively, and more than 26 % sequence identity with those of the eukaryotic enzymes. Finally, this alignment emphasizes the presence of highly conserved and potentially reactive residues (Fig. 1), in which Arg-107, Glu-108, His-383, Ser-451, and Ser-452 (numbering in human GGT) have already been proposed to play a role in the catalytic function of human GGT (Ikeda et al. 1993; Ikeda et al. 1995; Ikeda et al. 1996).

Expression optimization and purification of BLrGGT

The expression of His_6-tagged enzyme in *E. coli* M15 (pQE-BLGGT) was carried out in the presence of different IPTG concentrations and in various cultivation temperatures. After 3-, 6-, 9-, 12-, 24-, 48-, and 54-h inductions, 1 ml of the bacterial culture was centrifuged and the harvested cells were resuspended in 100 μl of loading buffer and the total cellular proteins were separated by 12 % SDS-PAGE. The protein patterns of the total cell extracts are shown in Fig. 2a. SDS-PAGE analysis of the total proteins from 0.025 mM IPTG-induced *E. coli* M15 (pQE-BLGGT) exhibited three additional bands, compared with the recombinant cell carrying pQE-30, with apparent molecular masses of approximately 62, 41, and 22 kDa, respectively. Many efforts have been made in heterologous gene expression in *E. coli* on the enhancement of protein quality (Olins and Lee 1993). In this study, several different growth temperatures were tried for the overexpression of active BLrGGT. As shown in Fig. 2b, the optimum temperature for the production of biologically active enzyme was 20 °C. The optimum IPTG concentration for the expression of BLrGGT was 0.025 mM. There is a significant reduction in the level of active enzyme when IPTG concentration exceeded 0.5 mM. For maximum production of BLrGGT, IPTG at a final concentration of 0.025 mM, and induction temperature and time of 20 °C and 48 h, respectively, were used in the subsequent experiments. Under these conditions, a specific activity of 6.5 U mg^{-1} $protein^{-1}$ was obtained for the crude extract.

The recombinant enzyme in the crude extract was further purified by nickel-chelate chromatography. As shown in Fig. 3, the molecular masses of pro-BLrGGT and the subunits were 62, 41, and 22 kDa, respectively. The purified scheme for the recombinant enzyme is summarized in Table 1. The transpeptidase activity for the purified enzyme was 185.6 U mg^{-1} $protein^{-1}$, indicating that the protein was purified approximately 29-fold by single-step purification.

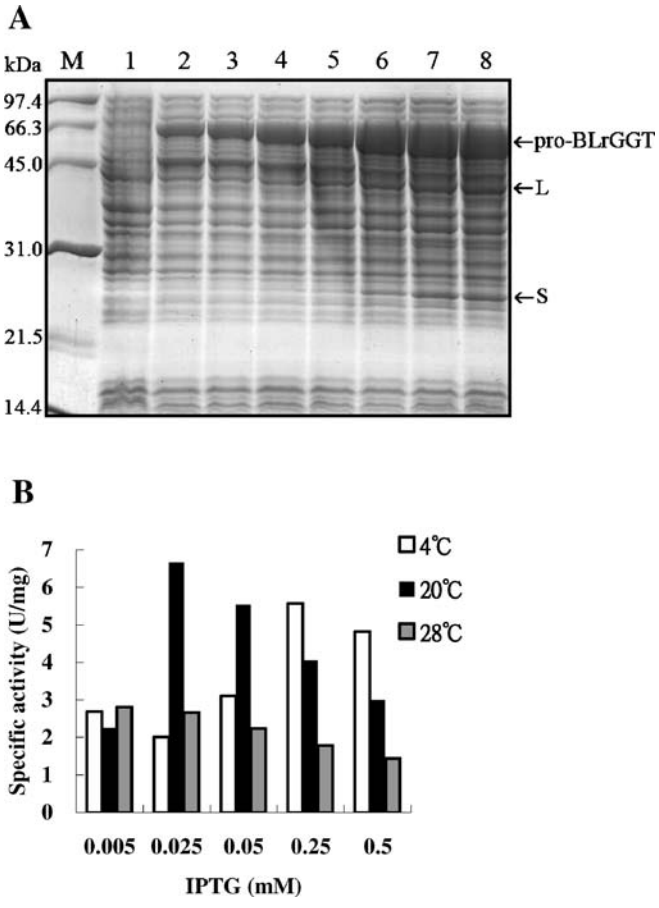

Fig. 2 Analyses of the soluble proteins and the specific activity of *E. coli* M15 (pQE-BLGGT) under a specific condition. **a** The crude extracts were separated on 12 % polyacrylamide-SDS gels and visualized by Coomassie brilliant blue staining. *M* protein size marker; *1* after 3-h induction in the absence of IPTG; *2* after 3-h induction; *3* after 6-h induction; *4*, after 9-h induction; *5* after 12-h induction; *6* after 24-h induction; *7* after 48-h induction; and *8* after 54-h induction. **b** Effect of temperature and IPTG concentration on the production of active BLrGGT. The amount of active enzyme was determined by measuring the specific transpeptidase activity of the soluble extract. The data is a representative of three independent measurements

