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MOLECULAR CLONING OF ESCHERICHIA COLI K-12 ggt AND RAPID ISOLATION OF γ-GLUTAMYLTRANSPEPTIDASE

Hideyuki SUZUKI, Hidehiko KUMAGAI, Takashi ECHIGO and Tatsurokuro TOCHIKURA

Department of Food Science and Technology, Faculty of Agriculture,
Kyoto University, Kyoto 606, Japan

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Based on the results of mapping of ggt, eight strains were selected from a gene library of E. coli. One of the strains harboring pLC9-12 was found to show 14 times higher γ-glutamyltranspeptidase activity per cell than the wild type strain. The ggt was subcloned to the BamHI site of pUC18 and the recombinant plasmid pSH101 was obtained. Ggt⁻ phenotype of γ-glutamyltranspeptidase-deficient mutants was complemented by pSH101. The specific activity of the enzyme in cells harboring pSH101 was 37-fold higher than that in the wild type cells. γ-Glutamyltranspeptidase was isolated from the periplasmic fraction of the cells by simple two steps and crystallized.

γ-Glutamyltranspeptidase (EC 2.3.2.2) catalyzes the transfer of γ-glutamyl residue from γ-glutamyl compounds such as glutathione to amino acids and peptides, and hydrolysis of γ-glutamyl compounds. Although extensive studies of the enzyme from eukaryotes have been performed (1, 2, 3), its physiological role is still controversial. Recently, Laperche et al. (4), and Coloma and Pitot (5) cloned the cDNA of rat renal γ-glutamyl-transpeptidase and determined its nucleotide sequence. We (6) and Nakayama et al. (7) used isolated enzyme preparations to elucidate the physiological role of bacterial γ-glutamyltranspeptidase. Previously, we isolated and characterized γ-glutamyltranspeptidase-deficient mutants of E. coli K-12, and performed genetic mapping of the mutations (ggt) onto the E. coli K-12 linkage map (8). These mutants did not require nutrient, and they released more glutathione into the medium than the wild type cell.

ABBREVIATIONS

X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside.

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For further enzyme chemical and crystallographical studies, it is essential to develop a rapid purification method of the enzyme. One way is to obtain starting material with high specific activity by constructing an overproducing strain of γ-glutamyltranspeptidase by genetic engineering.

Herein we report the construction of an overproducing strain of E. coli γ-glutamyltranspeptidase and a rapid purification method.

MATERIALS AND METHODS

Reagents. Restriction enzymes, T4 DNA ligase and DNA molecular weight markers were purchased from Nippon Gene Co. and Takara Shuzo Co., RNase A and alkaline phosphatase from calf intestine were from Sigma Chemical Co.

Bacterial strains and plasmids. All strains used in this study were E. coli K-12 derivatives. pUC18 (9) was kindly donated by T. Kouchi and K. Oyama. Other plasmids were purified from the cells harboring each plasmid as described (10).

Growth media and cultivation of bacteria. LB broth (11) was usually used. The solid media contained 1.5% agar. M9 minimum plates (8) containing 40 μg/ml of X-gal, 0.2 mM IPTG and 50 μg/ml of ampicillin, or LB plates with 50 μg/ml of ampicillin were used when transformants harboring pUC18 derivatives were grown. Cells were grown in water baths or in a warm room with reciprocal shaking, or in incubators at appropriate tempertures.

Assay of γ-glutamyltranspeptidase activity. γ-Glutamyltranspeptidase activity was measured as described (12).

Manipulation of DNA. Restriction endonuclease cleavage and ligation were performed according to the specifications of the manufactures, except for the addition of 70 μg/ml of bovine serum albumin to the ligation buffer according to Burns and Beacham (13). After cleavage with an appropriate restriction endonuclease, pUC18 was treated with alkaline phosphatase(14). Transformations and gel electrophoresis were performed as described (14, 15).

Purification and crystallization of γ-glutamyltranspeptidase. γ-Glutamyltranspeptidase was purified from periplasmic fraction of SH642 by ammonium sulfate precipitation and Chromatofocusing, and crystallized as described (6).

Gel electrophoresis of proteins. Periplasmic fractions were prepared as described (12) and subjected to the modified Laemmli gel (6).

