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# *ESCHERICHIA COLI* γ-GLUTAMYLTRANSPEPTIDASE MUTANTS DEFICIENT IN PROCESSING TO SUBUNITS

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SUMMARY Arginyl residues 513 and 571 of *Escherichia coli* K-12 γ-glutamyltranspeptidase (EC 2.3.2.2) were substituted with alanyl and glycyl residues, respectively, by oligonucleotide-directed *in vitro* mutagenesis. Both mutants were devoid of the enzymatic activity. On Western blot analysis, we found that both mutants accumulated a γ-glutamyltranspeptidase precursor which was not processed into large and small subunits in the periplasmic space of *Escherichia coli*. 

INTRODUCTION γ-Glutamyltranspeptidase (GGT, EC 2.3.2.2) is widely distributed in living organisms and is designated as the key enzyme in glutathione metabolism. It catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, and the transfer of their γ-glutamyl moieties to amino acids and peptides (1). We investigated the enzyme of *E. coli* K-12, and reported its low temperature (20℃) dependent biosynthesis (2), its localization in the periplasmic space (2), characteristics of the purified enzyme (3), the isolation and characterization of GGT-less mutants (4), molecular cloning and sequencing of *ggt*, the gene which codes for GGT (5), and the enzymatic synthesis of γ-glutamyl peptides with GGT (6,7,8,9). The amino acid sequence deduced from the nucleotide sequence of *ggt* indicated the formation of prepro-GGT as the translation product and the occurrence of post-translational processing, in which the signal peptide (25 amino acid residues) is released, and the cleavage between the large (365 amino acid residues, 39.2kDa) and the small subunit (190 amino acid residues, 20.0kDa) takes place.

The amino acid sequence of *E. coli* GGT shows 33.0% homology with those of rat GGT (10) and 32.4% with human GGT (11), and the other amino acid residues are almost all conserved through the conservative substitutions of amino acids.

ABBREVIATIONS:
GGT, γ-glutamyltranspeptidase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidenedifluoride.

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On amino acid sequence alignment for examination of the similarity among the *E. coli*, rat and human GGTs, we found that two arginyl residues, 513 and 571, located on the small subunit are conserved in all of them (5).

Treatment of mammalian GGTs with chemical modifying agents for arginyl residues was reported to inactivate them, and it was proposed that an arginyl residue in the small subunit is involved in the recognition of an anionic moiety of an acceptor in the transfer reaction by way of an electrostatic interaction (12,13).

In this study, we constructed mutant *E. coli* GGTs with arginyl residues 513 and 571 substituted with an alanyl and a glycyl residue, respectively, by oligonucleotide-directed *in vitro* mutagenesis. The mutant GGTs were compared with the wild-type GGT.

## MATERIALS AND METHODS

Materials. Restriction endonuclease, T4 DNA ligase and DNA molecular weight markers were purchased from Takara Shuzo Co., Toyobo Co., and Nippon Gene Co., respectively. Anti-rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey) was purchased from Amersham International plc. PVDF membrane was purchased from Nihon Millipore Kogyo K.K.

Bacterial strains. The strains of *E. coli* K-12 used in this work are presented in Table 1.

Strain construction. The P1*vir* phage was grown on *E. coli* strain SH682. The lysate was used to transduce KS474 to obtain Ggt$^-$ Tet$^r$ transductant NS453. A spontaneous tetracycline-sensitive derivative of the transductant was selected according to Bochner *et al.* (14) and rendered *recA* with JC10240, as described (15). The *recA* exconjugant was screened on an LB plate containing 2μg/ml nitrofurantoin (16).

Table 1. *E. coli* strains used

| Strains | Genotype | Source and reference |
|---|---|---|
| MV1184 | Δ(*srl-recA*)*306*::Tn*10* Δ(*lac-proAB*) *ara thi rpsL* φ80d*lacZ*ΔM*15* [F' *proAB lacI*$^q$ *lacZ*ΔM*15 traD*36] | Takara Shuzo Co. (24) |
| KS474 | F$^-$ *degP41*(ΔPstI-Kan$^r$) Δ*lacX74* *galE galK thi rpsL* Δ*phoA*(PvuII) | K. L. Strauch (25) |
| SH682 | F$^-$ *ggt-2 zhg*::Tn*10* | Laboratory stock (4) |
| NS453 | F$^-$ *degP41*(ΔPstI-Kan$^r$) Δ*lacX74* *galE galK thi rpsL* Δ*phoA*(PvuII) *ggt-2 zhg*::Tn*10* | This work |
| JC10240 | HfrP045 *srl300*::Tn*10 recA56* *thr300 ilv318 rpsE300* | A. J. Clark (15) |
| NS471 | F$^-$ *degP41*(ΔPstI-Kan$^r$) Δ*lacX74* *galE galK thi rpsL* Δ*phoA*(PvuII) *ggt-2 srlC300*::Tn*10 recA56* | This work |

Growth medium and cultivation of bacteria. The growth medium was LB broth containing ampicillin at 100 μg/ml. Overnight cultures containing recombinant plasmids were subcultured at a dilution of 1:100 in LB medium and grown at 20℃ two overnights.

Subcloning, transformation and gel electrophoresis. Subcloning, transformation and gel electrophoresis were performed as described (17,18).