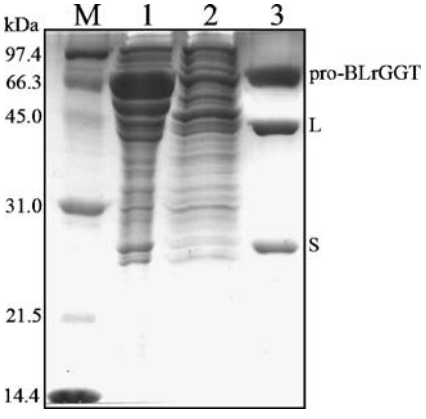

Fig. 3 SDS-PAGE analysis of BLrGGT. *M* protein size marker; *1* the crude extract of IPTG-induced *E. coli* M15 (pQE-BLGGT); *2* the filtrate after a nickel-chelate chromatography; and *3* the purified BLrGGT

Table 1 The purification scheme for BLrGGT

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	6,625±97	1,032±48	6.4	–	1
Ni^{2+}-NTA	4,826±72	26±3	185.6	73	29

Cell pellet from 1 l culture was resuspended in 300 ml of the binding buffer and subjected to protein purification as described in the "Materials and methods" section

Biochemical properties of BLrGGT

GGT activity against L-γ-Glu-*p*-NA was optimal at pH 6–8, and strong activity was still detectable at pH 9 (Fig. 4a). However, activity rapidly declined under acidic conditions (below pH 5). BLrGGT was stable in the neutral to alkaline pH range (Fig. 4a). The apparent temperature dependence of the purified enzyme in a 10-min assay was examined. The enzyme was most active at 40 °C, and more than 85 % maximum activity was found from 35 to 45 °C (Fig. 4b). BLrGGT was stable at temperatures below 40 °C and approximately 40 % of the original activity was retained at 45 °C. The K_m and k_{cat} values of purified enzyme were estimated to be 21 μM, 105 s^{-1}, respectively.

To determine the effects of metal ions on the activity of BLrGGT, the purified enzyme was dematalized by dialyzing against a buffer containing 50 mM Tris–HCl (pH 8) and 1 mM EDTA, and then against the above buffer without EDTA. In the absence of metal ions, the specific transpeptidase activity of the purified enzyme was 73 U/mg protein. The addition of $CoCl_2$ to a final concentration of 1 mM had no effect on the enzyme activity, while the chloride salt of Hg^{2+}, Zn^{2+}, Pb^{2+}, and Ni^{2+} ions had an inhibitory effect on the GGT activity (Table 2). The enzyme activity was strongly enhanced by chloride salt of Mg^{2+}, K^+, and Na^+ ions, especially when the concentrations of these ions exceed 10 mM.

The transpeptidation to ethylamine from various γ-glutamyl compounds including glutathione (GSH), *S*-methyl-GSH, oxidized GSH, L-Gln, D-Gln, γ-L-Glu-Leu, γ-L-Glu-His, γ-L-Glu-monohydroxamate, γ-L-Glu-α-napthylamide, γ-L-Glu-NA, L-Glu-γ-(3-carboxy-4-hydroxy-anilide), L-Glu-γ-monomethyl ester, and L-Glu-γ-monoethylester was evaluated (Table 3). The reaction rate for the glutamyl transfer from γ-Glu-*p*-NA to ethylamine was 0.314 nmol min^{-1}. BLrGGT showed a lower relative rate toward GSH, L-Gln and oxidized GSH than γ-Glu-*p*-NA. In contrast, *S*-methyl-glutathione, γ-L-Glu-monohydroxamate, γ-L-Glu-Tyr, γ-L-Glu-Leu, L-Glu-γ-monomethyl ester, L-Glu-γ-monoethyl ester, and L-Glu-γ-(3-carboxy-4-hydroxy-anilide) were not suitable for the transpeptidation reaction.

