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Site-directed mutagenesis of conserved Thr407, Asp433 and Met464 residues in small subunit of *Escherichia coli* γ-glutamyltranspeptidase

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Sequence comparison showed that residues Thr407, Asp433, and Met464 in the small subunit of *Escherichia coli* γ-glutamyltranspeptidase (EcGGT) were conserved in the aligned enzymes. In this study, we further investigated the functional significance of these conserved residues by site-directed mutagenesis. The wild-type and mutant enzymes were overexpressed in the recombinant *E. coli* M15 cells and purified to near homogeneity by Ni^{2+}-NTA resin. Except M464L, other mutants had shown no GGT activity under enzyme assay conditions and activity staining. Furthermore, mutations on these residues impaired the capability of autocatalytic processing of the enzyme. Based on these observations, it is concluded that these residues play an important role in the enzyme maturation.

Keywords: *Escherichia coli*, γ-Glutamyltranspeptidase, Site-directed mutagenesis, Autocatalytic processing

The reclamation of extracellular glutathione and its conjugates are initiated by γ-glutamyltranspeptidase (GGT; EC 2.3.2.2). The enzyme cleaves the γ-glutamyl amide bond of glutathione to liberate cysteinylglycine and the catalytic mechanism proceeds via a γ-glutamyl-enzyme intermediate[1-5]. The γ-glutamyl group can be transferred to water (hydrolysis) or an amino acid or short peptide (transpeptidation) (Fig. 1A). Mammalian GGTs have been found to be embedded in the plasma membrane by a single N-terminal transmembrane anchor and are heterologously glycosylated, whereas bacterial homologs are soluble and localized in the periplasmic space. Overall, the GGTs are highly conserved in mammalian and bacterial homologs, often sharing greater than 25% sequence identity.

A post-translational modification is often required for the maturation of precursor protein. *Escherichia coli* GGT (EcGGT) is synthesized as a 61.8 kDa polypeptide and after removal of the signal sequence, the precursor EcGGT is cleaved to yield a heterodimer comprising 39.2 and 20 kDa subunits[6] (Fig. 1B). Processing of EcGGT is believed to be an

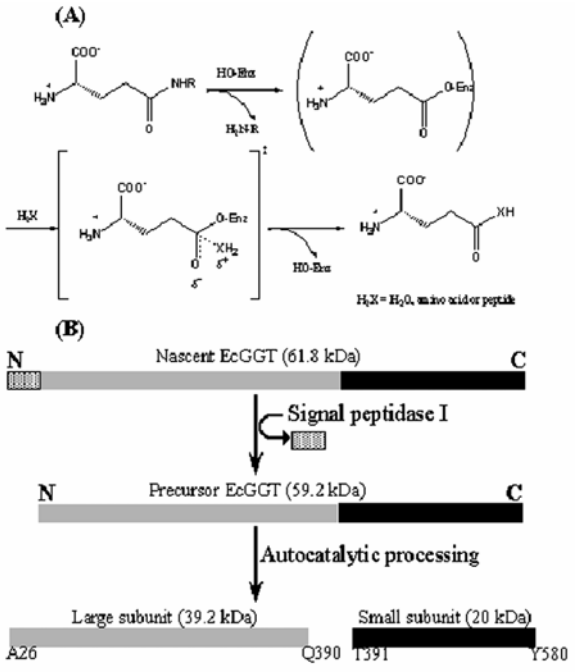

Fig. 1—(A): Reaction mechanism of GGT (adapted from ref. 13); and (B): Schematic diagram of two-step maturation of EcGGT [The 61.8 kDa precursor enzyme is activated through the removal of signal peptide and a subsequent intermolecular cleavage between Gln390 and Thr391 to generate a native $(L/S)_2$ heterodimer]

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Abbreviations: GGT, γ-glutamyltranspeptidase; EcGGT, *Escherichia coli* GGT; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni^{2+}-NTA; nickel nitrilo-triacetate; PAGE, polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate-PAGE; *p*-NA, *p*-nitroaniline; L-γ-Glu-*p*-NA, L-γ-glutamyl-*p*-nitroanilide.

intramolecular autocatalytic event proceeds via a nitrogen → oxygen acyl shift with a conserved threonine residue serving as the nucleophile[7]. Based on the enzymatic function and autoprocessing activity, GGT has been classified as an Ntn (N-terminal nucleophile) hydrolase. The members of Ntn hydrolase family share a similar αββα-core structure and the precursors are autocatalytically processed to generate an active enzyme[8,9]. The new N-terminal residue of processed enzyme, typically a serine, threonine or cysteine residue then serves as a nucleophile in the catalytic mechanism[10-14]. In EcGGT, N-terminal residue is Thr-391 and its replacement with Ala results in failure of post-translational processing, thereby yielding the unprocessed precursor peptide[15]. Moreover, substitutions of Arg513 and Arg571 of EcGGT with Ala and Gly, respectively abolish the GGT activity and impair the autocatalytic processing of the enzyme[16].

