Review

Bacterial $\gamma$-glutamyltranspeptidases, physiological function, structure, catalytic mechanism and application

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Abstract: $\gamma$-Glutamyltranspeptidase (GGT) has been widely used as a marker enzyme of hepatic and biliary diseases and relations between various diseases and its activity have been studied extensively. Nevertheless, several of its fundamental enzymatic characteristics had not been elucidated. We obtained homogeneous preparation of GGTs from bacteria, characterized them, and elucidated its physiological function that is common to mammalian cells, using GGT-deficient *E. coli*. Prior to GGT of all living organisms, we also identified catalytic nucleophile of *E. coli* GGT and revealed the post-translational processing mechanism for its maturation, and also its crystal structure was determined. The reaction intermediate was trapped and the structure-based reaction mechanism was presented. As for its application, using its transferase activity, we developed the enzymatic synthesis of various $\gamma$-glutamyl compounds that are promising in food, nutraceutical and medicinal industries. We found GGT of *Bacillus subtilis* is salt-tolerant and can be used as a glutaminase, which is important in food industry, to enhance umami of food, such as soy sauce and miso. We succeeded in converting bacterial GGT to glutaryl-7-aminocephalosporanic acid acylase, which is an important enzyme in cephem antibiotics production, by site-directed and random mutagenesis.

Keywords: $\gamma$-glutamyltranspeptidase, glutathione, glutathione-cycle, production of $\gamma$-glutamyl-compounds, three-dimensional structure, *Escherichia coli*

Introduction

$\gamma$-Glutamyltranspeptidase (GGT) is widely distributed in living organisms. The transpeptidation activity of GGT with glutathione (GSH) as the substrate was discovered in 1950 in sheep kidney. Since then, GGTs have been found in various tissues of mammals including kidney, liver, pancreas, and brain, and also found in other organisms such as higher plants and bacteria. GGT catalyzes the transfer of the $\gamma$-glutamyl residue of various $\gamma$-glutamyl compounds to water (hydrolysis reaction) and to amino acids or dipeptides (transpeptidation reaction) as shown in the following scheme.

\[
\gamma\text{-Glu-X} + \text{H}_2\text{O} \rightarrow \text{Glu} + \text{X} \\
\text{(hydrolysis reaction)}
\]

\[
\gamma\text{-Glu-X} + \text{Y} \rightarrow \gamma\text{-Glu-Y} + \text{X} \\
\text{(transpeptidation reaction)}
\]

\[
(\gamma\text{-Glu-X}, \gamma\text{-glutamyl compound}; \text{Y: amino acid or dipeptide})
\]
Above two reactions are chemically equivalent, but this enzyme is officially classified among the transferases, but not the hydrolases. The systematic name is (5-L-glutamyl)-peptide: amino-acid 5-glutamyl transferase (GGT; EC 2.3.2.2). The enzyme is also referred to as γ-glutamyltransferase (recommended by NC-IUBMB), γ-glutamyl peptidyltransferase and glutamyl transpeptidase. The name is often abbreviated as γ-GTP, GGT or γ-GT. The abbreviation γ-GTP is widely used in the diagnosis of liver diseases. Elevation of GGT activity in serum is associated with liver carcinogenesis, and is also induced significantly by other diseases such as alcoholic hepatitis, cholestasis and pancreatic disorders.

GGT plays an important role in GSH metabolism. A major physiological role of the enzyme is to cleave the extracellular GSH to use as a source of Cys, whose intracellular concentration is kept the lowest among amino acids because of its toxicity to the cell. The sulphydryl group of Cys easily dissociates at neutral pH, resulting in the thiolate anion. This thiolate anion generates active oxygen species including superoxide, and itself becomes a thyl radical to cause a radical reaction. On the other hand, the SH group of the Cys residue in GSH has a higher $pK_a$ value than free Cys and does not dissociate so easily, resulting in a safe thiol with low reactivity. Cys is stored in extracellular GSH and is supplied to the inside of cell when it is necessary. Another important role of GGT is to cleave glutathione-S-conjugates as a key step in detoxification of xenobiotics or in drug metabolism. This reaction is the first step in the conversion of glutathione-S-conjugates to the corresponding mercapturic acids, which are excreted in urine. These studies on GSH and GGT have been mainly conducted using mammals or their tissues. At the same time, enzymatic researches on mammalian origin GGTs were also actively pursued. However, mammalian GGTs exist as anchored state outside of cell-membranes and it is necessary to be solubilized by treatment with a detergent, organic solvent or proteinases. Furthermore, they have sugar chains attached to them in a fairly complex manner, which makes it difficult to obtain a homogeneous preparation.

Although the existence of GGT in bacteria was known, little had been studied regarding relationship with its physiological functions and GSH metabolism when we began the research on bacterial GGTs in 1984. We could obtain homogeneous preparation of bacterial GGTs because they existed in a soluble form and were not modified with sugar chains. Their enzymatic characteristics and physiological functions were studied using the purified enzyme preparations and with GGT deficient mutant of E. coli K-12. Not only we could elucidate processing and catalytic reaction mechanisms of GGT using bacterial GGTs prior to mammalian GGTs, but also we could determine the three-dimensional structure of GGT for the first time.

As a result of basic research on the bacterial GGT, it became possible to obtain a large amount of enzyme by their gene cloning and this enabled us to use GGTs for their application. The reactions catalyzed by EcGGT are quite pH dependent. Hydrolysis reaction proceeds at acidic pH, while transpeptidation reaction proceeds at alkaline pH. By adjusting the pH of the reaction solution, we can let the GGT catalyze one of the reactions selectively. When GGT hydrolyzes glutamine to glutamate, it is a glutaminase reaction important in food industry to increase umami taste. γ-Glutamylation can increase the solubility in water, stability in serum, and preferable taste of compounds. Taking advantage of the broad substrate specificity of bacterial GGTs, we developed the enzymatic synthesis methods of various γ-glutamyl compounds.

This review describes the summary of our research on bacterial GGTs.

**Purification of GGT from Proteus mirabilis, characterization and physiological function**

A homogeneous crystalline preparation of GGT was obtained for the first time from *Proteus mirabilis* (Pm) which belongs to *Enterobacteriaceae*. Since it has higher GSH metabolizing activity than many other bacterial strains,2) it was chosen as the source of GGT. GGT in mammalian tissues was reported to exist on the outer surface of the plasma membranes anchoring its N-terminal in the membrane and to exhibit secretory or absorptive functions. It can be solubilized from the membrane by treatment with a detergent, organic solvent or proteinases.3,4) On the other hand, *Pm*GGT could be easily solubilized by ultrasonic treatment of the cells, suggesting that it exists in soluble form in the cells as reported for other bacteria.5,6) The cellular localization of GGT was investigated by immunocytochemical and cell fractionation experiments. The antiserum prepared against the purified *Pm*GGT was found to inactivate GGT activity of both purified enzyme and intact cells. Native cells of *P. mirabilis* were aggregated...
with the polyclonal antibody. Indirect immunofluorescence and electron microscope analysis suggested that GGT is localized on the surface of the cell. Its distribution in the cell wall or periplasmic space or both was also confirmed by the treatment of cells with lysozyme and EDTA. That the addition of membrane phospholipids isolated from the same bacterium activated its hydrolytic activity four-fold indicates its loose binding to the membrane.\(^7\)

\(Pm\)GGT has an estimated molecular weight of 75K, consisting of two different subunits with molecular weight of 47K and 28K, which are similar to the subunits of rat kidney GGT.\(^3\),\(^4\) \(Pm\)GGT catalyzes both transpeptidation and hydrolysis of various \(\gamma\)-glutamylpeptides and uses many amino acids as acceptors of the \(\gamma\)-glutamyl residues as reported for hog kidney GGT.\(^8\) Orlowsky and Meister\(^9\) proposed that \(\gamma\)-glutamylcycle plays a role in the amino acid uptake through the cell membrane into cytoplasm and that GGT is a key enzyme in this system. Although \(K_m\) values for the \(\gamma\)-glutamyl donors, GSH and \(\gamma\)-glutamyl-p-nitroanilide, were low in the transpeptidation reaction as \(1.8 \times 10^{-4}\) and \(4.0 \times 10^{-4}M\), respectively, \(K_m\) values for the \(\gamma\)-glutamyl acceptors, Gly-Gly and L-Phe, were very high as \(2.9 \times 10^{-3}\) and \(3.2 \times 10^{-2}M\), respectively. These high \(K_m\) values for the acceptors suggest that this enzyme does not transport amino acid since amino acids do not exist at such high concentrations in natural environment.