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed with a T7-GEN *In Vitro* Mutagenesis Kit (United States Biochemical Co.) and with an Oligonucleotide-directed *in vitro* Mutagenesis System Version 2 (Amersham International plc) according to the manufacturers' directions.

DNA sequencing. The DNA sequence was determined by the M13 dideoxy sequencing method (19,20) with a Sequenase Kit (United States Biochemical Co.) using [$\alpha$-$^{32}$P]dCTP (Amersham International plc).

Bacterial extract preparation, SDS-PAGE and Western blotting. Periplasmic fractions were prepared as described (2). Periplasmic fractions were separated by 12.5% SDS-PAGE according to Laemmli (21). After electrotransfer to a PVDF membrane, the membrane was blocked with TBS-T (tris-buffered saline, pH7.6, and 0.1% Tween 20) containing 5% skim milk. GGT was detected with anti-*E.coli* GGT antibody and anti-rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey).

GGT activity. GGT activity was measured as described (2).

## RESULTS AND DISCUSSION

The MV1184 strain harboring pSH253 (22) was infected with the M13KO7 helper phage and single-stranded pSH253 was purified from the phage particles. This

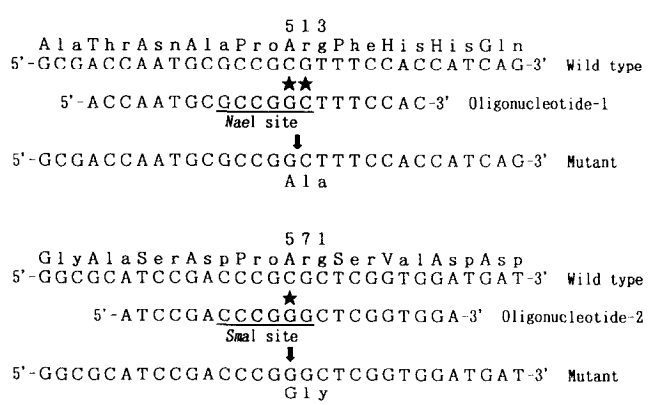


Fig. 1. Oligonucleotides used in this study.
Oligonucleotide-1 was designed to substitute Arg-513 with Ala and oligonucleotide-2 was designed to substitute Arg-571 with Gly.

ssDNA was used as the mold and the oligonucleotides shown in Fig. 1 were used as primers. Mutant plasmids were constructed by oligonucleotide-directed *in vitro* mutagenesis. Since the oligonucleotides were designed so as to make new restriction endonuclease cleavage sites for *Nae*I and *Sma*I when arginyl residue-513 and arginyl residue-571 were substituted with an alanyl residue and a glycyl residue, respectively, the mutations were confirmed by the restriction pattern of the plasmids on agarose gel electrophoresis. We also confirmed the mutations by DNA sequencing. The mutant plasmid whose arginyl residue-513 was substituted with an alanyl residue was designated as pGGT513RA and that whose arginyl residue-571 was substituted with a glycyl residue as pGGT571RG.

DegP protease deficient strain NS471 was transformed with pSH253, pGGT513RA and pGGT571RG. Periplasmic fractions were obtained from each transformants and their GGT activity was measured. The strains harboring pGGT513RA and pGGT571RG showed no GGT activity, which is compatible with the results of a chemical modification study.

The periplasmic fractions were subjected to SDS-PAGE, followed by Western blot analysis (Fig. 2). The strain harboring pSH253 gave bands corresponding to

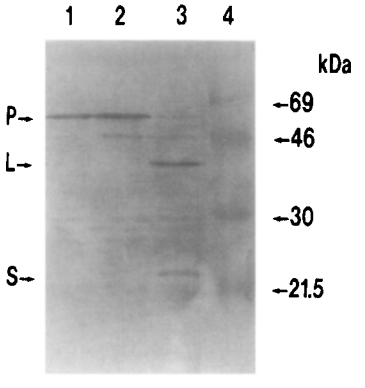


Fig. 2. Western blot analysis of periplasmic fractions.
Lane 1, periplasmic fraction of NS471 harboring pGGT571RG., 2, periplasmic fraction of NS471 harboring pGGT513RA., 3, periplasmic fraction of NS471 harboring pSH253., 4, rainbow coloured protein molecular weight markers (Amersham International plc). L, S and P indicate the positions of the large subunit, small subunit and precursor, respectively.

the large and small subunits. However, the strains harboring pGGT513RA and pGGT571RG only gave a single band corresponding to molecular weight of 59kDa. A similar results were obtained when whole cells were subjected to SDS-PAGE, followed by Western blot analysis (data not shown).

Since mature GGT consists of one large (39kDa) and one small (20kDa) subunit (5), the results show that the latter two strains harboring mutant plasmids synthesize pro-GGT.

A GGT-processing enzyme is not known yet, but GGT itself was reported to have latent protease activity (23), though it is not known if GGT is autoprocessed. Therefore, further study is needed to determine that the mutant GGTs are not processed whether because the structural change of the mutant GGTs makes it difficult for a processing-enzyme to cleave pro-GGT or because arginyl residues 513 and 571 are the active site residues for autoprocessing.

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