EcGGT has been used for the synthesis of industrially important compounds, including γ-glutamyl-L-DOPA[17], γ-glutamyl-Phe[18], and γ-glutamyl-Trp[19]. To obtain large amount of GGT for industrial applications, earlier, we have constructed a truncated EcGGT gene lacking the first 48-bp coding sequence for part of the signal sequence and cloned the PCR-amplified fragment into expression vector pQE-30. The overexpressed enzyme has been purified to homogeneity by one-step chromatography to a specific transpeptidase activity of 4.25 U/mg protein and a final yield of 83%[20].

In this study, we have aligned the EcGGT sequence with GGTs of *Bacillus licheniformis*, *Helicobacter pyroli*, *Homo sapiens*, *Sus scrofa*, and *Mus musculus* (Fig. 2). We have found 30-49% sequence identities of EcGGT with other GGTs and residues Thr407, Asp433 and Met464 in the small subunit of the enzyme are fully conserved. Based on the alignment, we have replaced the conserved Thr407, Asp433, and Met464 residues of EcGGT with other amino acids, in order to have insight into the function of these residues. Mutations on these residues have been found to impair the autocatalytic processing of the enzyme, indicating that they might play a crucial role on the enzyme maturation.

Materials and Methods

Materials, bacteria strains and growth conditions

Growth medium components were acquired from Difco Laboratories (Detroit, MI, USA). Restriction and modification enzymes were obtained from Promega Life Sciences (Madison, WI, USA) and used in accordance with the supplier's instructions. Oligonucleotides for DNA sequencing and mutagenesis were obtained from the Mission Biotechnology Inc. (Taipei, Taiwan). Nickel nitritriacetate (Ni^{2+}-NTA) resin was purchased from Qiagen Inc. (Valencia, CA, USA). All other chemicals were commercial products of analytical or molecular biological grade.

Escherichia coli NovaBlue (Novagen Inc., Madison, WI, USA) was used for the preparation of plasmids and *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) for the site-directed mutagenesis. T5 RNA-polymerase-mediated gene expression was performed in *E. coli* M15 (Qiagen). Cells were grown in Luria-Bertani (LB) medium (10, 10 and 5 g/L of tryptone, NaCl and yeast extract, respectively) at 37 or 28°C supplemented with 100 μg ampicillin/mL for NoveBlue and XL1-Blue strains or 100 μg ampicillin/mL and 25 μg kanamycin/mL for M15 strain.

Molecular techniques

All DNA manipulations were essentially performed as described previously[21]. Mutagenesis was performed on plasmid pQE-EcGGT[20] with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions and a pair of complementary mutagenic primers (Table 1). Polymerase chain reaction was performed under the following conditions: Initial denaturation for 30 s at 95°C, 16 cycles of 30 s at 95°C, 1 min at 55°C and 30 min at 68°C. After digestion of parental DNA with *Dpn*I, amplified plasmids were transformed into *E. coli* XL1-Blue supercompetent cells. The presence of intended mutation was confirmed by DNA sequencing. The mutated plasmids were designed as pQE-EcGGT/T407D, pQE-EcGGT/T407K, pQE-EcGGT/T407S, pQE-EcGGT/D433E, pQE-EcGGT/D433G, pQE-EcGGT/D433K, pQE-EcGGT/M464E, pQE-EcGGT/M464K, and pQE-EcGGT/M464L, respectively.