The authors found that inhibition of \(Pm\)GGT during cultivation resulted in leakage of GSH into the medium.\(^10\) This phenomenon and cell localization of GGT and its affinity for the substrates suggest that intracellular GSH is transported to the outside of cell membrane and hydrolyzed by GGT. The function of GGT in bacterial cells is not the uptake of amino acids through cell membrane but the hydrolysis of GSH at the outside of cell membrane. A possible role of GSH may be the protection of cell membrane from oxidation agents including peroxides and radicals.\(^10\)

**Purification and characterization of GGT from \(E.\ coli\)**

\(E.\ coli\) K-12 was much more convenient and useful to perform physiological, enzymatic and genetic studies of GGT. All previous studies showed that \(E.\ coli\) had only weak or negligible GGT activity.\(^11\),\(^12\) We found that GGT activity in \(E.\ coli\) was greatly affected by the growth temperature, while that of \(P.\ mirabilis\) was not.\(^13\) The maximum transpeptidation activity per cell in \(E.\ coli\) at 20°C was more than twice of that in \(P.\ mirabilis\). Therefore, we decided to start studies on \(E.\ coli\) GGT.

Usually \(E.\ coli\) cells are cultivated at 37°C, but they showed the maximum activity of GGT when they were grown at 20°C, 14% of that at 37°C, and none at 43°C. As is described later, this is caused by the stability of mRNA of GGT at the low temperature. The enzyme activity of intact cells grown at 20°C was stably maintained even after the temperature was shifted up to 45°C. The activity increased during exponential phase, and the maximum activity was found at stationary phase. To investigate the intracellular localization of GGT, \(E.\ coli\) cells were fractionated by osmotic shock treatment and by lysozyme treatment, and both hydrolysis and transpeptidation activities of GGT were found in the periplasmic fraction in both cases.\(^13\) The result of lysozyme treatment is shown in Table 1.

We prepared periplasmic fraction and purified \(Ec\)GGT from this fraction, and obtained homogeneous preparation at the purification magnitude of 76-fold.\(^14\) In contrast, \(Pm\)GGT needed tremendously high (15,200-fold) purification since it was purified from whole cell homogenate.\(^1\) \(Ec\)GGT has the molecular weight of 61K consisting of two different subunits with molecular weight of 39.2K and 22K. The apparent \(K_m\) value for GSH as \(\gamma\)-glutamyl donor

| Cell fraction         | Protein | \(\gamma\)-Glutamyl transpeptidase | \(\gamma\)-Glutamyl hydrolase | 5' -Nucleotidase | \(\beta\)-Galactosidase |
|-----------------------|---------|-----------------------------------|----------------------------|----------------|----------------------|
| Periplasmic space     | 4.1     | 98.8                              | 96.7                       | 99.1           | 0.7                  |
| Outer membrane        | 1.2     | 0                                 | 0.6                        | 0.6            | 0                    |
| Cytoplasm             | 35.2    | 1.2                               | 0                          | 0.3            | 70.9                 |
| Spheroplast goast     | 59.5    | 0                                 | 2.7                        | 0              | 28.4                 |

Protein and enzyme activities were shown as % of total amount. Cells were grown in LB broth with 0.2% galactose at 20°C for 40h and then treated with lysozyme. 5'-Nucleotidase and \(\beta\)-galactosidase are marker enzymes for periplasmic and cytosol fractions, respectively. This table is reproduced from H. Suzuki et al. (1986) J. Bacteriol. **168**, 1332–1335 (ref. 13) with some modifications.
was $3.5 \times 10^{-5}$ M and those for Gly-Gly and L-Arg as acceptors were 5.9 and $2.1 \times 10^{-1}$ M, respectively. These high $K_m$ values for acceptors indicate that the transpeptidation reaction has no physiological role in the cells.

**GGT-deficient mutants and GSH cycle in *E. coli***

a) **Isolation of mutants and genetic mapping of the mutation.** By independent two mutation operations with ethylmethanesulfonate, *E. coli* mutants lacking GGT were isolated. In each screening, we got a few candidates from 624 and 540 isolates. And we left most likely ones from each screening and they are ggt-1 and ggt-2, respectively. Periplasmic fractions of two strains, which lost GGT activity completely, gave no precipitin line in Ouchterlony fusion test with antiserum against purified *Es*GGT generated in a rabbit. The mutations of two mutants that lost the enzyme activity completely were mapped by means of Hfr (high frequency of recombination) conjugation and P1 phage-mediated transduction. ggt was mapped at 76 min between glpD and uppA on the *E. coli* genome (map position recalculated after the genome project is position 77.24 min). 17)

The mutations occurred in ggt gene region of the genomes of the mutant strains were identified by DNA sequencing. The ggt-1 has one base deletion (deleted 3,586,403₁⁷A) and three base substitutions (substituted 3,586,078₁⁷T to A, 3,585,911₁⁷T to A, and 3,585,347₁⁷G to A) on the *E. coli* K-12 substr. MG1655 genome. And it is suggested to produce a truncated protein consists of 128 amino acids. On the other hand, ggt-2 deleted 2,714 bases from 3,584,196₁⁸ to 3,586,909₁⁸ of the genome and completely lost the coding region of ggt (H. Suzuki, N. Miyakawa and H. Kumagai, unpublished result).

b) **Elucidation of the physiological role of GGT in Cys salvage pathway using GGT-deficient mutants.** Since *E. coli* showed the maximum GGT activity when it was grown at 20°C but no activity above 43°C, the GGT-deficient mutant was expected to be low-temperature sensitive as to growth or nutrient requirement. However, GGT-deficient mutants grew normally on rich and minimal medium plates at all temperatures tested. Both the wild type and GGT-deficient mutant (Δggt-2) leaked more GSH into the media at around 18 and 40°C than other temperatures, but at all temperatures tested about three-fold GSH was found in the medium of GGT-deficient mutant comparing with that of the wild-type strain. The wild-type *E. coli* accumulated the maximum amount of GSH in the medium during the early stationary phase, but its concentration decreased rapidly during stationary phase. On the other hand, its concentration in the medium of GGT-deficient mutant decreased much more gradually during stationary phase (Fig. 1). 16)

We speculated that some enzymes besides GGT that cleave GSH in the medium and/or some transporters that take GSH up from the medium might exist. As is described later, a novel GSH importer was found.

The physiological role of GGT in GSH metabolism of *E. coli* cells was investigated using GGT-deficient mutants. A Cys auxotroph and a Gly auxotroph were examined to determine if they could utilize GSH as a Cys source and a Gly source, respectively. The Cys auxotroph grew on M9 glucose minimal medium plates supplemented with GSH as well as with Cys, Cys-Gly or γ-Glu-Cys. However, a Cys auxotroph with GGT-deficient mutation did not grow on M9 glucose plates supplemented with GSH or γ-Glu-Cys, while it grew on ones supplemented with Cys or Cys-Gly. Similar results were obtained in the case of the Gly auxotroph. These results indicate that *E. coli* can utilize exogenous GSH as a Cys
source and a Gly source, and that GGT takes an essential role for this. One of the important physiological roles of GGT in E. coli cells could be the catalysis of the initial step of salvage or recycling of Cys. It was also shown in mammalian cell line and in yeast that GGT catalyzes the initial step of the cleavage of extra cellular GSH to utilize as a Cys source and nitrogen source, by other researchers. When the GGT activity of intact cells of wild-type E. coli was measured, the transpeptidation activity was negligible. Besides, since the $K_m$ values for $\gamma$-glutamyl acceptors in the transpeptidation reactions were extremely high, it is hard to imagine that the transpeptidation reaction has any physiological role in E. coli cells. Therefore, we suggested that GGT locates in the periplasmic space hydrolyzes the $\gamma$-glutamyl linkage of a $\gamma$-glutamyl amino acid and thus released amino acid is taken up and utilized by E. coli.

c) Identification of E. coli enzymes with cysteinylglycinase (CGase) activity. After cleavage of GSH by periplasmic GGT, the Cys-Gly released is thought to be taken into the cells and cleaved to Cys and Gly by the action of some peptidases with CGase activity to utilize it as a Cys or Gly sources, respectively. We investigated the peptidases which cleave Cys-Gly in E. coli, because there had been no report on CGase activity of E. coli, since McCroquodale reported it in 1963. Although there were several peptidases known in E. coli, it has never been reported whether these peptidases can cleave the peptide bond of Cys-Gly or there is an unknown CGase. We compared the CGase activities of several strains deficient in aminopeptidases with broad substrate specificities, and found that cytoplasmic aminopeptidases A, B and N, and dipeptidase D to be cooperatively responsible for this activity.