In vitro maturation of BLrGGT

To investigate the in vitro maturation of pro-BLrGGT, an IPTG-induced *E. coli* M15 (pQE-BLGGT) was grown on 100 ml LB medium at 20 °C for 3 h and the cells were harvested by centrifugation, and pro-BLrGGT purified as described in "Materials and methods". The maturation of the purified enzyme in the Tris–HCl buffer (pH 8) was monitored at 4 and 20 °C. At each sampling time, the amount of pro-BLrGGT was analyzed by SDS-PAGE and the GGT activity was simultaneously assayed. As shown in Fig. 5, the processing of pro-BLrGGT was a time-

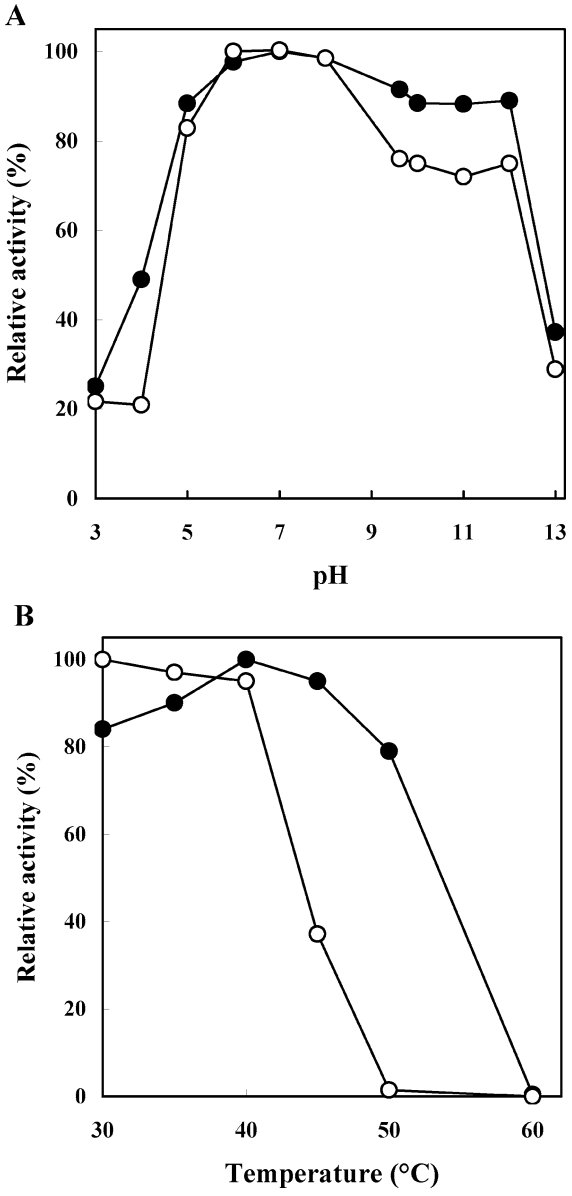

Fig. 4 Effects of temperature and pH on activity (●) and stability (○) of BLrGGT. GGT activity was measured at various pH values (**a**) and temperatures (**b**) under standard assay conditions. The data is a representative of three independent experiments

Table 2 Effect of chloride salt of metal ions on the activity of the purified BLrGGT

Metal ions	Concentration (mM)	Relative activity (%)
None	1	100
Ca^{2+}	1	78
Co^{2+}	1	101
Ni^{2+}	1	70
Zn^{2+}	1	76
Pb^{2+}	1	32
Hg^{2+}	1	85
K^{+}	1	118
	10	120
	100	154
	200	171
	400	161
Na^{+}	1	112
	10	129
	100	161
	200	174
	400	159
Mg^{2+}	1	126
	10	154
	100	244
	200	242
	400	214

The data is a representative of three independent experiments

dependent event and could proceed autocatalytically. When the incubation at 20 °C extended more than 20 days, most of the pro-BLrGGT was processed into the large subunit and the small subunit (Fig. 5a). It is worth noting that the large subunit was quite stable during the maturation and the small subunit was found to further degrade at 20 °C leading to the decrease of the GGT activity, while there was no obvious degradation that occurred upon the small subunit when the incubation temperature was set at 4 °C. It is interesting to note that the specific activity of the purified enzyme was less than 10 U mg^{-1} $protein^{-1}$ (data not shown). The activity increased rapidly during the first week and reached a plateau after incubating the purified enzyme at 20 °C for 10 days, while a sharp decrease in the activity was observed in the sample incubated at 20 °C for more than 7 days (Fig. 5b). These results clearly indicate that the maturation is essential for the activation of BLrGGT. Also, the degradation of small subunit of the processed enzyme could be responsible for the decrease in enzyme activity.