The amino acid sequences were analyzed using 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* GGT

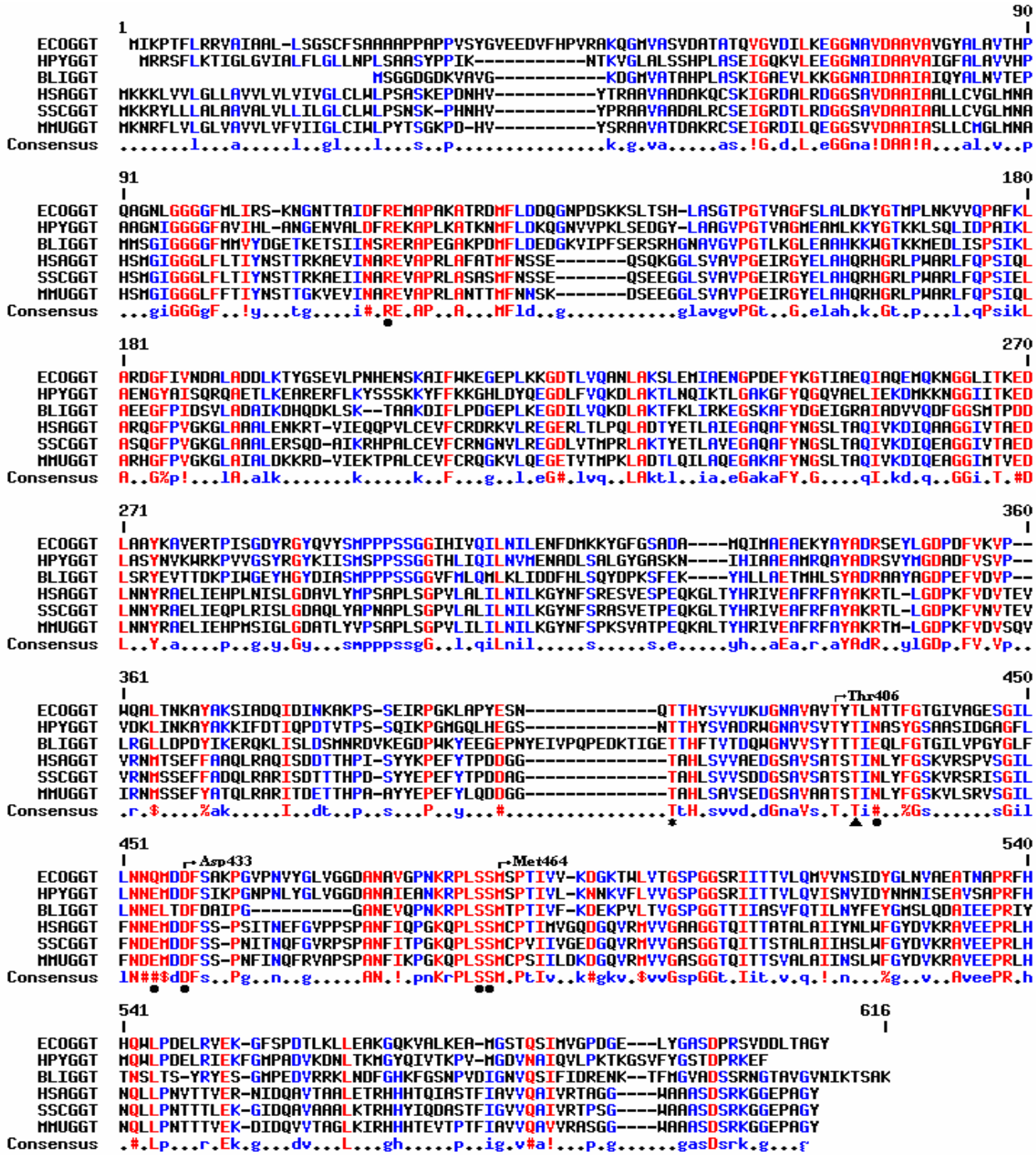

Fig. 2—Sequence alignment of microbial and animal GGTs [The deduced amino acid sequence for *E. coli* GGT (ECOGGT), *H. pylori* GGT (HPYGGT), *B. licheniformis* GGT (BLIGGT), *H. sapiens* (HSAGGT), *S. scrofa* GGT (SSCGGT), and *M. musculus* GGT (MMUGGT) are shown. Gaps in aligned sequences (dashes) were introduced to maximize similarities. The essential residues for catalytic activity, stabilizing the nucleophile and binding of substrate are marked by a star, a triangle, and solid circles, respectively]

Table 1—Oligonucleotides used to create substitutions on the conserved residues of EcGGT