Among these peptidases, the gene of aminopeptidase B, which is the least known peptidase, was cloned. Then the enzyme was purified and characterized. It was suggested to be a homo-hexameric metal peptidase exhibiting high homology with lencine amino peptidase of mammals and aminopeptidase A of E. coli. We proposed GSH cycle system in E. coli and that this cycle acts as a salvage system of Cys and Gly in the cells (Fig. 2). E. coli synthesizes GSH by the sequential actions of $\gamma$-Glu-Cys synthetase (the product of gshA) and GSH synthetase (the product of gshB) as in other organisms. It excretes GSH into culture medium during the exponential phase and the concentration of GSH in the culture medium reaches the maximum in the early stationary phase, but thereafter it is hydrolyzed by GGT in the periplasm to liberate Glu and Cys-Gly. Cys-Gly is taken up into the cytoplasm and then cleaved into Cys and Gly by aminopeptidases A, B and N, and dipeptidase D to be utilized as a source of Cys and Gly.

d) First discovery of an ABC transporter that imports GSH into cells through all organisms. Even in the case of a GGT-deficient strain of E. coli, the concentration of GSH in the culture medium decreased gradually after long-time incubation (Fig. 1). This finding prompted us to search for a GSH transporter which had never reported in bacteria. The ybiK gene of E. coli was reported as a member of the CysB regulon and it was suggested to encode a protein involved in GSH transport of metabolism, but its mechanism was unclear. GenBank suggested that four genes, yliA, yliB, yliC, and yliD, located downstream of ybiK, are transcribed with ybiK. EcoCyc data base suggested that YliA, B, C, and D constitute an uncharacterized member of the ATP-binding cassette superfamily transporters. It suggested that yliA and yliB encode the ATP binding component and periplasmic binding protein, respectively, and that yliC and yliD encode the plasma membrane components. From the above information, we speculated that YliA, B, C, and D might compose the GSH transporter.

Since the wild-type E. coli accumulates only several µM of GSH in the medium even at the maximum, it is difficult to measure such a low concentration of GSH. Therefore, the strains transformed with a plasmid harboring the gshA and gshB genes on pBR322 were constructed to overproduce GSH-synthesizing enzymes. There was little difference in the growth among the strains used. The
effects of \( \Delta ggt \) and \( \Delta yliAB \) on the concentration of extracellular GSH were compared in this GSH overproducing construct. The concentration of extracellular GSH decreased after reaching the maximum during the early stationary phase when either of \( ggt \) or \( yliAB \) was normal. That \( \Delta ggt \) \( yliA^+ \) \( yliB^+ \) strain accumulates much more GSH in the medium than the \( ggt^+ \) \( yliAB \) strain indicates that GGT is more effective in reducing the concentration of extracellular GSH than the YliABCD transporter. On the other hand, when both \( ggt \) and \( yliAB \) were deleted, the concentration of the extracellular GSH was kept high up to 55 h. Complementation of the \( \Delta ggt \) \( yliAB \) strain with pACYC177 containing the \( ybiK^+ \) \( yliA^+ \) \( yliB^+ \) \( yliC^+ \) \( yliD^+ \) opeon dramatically decreased the extracellular GSH. These results indicate that GGT and the YliABCD transporter are the two determinants that decrease the extracellular GSH. Then, the ability of YliABCD transporter was confirmed using \([35S] \) glutathione and GGT-deficient derivatives (Fig. 3).28) The \( yliAB \) strain transported practically no GSH, while its \( yliA^+ \) \( yliB^+ \) derivative obviously transported GSH. Moreover, \( yliAB \) strain transformed with pACYC177 containing \( ybiK^+ \) \( yliA^+ \) \( yliB^+ \) \( yliC^+ \) \( yliD^+ \) (SH1552 and SH1617) complemented the GSH transport phenotype. However, the same host strain transformed with pACYC177 containing \( ybiK^+ \) \( yliA^+ \) \( yliB^+ \) \( yliC^+ \) (SH1554) and the same strain transformed with pACYC177 containing \( ybiK^+ \) \( yliA^+ \) \( yliB^+ \) \( yliD^+ \) (SH1620) (Fig. 3B) almost completely lost GSH transport activity, albeit they uptake slightest GSH. And this slightest GSH transport activity may have been observed because a little read through to \( yliC \) and \( yliD \) caused by the small insertion sequence left. That transport of GSH was inhibited by verapamil, but not by carbonylcyanide-m-chlorophenylhydrazone, indicates that this transport system depends on ATPase activity and not on membrane potential. The results indicate that YliABCD composes an ATP-binding cassette transporter, YliA and -B being an ATP-binding component and periplasmic binding protein, respectively, and YliC and -D being plasma membrane components. The role or involvement of YliK in this GSH transport system is not clear yet.

This was the first finding not only as bacterial GSH transporter but also as a GSH importer with an ATP binding cassette among all organisms.28)

**Cloning and sequencing of E. coli K-12 ggt**

When we cloned the genomic \( ggt \) gene of \( E. coli \), the cDNA of rat renal GGT29),30) and the cDNA of human hepatic GGT,31) human placental GGT32) and human hepatoma GGT33) had already been cloned and their nucleotide sequences had been determined. Amino acid sequences of human GGTs from different sources were essentially the same. Mature GGT consists of one large and one small subunit.1),14),34) Mammalian GGTs were reported in these papers to be synthesized as single polypeptides and then processed into large and small subunits. We cloned \( E. coli \) \( ggt \) gene and determined its DNA sequence to compare with those of mammals for more information about structure and function of GGTs.

Since \( E. coli \) \( ggt \) was mapped at 76 min on its genome,16) eight strains of Clarke-Carbon colony bank35) known to harbor pLC plasmid with \( htpR, \) \( fam, \) \( fisE, \) \( livH, \) \( livK \) or \( livJ \) gene(s) mapped at 76 min of \( E. coli \) genome, were screened for pLC plasmids with \( ggt \). The strain harboring pLC9-12 had 14-fold higher GGT activity than the wild type. The \( ggt \) was subcloned to pUC18 and plasmid pSH101 was obtained. Ggt- phenotype of GGT-deficient mutant was complemented by pSH101. The specific activity of the enzyme of the cells harboring pSH101 was 37-fold higher than that of the wild-type cells. GGT was isolated from the periplasmic fraction of the cells by simple two steps and crystallized.36) We also employed \( E. coli \) tolA mutant strain that pleiotropi-
cally releases periplasmic enzymes, transformed it with plasmids carrying the ggt gene and obtained GGT-leaky strain which leaks about 70% of its GGT into the culture medium.\cite{37,38} The excreted GGT was purified to electrophoretic homogeneity by a simple two-step method with a yield of 51\%.\cite{39}

The DNA sequence of ggt of E. coli was determined.\cite{40} The sequence contains a single open reading frame encoding the signal peptide, and the large and small subunits, in this order (Fig. 4). The gene structure indicated that EcGGT is synthesized as pro-GGT and processed later into the large and small subunits, as in the case of mammalian GGTs. The molecular weight of the mature EcGGT was calculated to be 59,207.89 consisting of one large subunit (molecular weight, 39,197.68; 365 amino acid residues) and one small subunit (molecular weight, 20,010.21; 190 amino acid residues). The amino acid sequence of EcGGT was compared with that of the rat renal GGT and with that of the human hepatic GGT. Although the identity with the E. coli and mammalian GGTs are relatively low, most parts of the proteins are conserved through conservative substitutions of amino acids. The exception was the first 50 amino acids of the EcGGT, with which mammalian GGTs do not show any similarity at all. This is because the N-terminal of the large subunit of mammalian GGTs are the anchor region buried in the plasma membrane, while the N-terminal of E. coli GGT is a signal peptide cleaved off when GGT is translocated into the periplasmic space.

**Analysis of low-temperature dependent expression of EcGGT**

As described above, the enzyme activity of intact cells grown at 20°C was stably maintained even after the temperature was shifted up to 45°C.\cite{13} This result supported a hypothesis that EcGGT is synthesized only at lower temperature. Therefore, low-temperature inducible mechanism of EcGGT was investigated.\cite{41} Western blot analysis showed that E. coli cells cultured at 20°C produced 6-fold more GGT protein than those cultured at 37°C. That the quantity of GGT precursor in the cells cultured at 20°C and 37°C was very low indicates that the difference in the GGT activity by the cultivation temperature is due to the amount of mature GGT protein, not the processing rate, and that once GGT protein is translated, it is processed immediately. The same phenomena were also observed in E. coli B and Salmonella typhimurium. We obtained results suggesting that the higher GGT activity in E. coli cells grown at 20°C was due to higher amount of GGT protein at 20°C. This is caused by higher amount of ggt mRNA exists in the cells grown at 20°C depending on a low-temperature induced ggt promotor and the stability of ggt mRNA at 20°C.\cite{41} Physiological significance of GGT inducibility at low temperature is not known.