RESULTS AND DISCUSSION

Since we mapped ggt mutations at 76 min between glpD and ugpA (8), eight strains of Clarke-Carbon colony bank (16) harboring pLC plasmids with htpR, fam, ftsE, livH, livK or livJ gene(s) (17), which are mapped at 76 min (18), were screened for pLC plasmid with ggt gene. γ-Glutamyltrans-

Table 1. Screening of Clarke-Carbon colony bank

Strain No.	Genotype	Relative transferase activity per cell (%)	Genes on a pLC plasmid
SH50 (MG1655)	F^- prototrophic	100	
SH664	F^- *ggt*	0	
SH181 (JA200)	F^+/Δ *trpE5 recA1 thr leuB6 lacY*	100	
SH206	pLC2-37 /SH181	69	*livH*
SH207	pLC2-38 /SH181	136	*livH*
SH208	pLC6-37 /SH181	126	*ftsE*
SH209	pLC9-12 /SH181	1440	*livH*
SH210	pLC19-48/SH181	124	*ftsE*
SH211	pLC31-16/SH181	110	*livK livJ ftsE htpR fam*
SH212	pLC31-32/SH181	98	*livK livJ ftsE htpR fam*
SH213	pLC41-22/SH181	156	*livP*

peptidase activity per cell of the eight strains was measured. The strain harboring pLC9-12 (SH209) had 14-fold higher γ-glutamyltranspeptidase activity than the wild type (Table 1). Periplasmic fractions were prepared from strains and subjected to native polyacrylamide gel electrophoresis and SH209 harboring pLC9-12 was found to overproduce γ-glutamyltranspeptidase protein (Fig. 1-A). pLC9-12 was isolated from 1 L culture, cleaved with restriction endonucleases and electrophoresed on agarose gel and polyacrylamide gel. Its restriction map was then established (Fig. 2).

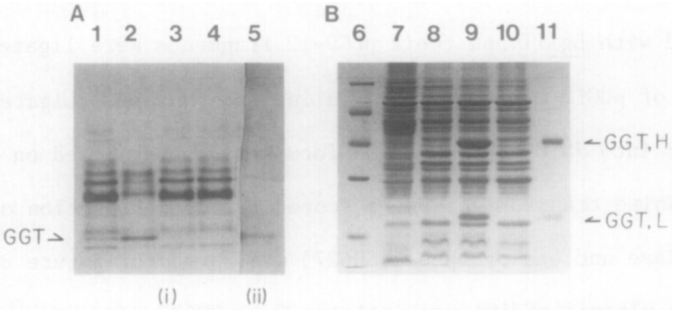

Fig. 1. (A) Native polyacrylamide gel electrophoresis of periplasmic fractions. Left half (i) was stained with Coomassie blue R-250 and right half (ii) was stained by γ-glutamyltranspeptidase activity staining (6). Arrows show the position of γ-glutamyltranspeptidase (GGT). Samples in lanes are as follows: lane 1 and 5, SH50 (wild type); 2, SH209 (pLC9-12/SH181); 3, SH684 (*ggt-1*); 4, SH703 (*ggt-2*)(ref. 8).
(B) SDS-polyacrylamide gel electrophoresis of periplasmic fractions. Gel was stained with Coomassie blue R-250. Positions of heavy subunit of γ-glutamyltranspeptidase (GGT, H) and that of light one (GGT, L) are shown by arrows. Samples in lanes are as follows: lane 6, molecular weight markers, 94, 67, 43, 30 and 20 kd from top to bottom; 7, SH50 (wild type); 8, SH641 (*ggt-2 recA*); 9, SH642 (pSH101/SH641); 10, SH643 (pUC18/SH641); 11, purified γ-glutamyltranspeptidase.