Mutant enzyme	Primer sequence (5'→ 3')[a]
T407D	GAGAGTAATCAAGATACCCATTACTCAG
T407K	GAGAGTAATCAAAAAACCCATTACTCAG
T407S	GAGAGTAATCAATCTACCCATTACTCAG
D433E	TAACCAGATGGATGAATTCTCCGCCAAAC
D433G	TAACCAGATGGATGGTTTCTCCGCCAAAC
D433K	TAACCAGATGGATAAGTTCTCCGCCAAAC
M464E	CCCGCTGTCGTCGGAGTCGCCGACCATTG
M464K	CCCGCTGTCGTCGAGGTCGCCGACCATTG
M464L	CCCGCTGTCGTCGCTGTCGCCGACCATTG

[a]Only sense sequences are shown and mismatches with the original sequence of the *ggt* gene are underlined

(Swiss-Prot Q62WE3), *H. pyroli* GGT (Swiss-Prot O25743), *H. sapiens* GGT (Swiss-Prot P19440), *S. scrofa* GGT (Swiss-Prot P20735) and *M. musculus* GGT (Swiss-Prot Q60928) were included in this study.

Expression and purification of recombinant enzymes

All the enzymes were prepared from *E. coli* M15 carrying either pQE-EcGGT or the mutated plasmids. Cells were grown at 37°C in 100 mL of LB medium containing the above-mentioned antibiotics to an optical density at 600 nm of approximately 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 m*M* and cultivation was continued at 20°C for 12 h. Cells were harvested by centrifugation at 12, 000 × *g* for 20 min, resuspended in 3 mL of binding buffer (5 m*M* imidazole, 0.5 *M* NaCl, and 50 m*M* NaH_2PO_4; *p*H 7.9) and disrupted by sonication. Debris was removed by centrifugation and supernatant was used as the crude enzyme solution, which was then mixed with Ni^{2+}-NTA resin. The His_6-tagged proteins were eluted from the resin with a buffer containing 0.5 *M* imidazole, 0.5 *M* NaCl, and 50 m*M* NaH_2PO_4 (*p*H 7.9).

Protein analyses

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli buffer system[22]. The gel was stained with 0.25% Coomassie Brilliant Blue R-250 dissolved in 50% methanol-10% acetic acid and destained in a solution of 30% methanol and 10% acetic acid. Protein size markers were phophorylase *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).

A 10% non-denaturing PAGE 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 m*M* 5-L-glutamyl-2-naphthylamide and 50 m*M* Gly-Gly in 100 m*M* Tris-HCl buffer (*p*H 9.0) and incubated at 37°C for 30 min. The gel was then transferred into a solution containing 0.05% Fast garnet GBC in 8% acetic acid until blue dark GGT band appeared.

Protein concentrations were determined by the Bradford method[23] using bovine serum albumin as the standard.

Enzyme assay

GGT activity was determined at 37°C according to previously described method[24] and formation of *p*-nitroaniline (*p*-NA) was recorded by monitoring the absorbance changes at 410 nm. Unless otherwise indicated, reaction mixture contained 1.25 m*M* L-γ-glutamyl-*p*-nitroanilide (L-γ-Glu-*p*-NA), 30 m*M* Gly-Gly, 1 m*M* $MgCl_2$, 50 m*M* Tris-HCl buffer (*p*H 9.0), 20 μL of enzyme solution at a suitable dilution and enough distilled water to bring the final volume of 1 mL. One unit of GGT activity was defined as the amount of enzyme that produced 1 μmol of *p*-NA per min under the assay conditions.

The enzyme kinetic parameters K_m and V_{max} were obtained from Lineweaver-Burke plot and were subsequently used to estimate enzyme turnover and catalytic efficiency. The kinetic parameters were determined in 50 m*M* Tris-HCl buffer (*p*H 9.0) containing 1 m*M* $MgCl_2$ and 1 to 100 μ*M* L-γ-Glu-*p*-NA.

Results and Discussion

Based on homology between EcGGT and GGT-related enzymes, residues Thr407, Asp433 and Met464 in the small subunit of the enzyme were selected for amino acid replacements. After verification of altered sequences, recombinant plasmids were transformed into *E. coli* M15 for IPTG-induced gene expression. The conditions for expression and purification of wild-type and mutant enzymes were generated as described previously[20]. The overexpression of wild-type and mutant enzymes was assessed by analyzing protein profiles of the whole-cell lysate preparations.