**Subunit assembling of EcGGT**

EcGGT is synthesized as pro-GGT and processed later into large and small subunits. The relationship between the subunit construct and the catalytic function of GGT was investigated by once separating into each subunit and then reassembling them.\cite{42} Large and small subunit of EcGGT were separated from each other by reverse-phase HPLC and the masses of each subunit were determined by ion spray mass spectrometry to be 39,207 and 20,015, respectively. There was virtually no enzymatic activity in each subunit alone, but a mixture of the two subunits showed partial recovery of the enzymatic activity. Partial reassociation of the subunits was also verified by native-polyacrylamide gel electrophoresis followed by silver staining and the exhibition of the enzymatic activity of the re-associated band was observed by the activity staining. To investigate in vivo association of the subunits, two kinds of plasmid were constructed encoding the signal peptide and either the large or small subunit. A GGT-deficient mutant of E. coli was transformed with each plasmid or with both of them. A small amount of each subunit protein was observed in the periplasmic space in the strain harboring the plasmid encoding the corresponding
subunit but exhibited no enzymatic activity. The strain transformed with both plasmids together exhibited the enzymatic activity, albeit its specific activity was approximately 3% of that of a strain harboring a plasmid encoding the intact structural gene. These results indicate that a portion of the large subunit and the small subunit can associate in vitro and in vivo, and be folded into an active structure, though the specific activity of the subunits associated was much lower than that of native enzyme. This suggests that the synthesis of GGT in a single precursor polypeptide and the subsequent processing are more effective to construct the intact structure of GGT than the association of the separately synthesized large and small subunits.42) 

It was recently found by chance that the recovery of the enzyme activity after thermal denaturation of EcGGT by the long-time incubation at 4 °C. In case of EcGGT, 80–90% of the enzyme activity recovered after 2 days of incubation at 4 °C. In contrast, GGT of Bacillus subtilis (BsGGT) did not recover its activity, on the contrary the activity decreased further by incubation at 4 °C.43) It was suggested that dissociation of subunits occurred by heat-treatment but, in the case of EcGGT, electrostatic complementarity between the subunit interfaces of each subunit facilitates heterodimeric association.

Identification of the catalytic nucleophile of EcGGT

The amino acid residue with catalytic nucleophile of GGT had been studied for a long time using its potent inhibitor, acivicin, a heterocyclic glutamate analogue produced by Streptomyces albus. However, the adduct formed between GGT and acivicin is an unstable hydroximic ester. That hydrolysis of this ester bond or transesterification to other amino acid with OH group in the enzyme caused by the harsh chemical reactions used to identify the modified amino acid residue resulted in the misidentification of the active site residue (Scheme 1).

The reaction catalyzed by GGT consists of two steps: (i) the active Oγ atom of Thr-391 attacks the carbonyl carbon atom of the γ-glutamyl compound

![Scheme 1. Comparison of affinity-labeling of the enzyme by acivicin and AFPBA.](image-url)
to form the $\gamma$-glutamyl-enzyme intermediate via tetrahedral intermediate, and (ii) the $\gamma$-glutamyl moiety is transferred to another substrate or the $\gamma$-glutamyl-enzyme bond is hydrolyzed to reform the resting enzyme (Scheme 2). The chemistry of the reaction catalyzed by GGT was suggested to be essentially the same as that of the well-known Ser proteases. Therefore, it was predicted that electrophilic organophosphorus compounds could be used to trap the catalytic nucleophile of GGT as were used to trap the active-site of Ser proteases. A phosphonic acid monofluoride derivative of glutamic acid, 2-amino-4-(fluorophosphono)butanoic acid (AFPBA), was developed as a mechanism-based affinity labeling agent. GGT was inhibited by AFPBA in a time-dependent manner. Since the inactivation rate was decreased by increase of the substrate concentration, AFPBA was suggested to compete with the substrate for the active site of GGT. Since the activity of the inactivated enzyme was not recovered even after prolonged dialysis, AFPBA was suggested to bind covalently to the catalytic nucleophile in a mechanism-based manner, forming a transition-state like adduct in the active site of GGT (Scheme 1). The stability of the phosphonylated adduct of GGT enabled us to identify the catalytic nucleophile. Ion-spray mass spectrometry of the modified GGT revealed that AFPBA was covalently and stoichiometrically attached to the small subunit. Protease digestion with lysyl endopeptidase and LC/MS analysis of the labeled small subunit identified the N-terminal peptide Thr-391-Lys-399 as the phosphonylation site. Subjecting this peptide to the MS/MS analysis, we could identify the phosphonylated amino acid residue to be Thr-391, the N-terminal residue of the small subunit (Fig. 5). Several other studies also support this result. First, Thr-391 of EcGGT is conserved among all GGTs whose primary sequences were known. Second, GGT was predicted to be a member of N-terminal nucleophile (Ntn) hydrolases, because the preliminary crystal structure of E. coli enzyme has shown the presence of two antiparallel pleated $\beta$-sheets, which is unique to this family. EcGGT was also predicted to belong to Ntn hydrolase superfamily from the fact that this enzyme is post-translationally processed to the active

Scheme 2. Catalytic mechanism of GGT. Oxyanion hole is shown by circle.
mature enzyme from the catalytically inactive precursor,\(^{40,42}\) which is a characteristic of N-terminal nucleophile hydrolases. In all Ntn hydrolases, the N-terminal residues newly generated by the processing are the catalytic nucleophile, and Thr-391 of EcGGT corresponds to those. Thus, we concluded that oxygen atom of the side chain of the N-terminal Thr-391 is the catalytic nucleophile of EcGGT.\(^{45}\)

Crystallographic study of EcGGT

a) Classification of GGT as a member of Ntn hydrolase superfamily from its three-dimensional structure. Preliminary study on the crystallization and X-ray diffraction analysis of EcGGT was conducted.\(^{47,48}\) Since the detail structure could not be determined by the multiple isomorphous replacement method, we prepared Se-Met derivative of EcGGT to determine EcGGT structure by the anomalous dispersion method. The metA \(^{\Delta}ggt-2\) strain was constructed and it was transformed with the plasmid harboring *E. coli ggt* gene. This strain was grown in M9 glucose minimal medium supplemented with ampicillin and Se-Met, and GGT was induced by the addition of isopropyl-\(\beta\)-D-thiogalactopyranoside, but without any other complicated supplements unlike what was normally done by other researchers. Then, Se-Met incorporated GGT was purified from the cells. The GGT structure was refined at 1.95 Å resolution as shown in Fig. 6.\(^{49}\) One side of the small subunit is surrounded by the large subunit. The GGT heterodimer has a stacked \(\alpha_3\beta_3\)-core; the \(\beta\)-strands of the large and small subunits form the two central \(\beta\)-sheets lining like slotted boards, and these \(\beta\)-sheets are sandwiched by \(\alpha\)-helices. The topology of GGT is similar to those observed in Ntn hydrolase superfamily members such as aspartylglucosaminidase\(^{50}\) and penicillin acylase.\(^{51}\) Such topology is conserved among the members of this superfamily. The main chain atoms of the 66 residues comprising the core of EcGGT are superimposable on those of human aspartylglucosaminidase with an rms deviation of 1.3 Å.

b) The structure of \(\gamma\)-glutamyl-enzyme intermediate presenting comprehensive reaction mechanism from bacteria to mammals. The substrate-binding pocket is the cavity and the catalytic residue, Thr-391, is located at the entrance of the deep groove, and the binding pocket extends from Thr-391 into the enzyme (Fig. 7A). The \((F_o-F_c)\) map near the substrate-binding pocket of the flash cooled crystal after soaking in a GSH-containing solution for about 10 s is shown in Fig. 7B. The \(\gamma\)-glutamyl moiety bound in the pocket was clearly visible in the electron density map. Remarkably, a covalent bond existed between the carbonyl carbon of the \(\gamma\)-glutamyl moiety and the O\(\gamma\) atom of Thr-391. This result clearly indicated that the attack of Thr-391 O\(\gamma\) on the carbonyl carbon at the \(\gamma\)-position of GSH results in \(\gamma\)-glutamyl-enzyme intermediate. The \(\alpha\)-carboxyl and \(\alpha\)-amino groups of the \(\gamma\)-glutamyl moiety locates at the bottom of the enzymatic pocket are hydrogen-bonded with many residues of the enzyme (Fig. 7B). The side chain O\(\gamma\) of Tyr-444 which exists at the tip of the lid-loop is hydrogen-bonded with Asn-411 O\(\delta\) (Fig. 7A). This hydrogen bond is thought to stabilize a lid-loop to shield the pocket from solvent. The residue corresponding to Tyr-444 is Phe in mammalian GGTs, suggesting the lid-loops of mammalian GGTs are more flexible than that of EcGGT. The structural differences may be one of the reasons that EcGGT is several hundred-fold less active measured in transpeptidation reaction than mammalian GGTs. Unfortunately, the amino acid residues which recognize the acceptors have not

![Fig. 5. MS/MS analysis of the peptide Thr-391-Lys-399. (A) Predicted mono isotopic masses for product ions of type y derived from the sequence shown above. (B) CID spectrum of the m/z 350.8 precursor ion from the unmodified enzyme. (C) CID spectrum of the m/z 405.8 precursor ion from the modified enzyme. This figure is reproduced from M. Inoue et al. (2000) Biochemistry 39, 7764–7771 (ref. 45).](image-url)
been identified. The comparison of the acceptor sites will enable more discussion over the difference of the transpeptidation activity. The length and width of the pocket is sized exactly to accept the \(\gamma\)-glutamyl moiety, with the active site being solvent accessible at the \(\gamma\)-glutamyl linkage. Thus, when GSH is bound to EcGGT, its Cys-Gly moiety would be exposed in the solvent.