Table 3 Substrate specificity of BLrGGT for γ-glutamyl donors

Substrate	Relative activity (%)
γ-Glu-*p*-NA	100
S-methyl-glutathione	1.8
L-Glu-γ-monohydroxamate	0.5
GSH	56.7
γ-Glu-L-Tyr	5.2
L-Glu-γ-monomethyl ester	4.3
L-Glu-γ-monoethyl ester	1.9
Oxidized GSH	10.6
L-Gln	18.8
D-Gln	1.3
L-Glu-γ-(3-carboxy-4-hydroxy-anilide)	3.9
γ-Glu-Leu	3.4

Transpeptidase activity was measured as described in the "Materials and methods" section. Concentrations of 2.5 and 1 mM for donors and ethylamine, respectively, were used. Activity is expressed relative to that found with γ-Glu-*p*-NA (100 %). The data is a representative of three independent experiments

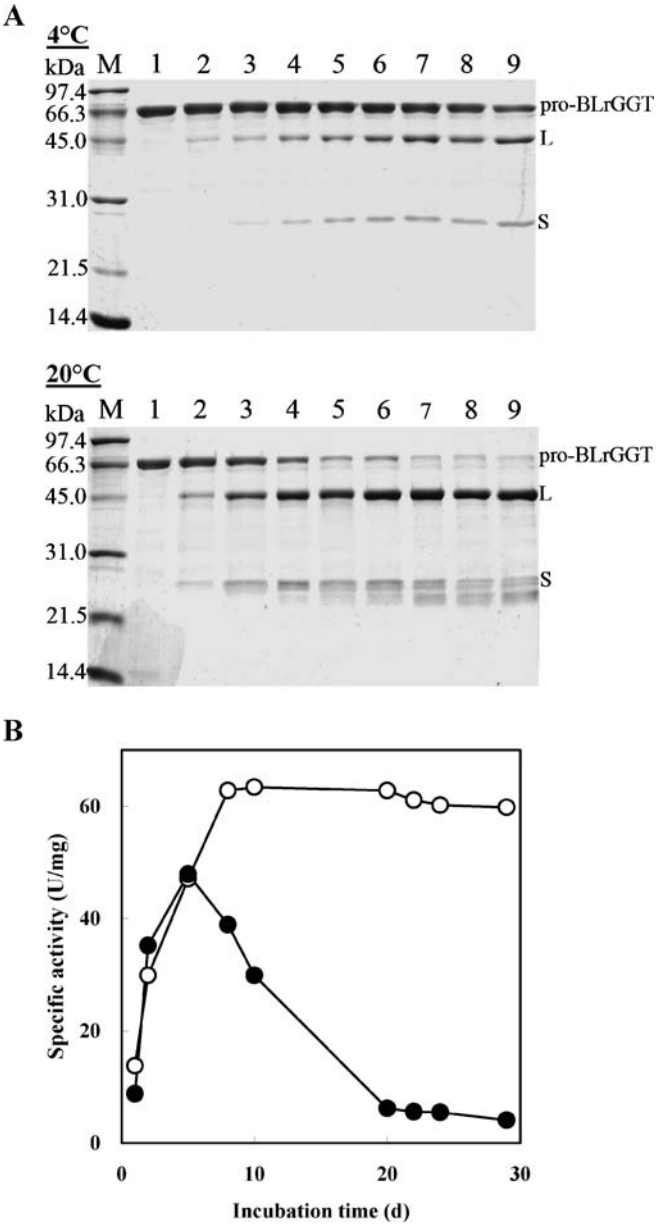

Fig. 5 Autocatalytic processing of pro-BLrGGT. Approximately 130 μg pro-BLrGGT ml^{-1} in 50 mM Tris–HCl (pH 8.0) was incubated at 4 or 20 °C for 0, 3, 5, 8, 10, 20, 22, 24, and 29 day (*lanes 1–9*). Aliquots, 20 μl, were withdrawn at the indicated intervals for SDS-PAGE analysis (**a**), and the enzyme activity was assayed under standard assay conditions and plotted against incubation time (**b**)