As shown in Fig. 3A, the wild-type enzyme and M464L (lanes 9 and 10) were expressed as two predominant bands with the molecular mass of 41 and 22 kDa, respectively, while other mutant proteins were present as a precursor (63 kDa), indicating that mutations on residues Thr407 and Asp433 impaired the autocatalytic processing of the enzyme. The wild-type and mutant enzymes in the crude extracts were further purified to near homogeneity by Ni^{2+}-NTA resin. Like the wild-type enzyme, the molecular masses of the subunits of purified M464L were determined to be 41 and 22 kDa, respectively, while the rest of the mutants existed in their precursor forms (Fig. 3B).

The wild-type EcGGT had the specific activity of 5.07 U/mg protein and the apparent K_m and k_{cat} values of 3.91×10^{-2} mM and 34.1 min^{-1}, respectively (Table 2). Among the mutant enzymes, only M464L retained 51.2% of the GGT activity and exhibited a decreased catalytic efficiency with an increased K_m value with respect to the wild-type EcGGT. This

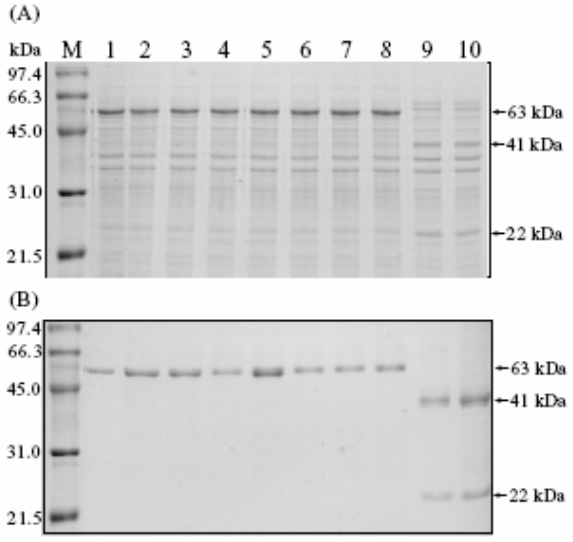

Fig. 3—SDS-PAGE analyses of (A): Whole-cell extracts from *E. coli* M15 transformants [Transformants were analyzed by 12% polyacrylamide-SDS gels and visualized by Coomassie brilliant staining. Lanes: M, protein size markers; 1, *E. coli* M15 (pQE-EcGGT/T407D); 2, *E. coli* M15 (pQE-EcGGT/T407K); 3, *E. coli* M15 (pQE-EcGGT/T407S); 4, *E. coli* M15 (pQE-EcGGT/D433E); 5, *E. coli* M15 (pQE-EcGGT/D433G); 6, *E. coli* M15 (pQE-EcGGT/D433K); 7, *E. coli* M15 (pQE-EcGGT/M464E); 8, *E. coli* M15 (pQE-EcGGT/M464K); 9, *E. coli* M15 (pQE-EcGGT/M464L); 10, *E. coli* M15 (pQE-EcGGT)]; and (B): Purified wild-type and mutant enzymes [Lanes: M, protein size markers; 1, T407D; 2, T407K; 3, T407S; 4, D433E; 5, D433G; 6, D433K; 7, M464E; 8, M464K; 9, M464L; 10, wild-type EcGGT]

Table 2—Specific activity and kinetic parameters of wild-type EcGGT and M464L

[Values represent mean of 3 measurements]

Enzyme	Specific activity (U mg^{-1})	k_{cat} (min^{-1})	K_m ($\times 10^{-2}$ mM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)
WT	5.07	34.1	3.91	87.2
M464L	2.60	4.53	10.8	4.19

implied that substitution of Met464 with Leu might cause a conformational change in the catalytic cleft of the enzyme. As expected, other mutants exhibited no detectable activity (data not shown).