The sequence alignment of several GGTs from typical organisms is shown in Fig. 8. The active residue Thr-391, Thr-409 forming hydrogen bond with Thr-391 O\(\gamma\), and Gly-483 and Gly-484 forming the oxyanion hole are completely conserved.

Arg-114, Asp-433, Ser-462, and Ser-463 in EcGGT correspond to Arg-107, Asp-423, Ser-451, and Ser-452 in human GGT, respectively, and these residues are strictly conserved in all GGTs (Fig. 8). Substitution of these residues of human GGT resulted in severe loss of the enzymatic activity.\(^{52-54}\)

Substitution of Arg-114 of EcGGT also reduced enzymatic activity significantly (H. Suzuki, Y. Morishita and H. Kumagai, unpublished result).

Figure 7B indicates that all of these residues are involved in the hydrogen bonds with the \(\alpha\)-amino and \(\alpha\)-carboxyl groups of the \(\gamma\)-glutamyl moiety. These results showed that the mechanism of substrate binding and formation of \(\gamma\)-glutamyl-enzyme intermediate is conserved among bacterial and mammalian GGTs, and that the slightest deviations in these critical residues dramatically affect enzymatic activity.

c) Development of GSH analogous peptidyl phosphorus esters. A series of GSH analogous peptidyl phosphorus esters, which are mechanism-
Based inhibitors of human GGT and EcGGT, were synthesized to evaluate the structural and stereochemical preferences of the Cys-Gly binding site of GGTs. Both enzymes were inhibited strongly and irreversibly by these inhibitors with phenoxide as a good leaving group. It was found that human GGT was highly selective for L-aliphatic amino acid such as L-2-aminobutyrate (L-Cys mimic) at the Cys binding site, whereas EcGGT significantly preferred L-Phe mimic at this site. That the C-terminal Gly and an L-amino acid analogue at the Cys binding site were necessary to inhibit human GGT, suggests that human GGT was highly selective for GSH. On the other hand, EcGGT was not selective for GSH, but still retained the dipeptide binding site.

**d) The reaction mechanism of EcGGT deduced from the structure of γ-glutamyl-enzyme intermediate.** The reaction catalyzed by GGT consists of two steps: (i) the active Oγ atom of Thr-391 attacks the carbonyl carbon atom of the γ-glutamyl compound to form the γ-glutamyl-enzyme intermediate via tetrahedral intermediate, and (ii) the γ-glutamyl moiety is transferred to another substrate or the γ-glutamyl-enzyme bond is hydrolyzed to reform the resting enzyme (Scheme 2). That the structure of γ-glutamyl-enzyme was observed by soaking the crystal of mature GGT in GSH solution (Fig. 7B) indicates that GGT is enzymatically active in the crystalline state. This also indicates that the second step of the reaction, the hydrolysis of the intermediate, is much slower than the first one.

It should be noted that the electron-density ascribable to a water molecule (W2) in the γ-glutamyl-enzyme intermediate was observed above the carbonyl group that connects the γ-glutamyl moiety and the enzyme (Fig. 7B). This water molecule most likely attacks the carbonyl carbon of the γ-glutamyl-enzyme intermediate as the nucleophile in the second step. In the γ-glutamyl-enzyme intermediate, Thr-391 Oγ is hydrogen-bonded to Thr-409 Oγ and the carbonyl oxygen atom is hydrogen-bonded to the nitrogen atoms of Gly-483 and Gly-484. The carbonyl carbon is likely to adopt a tetrahedral arrangement upon W2 attacking the carbonyl carbon atom. The structure of γ-glutamyl-enzyme intermediate appears analogous to that seen in the acyl intermediates of serine proteases, where the site surrounded by the carbonyl oxygen and the nitrogen atoms of Gly-483 and Gly-484 is the oxyanion hole.
Post-translational processing of EcGGT

a) Importance of N-terminal amino acid sequence of the small subunit on processing. Amino acid residues at the cleavage site for processing were substituted by site-directed mutagenesis to investigate the maturation of GGT.\textsuperscript{57} The processing phenotypes of mutants were examined by Western blot analysis, and their GGT activities were also measured. Substitution of amino acid residues, destined to become the N-terminal three amino acid residues of the small subunit (Thr-391, Thr-392 and His-393) after processing, prevented enzyme maturation and the immature mutant enzymes exhibited no enzymatic activity. A mutation at the C-terminal residue of the large subunit (Gln-390) had less effect on the processing and the enzymatic activity. These results suggested that the sequence of threonylhistidinyl residues at the N-terminal of the small subunit is very important for the processing of EcGGT and this post-translational processing is essential for EcGGT to acquire the enzymatic activity.\textsuperscript{57}

b) Evidence of autocatalytic processing of pro-GGT. The processing of four Ntn hydrolases, the \(\beta\)-subunit of 20S proteasome, glycosylasparaginase, penicillin acylase, and cephalosporin acylase, were experimentally shown to be autocatalytic, as originally predicted by Brammigan \textit{et al.}\textsuperscript{40} in structural studies. However, it was not shown whether this feature is common to all members of the Ntn hydrolase superfamily. Various evidences strongly suggested that GGT belongs to the Ntn hydrolase superfamily. It could not be classified to this superfamily because the three-dimensional structure of GGT was not available at that time. Supposing GGT is an Ntn hydrolase, it is possible that it processes autocatalytically. Mutant precursors, pro-MBP (maltose binding protein)-GGT, pro-T391S, and pro-T391C were purified to electrophoretic homogeneity, and the processing mechanism of EcGGT was investigated in \textit{vivo} using these precursors.\textsuperscript{58} Nonprocessing (pro-T391A) or slow processing (pro-T391C and pro-T391S) precursors of GGT as well as mature form MBP-GGT expressed from pMAL-p2-ggt, a plasmid carrying genes of MBP without signal peptide region and GGT, accumulated in the cytoplasm as an insoluble fraction. After collecting the insoluble fraction by centrifugation, it was denatured with guanidine HCl, and pro-MBP-GGT was purified with Sephacryl S-300 column equilibrated with guanidine HCl. When GGT precursors were incubated in the buffer (pH 6–8.5), they were processed into two subunits in the absence of another protein (protease). It was also shown that the processing of pro-MBP-GGT and pro-T391S took place at exactly the same position as where the wild-type GGT is processed \textit{in vivo}, \textit{i.e.} between residues corresponds to residues 390 and 391 in the wild-type EcGGT (Fig. 9).\textsuperscript{58} pro-T391C was also processed autocatalytically, albeit the rate of processing was extremely slow. In the case of mammalian GGT, Kuno \textit{et al.}\textsuperscript{59} suggested that a membrane-bound trypsin-like serine protease cleaved pro-GGT. The possibility of contaminated proteases in the precursor samples was excluded by the addition of the conventional protease inhibitors. Besides, first-order reaction kinetics of the processing reaction also supports that this is an intramolecular event as discussed by Guan \textit{et al.}\textsuperscript{40} Thus, autocatalytic processing of GGT was confirmed, and the involvement of proteases was excluded.

c) Identification of the catalytic nucleophile of the autocatalytic processing. Then, which residue(s) is responsible for the autocatalytic processing of GGT? The importance of Thr-391 was predicted from the effects of mutation of this residue to Ala, which yielded in a nonprocessing precursor.\textsuperscript{57} On the other hand, when Thr-391 was replaced by Ser or Cys, the derived precursors were processed, albeit slowly, as described above. There is no Cys residue in the large subunit or the small subunit of EcGGT.\textsuperscript{40} Neither pro-MBP-GGT nor pro-T391S have Cys residue, and the only Cys residue of pro-T391C is Cys-391, which was replaced by Thr-391. p-Chloromercuribenzoate (pCMB) did not affect the processing of pro-MBP-GGT or pro-T391S, whereas it completely abolished the processing of pro-T391C. This indicated that pCMB bound to the thiol group of Cys-391 of pro-T391C and thereby inhibited its processing (Fig. 10). We concluded that the catalytic nucleophile for the processing reaction of the wild-type EcGGT is the oxygen atom of the side chain of Thr-391 which is destined to be the N-terminal
residue of the small subunit and is also the catalytic nucleophile of the enzymatic reaction. This is compatible with the fact that the nucleophile of the enzymatic reaction is also the nucleophile of the processing reaction for several Ntn hydrolases.

d) Intramolecular autocatalytic processing of GGT via ester intermediate. Hydroxylamine, NH₂OH, is a strong nucleophile, and thioesters are particularly reactive with NH₂OH at neutral pH, whereas oxygen esters react slowly. Guan et al. showed that the initial step of autocatalytic processing of glycosylasparaginase, a member of Ntn hydrolases, is an N-O or N-S acyl shift at its residue 152 to yield an ester intermediate because the processing rate of the precursor, whose Thr-152 is replaced by Cys, was very much accelerated by the addition of NH₂OH at pH 7. The autocatalytic processing of pro-T391C of EcGGT was apparently accelerated much more than that of pro-T391S in the presence of NH₂OH at pH 7. This indicated that the processing of the wild-type GGT proceeds via the ester intermediate by N-O acyl shift. This result also shows that the autocatalytic processing of GGT is an intramolecular event. The proposed mechanism underlying intramolecular autocatalytic processing of GGT is summarized in Scheme 3.