Discussion

In this study, a truncated *B. licheniformis* ATCC 27811 GGT lacking part of the N-terminal sequence was constructed and expressed in *E. coli* M15. It is interesting that BLrGGT was fully active toward a range of substrates. This result indicates that the recombinant enzyme was correctly folded and retained a quaternary structure, allowing a normal substrate recognition and catalytic reaction. To date, *E. coli* has been extensively used for the production of secreted enzymes (Choi and Lee 2004). It should be noted that the endogenous or host-based signal sequences are essential for enzyme secretion. The role of signal peptides in the initiation of translocation process has been well documented (Izard and Kendall 1994). Most of the secreted enzymes possess an N- or C-terminal propeptide to preclude correct folding of the associated enzyme. The transformation of propeptide into a conformation ready for secretion is sometimes accomplished by interaction of the mature component with an accessory protein such as foldase and chaperone (Eder and Fersht 1995). In general, mutations and deletions within the signal peptide sequence result in the intracellular accumulation of precursor protein (Lammertyn and Anné 1998). However, it has been reported that the 70-kDa precursor of *Alteromonas halopanctis* α-amylase was directly secreted into the supernatant of *E. coli* and processed by a nonspecific protease into mature enzyme (Feller et al. 1998). In our case, BLrGGT might follow a secretion pathway similar to that of *A. haloplanctics* α-amylase when it is expressed in the recombinant host.

The choice of an expression system for the high-level production of recombinant proteins depends on many factors. These include cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications, and biological activity of the interested protein (Hodgson 1993; Hockney 1994). To obtain a functional protein, a number of strategies could be used for the soluble expression of recombinant proteins in *E. coli* (Sørensen and Mortensen 2005). It has been reported that formation of inclusion bodies in recombinant expression systems is the result of an unbalanced equilibrium between in vivo protein aggregation and solubilization (Carrio and Villaverade 2001). A well-known technique to limit the in vivo aggregation of recombinant proteins consists of cultivation at reduced temperature (Schein 1989). This strategy has proven to be effective in improving the solubility of a number of different proteins including subtilisin E, bacterial luciferase, β-lactamase, rice lipoxygenase, and kanamycin nuclotidyltransferase (Kiefhaber et al. 1991). As shown in Fig. 2, cultivation of *E. coli* M15 (pQE-BLGGT) at a temperature below its physiological condition also favored the soluble expression of BLrGGT. Under optimum conditions, the specific transpeptidase activity of the crude extract from *E. coli* M15 (pQE-BLGGT) reached 6.5 U mg^{-1} $protein^{-1}$. Further purification step led us to obtain approximately 26 mg l^{-1} of active enzyme.

The homogenous preparations of renal GGTs have been performed on various mammalian species. These enzymes have molecular weight of approximately 22 kDa for the small subunit, but are divided into two groups with respect to their large subunit: one group with molecular weights ranging from 40 to 50 kDa (rat and rabbit) and the other group with molecular weights of approximately 64 kDa (human, bovine, and sheep) (Tate and Meister 1981). The molecular weights of subunits for microbial GGTs are found to be within 39–47 and 22–28 kDa (Nakayama et al. 1984; Suzuki et al. 1986; Ogawa et al. 1991; Chevalier et al. 1999), respectively. The small subunit for BLrGGT has a molecular weight of 22 kDa, which is the same as those of mammalian renal GGTs, while the large subunit displays a molecular weight of 41 kDa that is much smaller than those of mammalian renal GGTs.

A base is generally proposed to be critical for the autoproteolytic activation of Ntn-hydrolase precursors into α- and β-subunits. It has been suggested that a His residue situated at a vicinity position of the processing site of *Flavobacterium* glycosylasparaginase is essential for its maturation and acts as a base in this reaction (Guan et al. 1996; Guan et al. 1998). However, X-ray crystallographical results revealed that the β-carboxyl group of the Asp residue just before the Thr residue, whose side chain is the nucleophile of the enzyme, acts as the base (Xu et al. 1999). In the case of the β-subunit of 20 S proteasome, the residue just before the catalytic Thr residue is the invariable Gly, which has no side chain to act as a base like Asp in glycosylasparaginase. These findings therefore rule out the involvement of Asp in the autocatalytic processing. On the contrary, Ditzel et al. (1998) showed that a water molecule acts as the base at the active center for the processing of β-subunit precursor of yeast 20 S proteasome. Also, a water molecule was found to be the general base in cephalosporin acylase (Kim et al. 2002) and glutaryl 7-aminocephalosporanic acid acylase (Kim et al. 2003). Therefore, it is likely that a water molecule plays a critical role in the autocatalytic action of pro-BLrGGT into α- and β-subunits.

In conclusion, this work highlights the power of using postgenomics techniques together with the *B. licheniformis* genome initiative to rapidly clone the industrial important genes. The truncated coding region of the *ggt* gene is subcloned downstream of the T5 promoter of pQE-30 and the production of active protein is improved by optimizing cultivation conditions. One-step purification of BLrGGT overexpressed in recombinant *E. coli* M15 cells allows us to easily obtain milligram quantity of fully active enzyme. The overproduced enzyme is beneficial for the synthesis of γ-glutamyl compounds for medical applications.

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