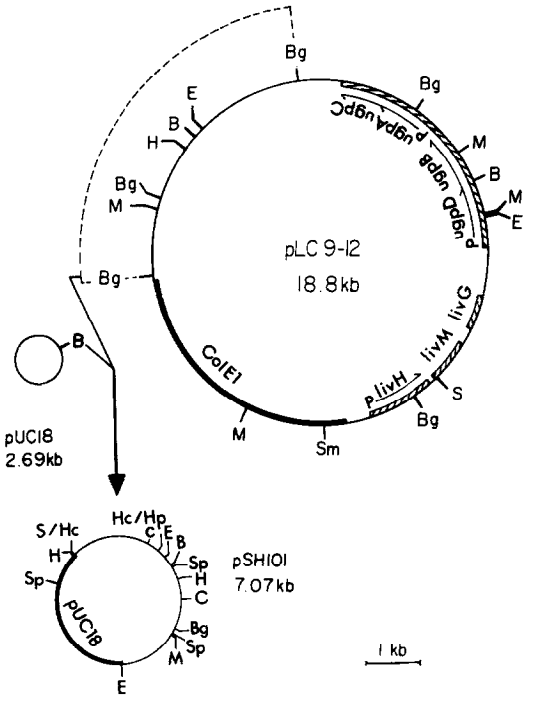

Fig. 2. Restriction maps of pLC9-12 and pSH101. DNA from ColE1 and pUC18 are shown by thick line in pLC9-12 and pSH101, respectively. Predicted locations of genes and promoters are shown by slashed boxes (▨) and letter P, respectively, as reported by other workers. Restriction endonuclease cleavage sites are shown by abbreviations as follows: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; Hc, HincII; H, HindIII; Hp, HpaI; M, MluI; S, SmaI; Sp, SspI.

From this restriction analysis we predicted that livH, M, G, and ugpD, B, A, C are on pLC9-12 in this order as reported by others (19-22). pLC9-12 was cleaved with BglII and their pLC9-12 fragments were ligated to the BamHI site of pUC18 treated with alkaline phosphatase. Ligated DNA was transformed into JM109 (9) and transformants were selected on X-gal plates at 37°C. White transformants were scored for overproduction of γ-glutamyl-transpeptidase and one of these (SH627) was saved for future use. The recombinant plasmid pSH101 was isolated from SH627 and a restriction map was established (Fig. 2). The 4.7kb BglII fragment of pLC9-12 was ligated

Table 2. Complementation test

Strain No.	Genotype	$Ggt^{+/-}$
SH646	F^- recA56 rpsL sr1300::Tn10	+
SH641	F^- ggt-2 recA56 rpsL sr1300::Tn10	-
SH642	pSH101/SH641	+++
SH643	pUC18/SH641	-

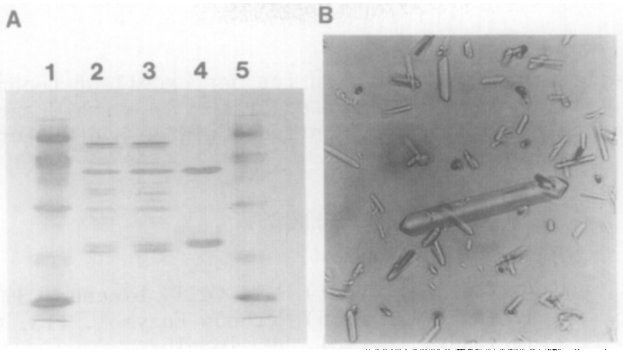

Fig. 3. (A) The stepwise purification of γ-glutamyltranspeptidase illustrated by SDS-polyacrylamide gel electrophoresis. Gel was stained by silver staining kit purchased from Wako Pure Chemical Co. Samples in lanes are as follows: lane 1 and 5, molecular weight markers as Fig. 1-B; 2, periplasmic fraction of SH642; 3, resuspended pellet from ammonium sulfate precipitation (60-80%); 4, γ-glutamyltranspeptidase after Chromatofocusing and concentration by ammonium sulfate precipitation.
(B) Crystals of γ-glutamyltranspeptidase. The photo was taken under a phase-contrast microscope.

to the BamHI site of pUC18. pSH101 complemented Ggt⁻ phenotype and the transformed cell (SH642) overproduced 37-fold γ-glutamyltranspeptidase compared to the wild type cells (Table 2 and Fig. 1-B). We developed a rapid method to purify γ-glutamyltranspeptidase from periplasic fraction of SH642 by ammonium sulfate precipitation and Chromatofocusing, and crystallized it (Fig. 3). By this method, the specific activity of the enzyme was increased 4.4-fold with a yield of 42% (Table 3). The minor small molecular weight γ-glutamyltranspeptidase fraction we obtained previously (6) was not observed this time. This may be because the proteolysis of γ-glutamyltranspeptidase that occurred previously during extended purification processes did not occur with this new rapid purification processes. It may be possible to obtain a much higher γ-glutamyltranspeptidase producing strain by connecting ggt to a tac promotor. We are in the process of DNA sequencing of ggt.

Table 3. Purification of γ-glutamyltranspeptidase from SH642

Steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
1. Lysozyme treatment	145.8	99.7	0.684	100
2. Ammonium sulfate (60-80%)	54.0	96.6	1.79	97
3. Chromatofocusing	13.9	41.6	3.00	42

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