The structure of EcGGT mutant, T391A, and the maturation mechanism inferred from the structure

As mentioned above, the T391A mutant protein lost intramolecular autocatalytic processing activity. T391A protein was expressed in E. coli,
purified and crystallized. The crystal structure of the protein was refined to 2.55 Å resolution. The core region of T391A protein is similar to the mature GGT as shown in Fig. 11B. Although the C-terminal residue of the large subunit, P-segment (residues 375–390), is far from the N-terminal Thr-391 of the small subunit in the mature GGT (Fig. 11B), the P-segment of the T391A protein occupied the active site groove (Fig. 12A). Displacement of the P-segment by the cleavage of the Gln-390-Thr-391 peptide bond caused the rearrangements of several adjacent segments. The P-segment itself flipped around Ile-378 and the P-segment directed opposite in the T391A protein and in the mature GGT (Fig. 12A). Displacement of the P-segment out of the pocket, the 411–416 segment shifts markedly (>1 Å) toward residues 482–485, narrowing the substrate-binding pocket to the size just fit for the \( \gamma \)-glutamyl moiety. These rearrangements complete the structure of the substrate-binding pocket, so that it is exactly able to recognize the \( \gamma \)-glutamyl moiety.

The structure analysis of several forms of GGT suggest that the substrate-binding pocket of mature GGT is open to the solvent for substrate introduction when the lid-loop is in the flexible form, and that the pocket is shielded by the lid-loop in the closed form when the substrate is bound to the pocket.

The structure of the T391A protein mimics the structure of the wild-type precursor. Based on the crystal structure of the T391A protein, Ala-391 was substituted by Thr on a computer, and the orientation of Thr-391 and the position of the water molecule (W4) found in the structure of the T391A protein were optimized to determine the wild-type precursor. Thus, we performed the modeling of the processing site of the precursor. It was predicted that the O\( \gamma \) atom of the Thr-391 would be located at the position that would easily attack the carbonyl carbon of Gln-390 (Fig. 13). A water molecule (W4) forms hydrogen bonds with O\( \gamma \) atom of Thr-391 and with Ser-388. The O\( \gamma \) atom of Thr-391, activated by this water molecule as a base, nucleophilically attacks the carbonyl carbon of Gln-390 to form an ester intermediate by N-O acyl shift via a tetrahedral intermediate, and the ester bond is hydrolyzed as we suggested before Scheme 3.)
Synthesis of γ-glutamyl compounds with GGT

There are various γ-glutamyl compounds including GSH, which play important roles in living organisms and they are produced chemically or enzymatically to use as pharmaceuticals or food additives. Since bacterial GGTs have low specificity for the acceptor in the transpeptidation reaction,\(^1\),\(^14\) it is a good biocatalyst to synthesize various γ-glutamyl compounds. In addition, while mammalian GGTs highly prefer GSH as a γ-glutamyl donor, bacterial GGTs can utilize relatively cheaper Gln besides GSH. As summarized in Table 2, we have developed the enzymatic methods to synthesize
various γ-glutamyl compounds using bacterial GGTs. These compounds were purified and identified by physiochemical methods.64–74)

a) γ-Glutamyl-L-dihydroxyphenylalanine (γ-Glu-DOPA) had been synthesized both chemically and enzymatically75) and reported to cause selective accumulation of dopamine in the kidney and to show the possibility of use as a specific renal vasodilator.76) γ-Glu-DOPA was synthesized with PmGGT and purified.64) By using thus obtained γ-Glu-DOPA as the substrate, we developed a new highly sensitive method to measure GGT activity in human serum with HPLC equipped with electrochemical detection device.77) The sensitivity limit of this method was 0.5 pmol of L-DOPA formed.

Fig. 13. Stereo view of the autocatalytic active site. This is the deduced model of the precursor according to the crystal structure of T391A protein. The oxygen atom of Thr-391 locates above the carbonyl carbon to attack it. Hydrogen bonds are shown as red or blue dashed lines.

Table 2. γ-Glutamyl compounds synthesized by GGT

| γ-Glutamyl compounds                             | Yield (%)* | Amount (g/L) | Ref. No. |
|-------------------------------------------------|------------|--------------|----------|
| γ-Glu-DOPA                                       | 79         | 51.5         | 64       |
| S-Benzyl-glutathione methyl ester                | 76**       | 31.2         | 65       |
| γ-Glu-Tyr methyl ester                          | 37         | 35.7         | 66       |
| γ-Glu-His                                       | 48         | 41.2         | 67       |
| γ-Glu-Phe                                       | 70         | 41.2         | 68       |
| γ-Glu-Val                                       | 88         | 4.3          | 69       |
| γ-Glu-taurine                                    | 22.5       | 11.4         | 70       |
| γ-Glu-ethylamide (Theanine)                     | 60         | 20.9         | 71       |
| γ-Glu-Gln                                       | 88         | 30.3         | 72       |
| γ-D-Glu-taurine                                 | 71         | 36.1         | 73       |
| γ-D-Glu-ethylamide                              | 74         | 25.6         | 73       |
| γ-D-Glu-Trp                                     | 66         | 11.0         | 74       |

*% of the amount of γ-glutamyl donor in the reaction mixture
**% of the amount of γ-glutamyl acceptor in the reaction mixture

Fig. 12. The structure of the P-segment. (A) Surface drawing of the region around the P-segment of the T391A protein. The surface of the P-segment is omitted for clarity. Ribbon models of the P-segments (residues 375–390) of the T391A protein (orange) and mature GGT (blue) are overlaid on the surface. This figure also shows a ribbon model (blue) of residues 431–458 of mature GGT, including the lid-loop (residues 438–449). All of these residues are disordered in the T391A protein. The 21 residues following Ile-378 extend in different directions in T391A protein and mature GGT. The substrate-binding pocket is colored green. (B) Close-up view of the P-segment and neighboring residues in the T391A protein shown as Corey-Pauling-Koltun and stick models. B is rotated by 90° relative to A around the vertical axis. Residues 411–416 and 482–485 are shown in gray, and the P-segment is in orange. Residues 411–416 and 482–485 form the sidewalls of the substrate-binding pocket in mature GGT and are involved in the recognition of the γ-glutamyl moiety. A ribbon model of these residues in mature GGT (blue) is superimposed on the T391A protein. This figure is reproduced from T. Okada et al. (2007) J. Biol. Chem. 282, 2433–2439 (ref. 63).
Furthermore, γ-Glu-DOPA was transformed to dopamine *in vitro* with brain homogenate by the sequential action of GGT and aromatic L-amino acid decarboxylase. Therefore, the usefulness of this γ-Glu-DOPA as a prodrug for L-DOPA was evaluated by tracing the changes of catecholamine levels in the brain after its intraperitoneal administration, and it increased the level of dopamine markedly and that of noradrenaline moderately in the brain. The maximal elevation of dopamine was observed within 30 min after administration of γ-Glu-DOPA, but a substantial increase of noradrenaline was observed 2 h after the administration. These results suggest that γ-Glu-DOPA may be applicable as a prodrug for Parkinson’s disease.

b) GSH is utilized as a medication for the treatment of hepatic diseases in Japan because of its physiological functions. It is industrially produced by chemical method or by extraction from yeast cells after enrichment of cellular GSH, but it was not produced enzymatically. So, we examined a new method for the production of GSH using *E. coli* GGT.