The crystal structure of EcGGT has been determined at 1.95 Å resolution[25]. Like other Ntn-hydrolase superfamily members, EcGGT heterodimer has a stacked αββα-core with the β-strands of L and S subunits, forming two central β-sheets, sandwiched by α-helices. Interestingly, C-terminus of L subunit and N-terminus of S subunit are quite distant from one another with Ser387 C and The391 N being 36 Å apart. Previous studies demonstrate that GGT catalytic mechanism involves an amino group[26], a carboxyl group[26,27], and an arginine residue[28,29]. In fact, α-carboxyl and α-amino groups of γ-glutamyl moiety of donor substrate are at the bottom of the substrate-binding pocket of EcGGT and this moiety is held in this position by many hydrogen bonds and salt bridges[25]. The carboxyl group of donor substrate is bonded with Arg114 Nη, Ser462 Oγ, Ser463 N and Ser463 Oγ via WAT1, and the amino group interacts with Asn411 O δ, Gln430 Oε and Asp433 Oδ. As carboxyl group of Asp433 plays an important role in the binding of donor substrate, it is expected substitution of this residue with either glycine or arginine would destroy one of the salt bridges.

To examine whether the amino acid substitutions affected the post-translational processing of the mutant enzymes, non-denaturing PAGE analysis of Thr407, Asp433 and Met464 variants as well as wild-type EcGGT was performed. As shown in Fig. 4A, the migration behavior of M464L (lane 9) was similar to that of the wild-type EcGGT (lane 10), but in other mutant proteins, a lower mobility shift was observed. Among the mutants, only M464L (lane 9) retained the GGT activity on the gel (Fig. 4B).

The crystal structure of EcGGT precursor protein has been determined at 2.55 Å resolution[30]. In precursor protein, the number of segments with high

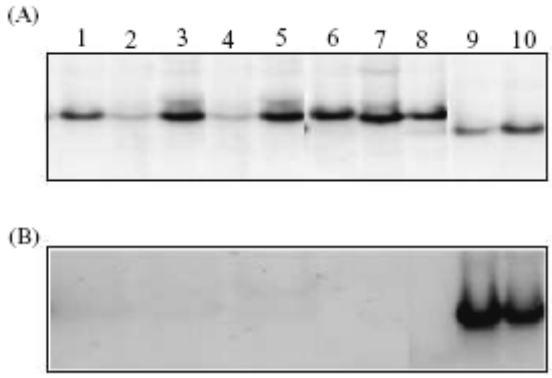

Fig. 4—Denaturing PAGE analysis of purified wild-type and mutant enzymes [Purified enzymes were separated by 10% polyacylamide gels and visualized by Coomassie brilliant blue staining (A) and activity staining (B). Lanes: 1, T407D; 2, T407K; 3, T407S; 4, D433E; 5, D433G; 6, D433K; 7, M464E; 8, M464K; 9, M464L; 10, wild-type EcGGT]

mobility is increased relative to the mature EcGGT and residues 375-390 (termed P-segment), takes on an extended conformation on the molecular surface. Except for the P-segment and residues located at its vicinity, the structure of precursor protein is similar to that of mature EcGGT[30]. Accordingly, mobility shift of Thr407, Asp433 and Met464 mutant enzymes in the non-denaturing gel might result from the major structural perturbation around the P-segment as the consequence of residue substitutions.

Recently, we noted that the precursor of *B. licheniformis* GGT could undergo *in vitro* maturation[31]. A water molecule was proposed to act as the base for autoproteolytic processing of Ntn-hydrolase precursors into L and S-subunits[32-34]. To investigate the *in vitro* maturation of Thr407, Asp433 and Met464 variants, the purified enzymes were incubated in 50 m*M* Tris-HCl buffer (*p*H 9.0) at 4°C. At each sampling time, the amount of precursor proteins was analyzed by SDS-PAGE and the GGT activity was simultaneously assayed. However, no significant maturation and GGT activity was observed for T407D, T407K, T407S, D433E, D433G, D433K, M464E and M464K mutant proteins (data not shown). At this stage, it is difficult to figure out the reason for the impact of these mutations on the autocatalytic event, since the EcGGT precursor processing has been demonstrated to be an intramolecular autocatalytic event and the catalytic nucleophile has been identified as the Oγ atom of Thr391[7]. Therefore, determination of three-dimensional structures of these mutants would contribute to our understanding the impact of these substitutions.

In conclusion, the present study showed that residues Thr407, Asp433 and Met464 were conserved in the aligned enzymes including bacterial GGT and human GGT-related enzymes (Fig. 2). Asp433 interacted with the carboxyl group of the γ-glutamyl moiety through an ionic bond and other two residues (Thr407 and Met464) might contribute to the wall formation of substrate-binding pocket of the enzyme[25]. However, the structural role of residues Thr407 and Met464 in EcGGT remains to be elucidated, since their substitutions would definitely affect the maturation of the enzyme.

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