GSH analogues were produced enzymatically from L-Gln as a γ-glutamyl donor and S-benzyl-Cys-Gly methyl ester as its acceptor by the transeptidation reaction of GGT. After the optimization of the reaction conditions, we obtained S-benzyl glutathione monomethyl ester, which can be easily demethylated by change of pH. The S-benzyl group also can be eliminated from the product by the catalytic hydrogenolysis (Scheme 4) and reduced GSH can be obtained. Since S-benzyl-Cys-Gly methyl ester is used as a starting material for the chemical production of GSH on an industrial scale, it is readily available. For chemical γ-glutamylation, protections of the reactive groups are necessary before the reaction, but it is not necessary in this enzymatic case. The other advantages of this method are that it involves an enzymatic reaction which does not require an energy source such as ATP, and that L-Gln, which is inexpensive and fairly soluble in water, can be used as a γ-glutamyl donor.

c) γ-Glu-Tyr. L-Tyrosine is used as an additive to the transfusing solutions for patients with uremia, but the problem is its exceptionally low solubility in water. Since the γ-glutamylation of amino acids and peptides were known to increase their solubility in water, γ-Glu-Tyr may be a good precursor of L-Tyr in the transfusing solution. Selective accumulation of L-Tyr and tyramine in kidney was also expected with this modification as observed in the case of γ-Glu-DOPA. So the enzymatic synthesis of γ-Glu-Tyr was examined with *E. coli* GGT. Because of low solubility of L-Tyr, we used L-tyrosine methyl ester as a γ-glutamyl acceptor in this reaction and the reaction conditions were optimized. The methyl residue can be removed from γ-Glu-Tyr methyl ester through selective hydrolysis, if necessary.

d) γ-Glutamylation of bitter amino acids. Some L-amino acids, such as aromatic, basic and branched-chain amino acids, are known as bitter amino acids, although several bitter amino acids are essential for humans. Therefore, the bitterness of these amino acids is a crucial problem when an amino acid mixture is administrated orally. On the taste of γ-glutamyl amino acids, Kirimura *et al.* reported that γ-Glu-Phe was not bitter, but sour and astringent. They reported that some γ-glutamyl amino acids tasted astringent. And thereafter, no one had tried to use γ-glutamylation to improve the taste of bitter amino acids. So the taste of several bitter amino acids, L-Phe, L-Val, L-Leu and L-His were tasted in the comparison with their γ-glutamyl derivatives by the panel members. The results clearly
indicated γ-glutamylation dramatically abolished their bitterness, produced souness and increased preference of amino acids tested. Several panel members answered γ-Glu-Phe had a lemon like refreshing souness. This might be a reason why they preferred γ-Glu-Phe. We have synthesized γ-Glu-Phe, γ-Glu-His and γ-Glu-Val by EcGGT.

e) γ-Glu-taurine was reported to exist in mammalian tissues and have several physiological functions in neural systems of mammals. The enzymatic production of γ-Glu-taurine from L-Gln and taurine through the transpeptidation reaction of EcGGT was examined. After the optimization of production condition, it was produced at the concentration of 55 mM in the yield of 22.5%.

The optimization of reaction conditions, the yields of expected compounds increased. The obtained γ-glutamyl compounds, however, are γ-D-glutamyl compounds. Fortunately, there is a latent demand for γ-D-glutamyl compounds. It has been reported that D-theanine (γ-D-Glu-ethylamide) exists in tea leaves and it has a taste similar to that of L-theanine and that γ-D-Glu-taurine has an antagonistic effect against excitatory amino acid. We examined enzymatic syntheses of D-theanine and γ-D-Glu-taurine, with D-Gln as a γ-glutamyl donor in the transpeptidation reaction involving EcGGT. After optimization of reaction conditions, the yields of these compounds improved better than those obtained with L-Gln as a donor. (Table 2).

i) γ-D-Glu-L-Trp (SCV-07, γ-D-Glu-Trp) is a prospective medicine for the treatment of tuberculosis. γ-D-Glu-L-Trp was reported to stimulate T-lymphocyte differentiation and specific immune response, and enhance interleukin 2 and interferon-γ production in mice. In phase 2 clinical trials, it was shown that γ-D-Glu-L-Trp in combination with standard chemotherapy was very effective in the treatment of tuberculosis. L-Trp is one of the preferable substrates for EcGGT among all γ-glutamyl acceptors investigated, although we did not identify the product by physicochemical methods. We have developed an efficient enzymatic method to synthesize γ-D-Glu-L-Trp from D-Gln and L-Trp employing EcGGT. After optimization of reaction conditions, 33 mM γ-D-Glu-L-Trp was obtained under the conversion rate of 66%.

Conversion of GGT to glutaryl-7-aminocephalosporanic acid (GL-7-ACA) acylase

Today semisynthetic cephalosporins, which are the best-sold antibiotics worldwide, are supplied using 7-ACA as a starting material by the chemical modification. Cephalosporin C produced by fungi is converted to 7-ACA by the sequential reactions of D-amino acid oxidase and GL-7-ACA acylase (Scheme 5).
Surprisingly high similarity was found between class IV cephalosporin acylases (which are practically GL-7-ACA acylases) and GGTs. Even though the \(-\text{glutamyl group and the glutaryl group have similar chemical structures and the linkage between the 7-}\)amino group of 7-ACA and the glutaryl group is an amide linkage (Scheme 5), \text{Ec} GGT does not deacylate GL-7-ACA. Asp-433 of \text{Ec} GGT is one of the residues that are completely conserved among GGTs but not with this class of GL-7-ACA acylases. The residue of human GGT, which corresponds to Asp-433, postulated to interact with the \(\alpha\)-amino group of the \(\gamma\)-glutamyl residue of its substrate,\(^{54}\) and the residue is Asn in this class of GL-7-ACA acylases. Therefore, the \text{Ec} GGT with the D433N mutation was obtained and confirmed this mutant enzyme has the GL-7-ACA acylase activity. Thus, single amino acid substitution of GGT, Asp-433 to Asn, converted GGT to GL-7-ACA acylase, albeit the activity was very weak.\(^{100}\)

By this time, the use of the three-dimensional structure of \text{Ec} GGT has become available.\(^{49}\) Based on three-dimensional structure, amino acid residues involved in substrate recognition were rationally replaced or randomized, and effective mutations were then combined. Novel screening methods either with whole cells or purified enzymes were developed, and it enabled us to obtain mutant enzymes with enhanced GL-7-ACA acylase activity.\(^{101}\)

It was found that the mutations obtained separately had synergistic effects in appropriate combinations. The \(K_m\) and \(k_{\text{cat}}\) values for GL-7-ACA of purified mutant enzymes were evaluated, and the D433N Y444A G484A enzyme had the best catalytic efficiency for GL-7-ACA. Its \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) values were 18- and 50-fold higher than those of the D433N enzyme, respectively.\(^{101}\) Although the reaction conditions used in studies by other researchers are varied and direct comparison is difficult, the \(k_{\text{cat}}/K_m\) value of this mutant enzyme is comparable to that of GL-7-ACA acylase from \textit{Pseudomonas} sp. strain V22 (one of the class IV CA).\(^{102}\)

Both the hydrolysis activity and transpeptidase activity of \textit{B. subtilis} 168 GGT are much higher than those of the wild-type \text{Ec} GGT.\(^{103}\) Although the wild-type \text{Ec} GGT had no detectable GL-7-ACA acylase activity, \textit{Bs} GGT was found to have inherent activity against GL-7-ACA,\(^{101}\) albeit very weak. \textit{Bs} GGT does not have a segment corresponding to the lid-loop presented in most GGTs from other sources that covers the bound \(\gamma\)-glutamyl moiety. The class IV CP acylases, which share high amino acid similarity with GGTs, also lack the segment corresponding to the lid-loop. As we described above,
the side chain $O_\eta$ of Tyr-444 at the tip of the lid-loop and Asn-411 $O_\delta$ is hydrogen-bonded and thought to stabilize the lid-loop (Fig. 7A). In fact, either the introduction of Y444A or N411G mutation, which disrupts this hydrogen bond, boosted up GL-7-ACA acylase activity of EcGGT.101) GL-7-ACA acylase activity of BsGGT was increased by means of site-directed and randomized mutagenesis, and the catalytic efficiency was improved up to about 1000-fold compared with the wild-type BsGGT.104)

**Purification and characterization of salt-tolerant Bacillus subtilis (Bs) GGT**

Soy sauce is a traditional Japanese seasoning, but it has been widespread all over the world. Its umami taste is mainly dependent on the amount of glutamic acid it contains. During the process of fermentation, soy proteins are digested into peptides by proteases from Koji mold, Aspergillus oryzae or A. sojae, followed by the cleavage of peptides to amino acids by peptidases. Liberated glutamine is further hydrolyzed to glutamic acid by glutaminase. When the level of glutaminase is insufficient, glutamine is converted spontaneously to tasteless or slightly sour pyroglutamic acid. Consequently, glutaminase is one of the most important enzymes for flavor enhancement in soy sauce manufacturing. Soy sauce is fermented in the presence of 18% NaCl at pH 5.5 to prevent contamination. In the presence of 18% NaCl, the activity levels of glutaminases of Koji mold are 10–18% relative to the original levels.105) Therefore, the salt-tolerance of glutaminases from various organisms had been studied for their application in food fermentation.

There is a long-lasting-eating habit of natto in Japan and its fermentative bacterium, B. subtilis, is easily accepted by people. BsGGT is an extracellular enzyme that exhibits glutaminase activity and is thus suitable for food fermentation. Since BsGGT is synthesized only during the mid-stationary phase, it is inconvenient for industrial use. To obtain large quantities of GGT, a strain overexpressing GGT for a sufficiently long period was generated. A plasmid vector, pHY300PLK, containing the B. subtilis ggt gene cloned from its genomic DNA was introduced into a spo0A abrB double mutant strain of B. subtilis, in which the level of GGT activity remains high after the mid-stationary phase. GGT activity of this strain increased steadily after the exponential phase and it became 15-fold higher than that in the parental strain. The recombinant GGT was purified by 252-fold with a yield of 30.4%. The enzyme is a heterodimer with one large subunit (45 K) and one small subunit (21 K). The salt-tolerance of GGTs purified from B. subtilis str. 168 and E. coli was compared at pH 5.5 which is used in soy sauce fermentation. Even in the presence of 18% NaCl, BsGGT showed 76% residual glutaminase activity, while EcGGT showed only 11%. This indicated that BsGGT is suitable for food fermentation under high salt conditions, such as the fermentation of soy sauce and miso (fermented soy bean, a Japanese traditional seasoning). This was the first report of salt-tolerant GGT.103)

As for the physiological role of BsGGT, we showed that GGT is important in utilization of extracellular GSH as a sulfur source in B. subtilis.106)

**Crystal structure of BsGGT**

a) Unique architecture of the solvent-exposed catalytic pocket. Structural studies of GGTs from E. coli and Helicobacter pylori (Hp)107) revealed detailed molecular mechanisms of catalysis and maturation. These two GGTs highly conserved the residues for the catalytic pockets, conferring the ability of the loop segment to shield the bound γ-glutamyl moiety from the solvent. The lid-loop is present not only in E. coli and H. pylori, but also in mammalian and plant GGTs. However, lid-loop is absent from BsGGT and this is also true for GGTs from other Bacillus species according to the amino acid sequence alignment. Another remarkable feature of BsGGT is its salt-tolerance. Crystal structure of BsGGT in complex with glutamate was determined at 1.95 Å resolution to understand these characteristics (Fig. 14A).108)

As compared with EcGGT (Fig. 14B),109) all of the interactions with glutamate are identical, except that Asn-411 and Gln-430 in EcGGT are replaced by Glu-423 and Glu-442, respectively, in BsGGT. BsGGT has neither the lid-loop nor the alternative segment that covers the active site pocket, while it has additional residues not present in most other GGTs at the C-terminal region of the large subunit. In EcGGT, upon autocatalytic cleavage of the peptide bond between Gln-390 and Thr-391, the C-terminal segment of the newly produced large subunit flips away as described above.63) Crystallographic analysis of BsGGT has revealed that the C-terminal segment of the large subunit in the mature BsGGT is located close to the catalytic pocket (Fig. 14A) although the seven C terminal residues (396–402) are invisible. BsGGT does not have the lid-loop motif, and the C-terminal segment
of the newly produced large subunit changed little after autocatalytic processing nor involved in shielding the active site pocket from the solvent. That is, the substrate/product is exposed to solvent when bound to the catalytic pocket. As described above, for EcGGT the catalytic reaction proceeds in the active site pocket, which is shielded from solvent by the lid-loop, as well as by release of the C-terminal segment from the active site pocket upon autocatalytic processing. The role of the lid-loop is made possible by its flexible nature, which allows it to adopt open or closed conformations. BsGGT has neither the lid-loop nor the alternative segment that covers the active site pocket. This raises questions about the role and significance of the lid-loop in GGT catalysis.

b) Halotolerance mechanism of BsGGT inferred from crystal structure of BsGGT. \(^{108}\)
Salt-tolerance is the most different characteristics of BsGGT from other GGTs. \(^{103}\) Since acidic surface of protein was reported to enhance its stability by increasing solvation through increased water-binding capacity, \(^{109}-^{111}\) its surface potential was compared with those of other GGTs. The water binding capacities of glutamate and aspartate have been reported to be 7.5 and 6.0 molecules per amino acid, respectively, whereas those for asparagine, serine and threonine have been estimated to be 2.0 molecules per amino acid. \(^{112}\) Without the presence of NaCl, BsGGT and EcGGT both have a negatively charged surface (Fig. 15A), whereas HpGGT has positively charged patches globally distributed across its molecular surface (Fig. 15C), consistent with the theoretical pI value (9.12) calculated from the amino acid sequence. We expected more negatively charged residues would be present on the molecular surface of BsGGT than on that of EcGGT, but there was no significant difference in surface potential between these two GGTs. The effect of salt concentration on the enzyme surface properties was assessed by solving the Poisson-Boltzmann equation. As a result, notable difference was observed in the electrostatic surface potentials between the two GGTs. Even in the presence of 3 M NaCl, the surface of BsGGT apparently charged negatively, while in EcGGT the negatively charged patches completely disappeared (Fig. 15B). \(^{108}\) The negatively charged areas of BsGGT under high-salt conditions increase the solvation, owing to increased water-binding capacity. This may allow the protein to remain in a hydrated state, preventing the binding of inorganic cations in the high-salt solution and self-aggregation. Because of this characteristic, BsGGT is highly applicable to the manufacture of fermented food.

Application of BsGGT to soy sauce and miso fermentations

A salt-tolerant wild-type BsGGT was added to the fermentation mixture of soy sauce aiming to increase the amount of Glu in the product. The concentration of Glu in the soy sauce was 36 mM higher with the addition of BsGGT than without GGT, in which Glu concentration was 60 mM. Nine out of ten panel members recognized the difference in intensity of umami and preferred the taste of the soy sauce fermented with BsGGT. The result indicated that BsGGT is competent for use in soy sauce fermentation as a glutaminase. \(^{113}\)

Although miso fermentation is a semi-solid fermentation, this was also the case. When BsGGT was added to miso “moromi” (fermentation mash), the glutamate concentration in moromi was higher and the umami became stronger than without the addition of BsGGT. In addition, the concentrations of γ-Glu-Val and γ-Glu-Val-Gly, known as “kokumi” peptides, in “tamari” (supernatant fluid of moromi) were significantly increased by the addition of BsGGT. These results indicate that BsGGT is able to improve the taste of miso. \(^{114}\)

GGT catalyzes not only a hydrolysis reaction, but also a transpeptidation reaction. BsGGT not only has glutaminase activity, but it also produces γ-glutamyl compounds as byproducts. Since BsGGT is salt-tolerant, which is a distinguished characteristic, we wondered if we could get a mutant BsGGT harboring only the hydrolysis activity. At that time, no three-dimensional structure of GGT was available. GGTs from various organisms were studied to identify the amino acid residues involved in substrate binding and catalysis. Site-directed mutagenesis studies of human GGT suggested that Asp-423 interacted electrostatically with an α-amino group of a γ-glutamyl substrate, \(^{54}\) and that Arg-107 was significant as to binding to the α-carboxyl group of a γ-glutamyl substrate. \(^{52}\) We obtained recombinant BsGGTs having mutation at Arg-113 and Asp-445, corresponding to Arg-107 and Asp-423 of human GGT, and investigated on their activities to obtain a mutant BsGGT harboring only the hydrolysis activity. At first, GGT-deficient E. coli strain with Δggt-2 mutation was transformed with plasmids harboring various mutant BsGGT genes. Cell-free extracts of these transformants were used as the enzyme preparations. In the wild type, the trans-
Peptidation activity was 2.38 times higher than the hydrolysis activity. The proportion of transpeptidation activities of Arg-113 and Asp-445 mutants (R113K, D445A, D445E, D445N, and D445Y) were lower than that of the wild type. In particular, the D445A mutant exhibited no detectable transpeptidation activity.115)

Fig. 14. Comparison of active sites of GGTs. (A) Electron density map for the bound glutamate in BsGGT. An omit Fo-Fc map for glutamate contoured at 2.0σ (orange) is overlaid on the stick models of GGT and the bound glutamate. (B) The glutamate-binding mode in EcGGT. The bound glutamate and catalytic threonine are shown in orange and cyan, respectively. Dashed lines indicate hydrogen bonds. This figure is reproduced from K. Wada et al. (2010) FEBS J. 277, 1000–1009 (ref. 108).

Fig. 15. Surface electrostatic properties of GGTs. (A) Electrostatic potentials of BsGGT (upper panels) and EcGGT (lower panels) calculated using parameters without taking into account ion strength. (B) Electrostatic potentials of BsGGT (upper panels) and EcGGT (lower panels) calculated for 3M concentration of monovalent ion. (C) The surface potential of HpGGT calculated using parameters without taking into account ion strength. The color scale ranges from −10kT per electron (red) to +10kT per electron (blue). The structures are graphically depicted with the viewpoint looking down the catalytic pocket (right panels) or rotated 90° from this view (left panels). This figure is reproduced from K. Wada et al. (2010) FEBS J. 277, 1000–1009 (ref. 108).
Summary

In summary, starting from the examination of GGT expression conditions in bacterial cells, we elucidated the properties of bacterial GGTs with purified preparation and also showed that the GSH hydrolysis reaction by GGT was the physiological reaction and the transpeptidation reaction did not function in bacterial cells. We could also show the existence and significance of the GSH cycle in bacteria using GGT-deficient mutants and as a result, we can indicate that a fairly similar system to mammals is working even in bacterial cells. In this system, the intracellular concentration of the highly reactive Cys seems to be kept low and it is supplied when necessary through the hydrolysis of extracellular GSH by the reaction of GGT. This seems to be a fine system which is common through wide range of living organisms. Further investigation is necessary to clarify precise molecular mechanism which controls the supply of Cys into the cell, including the physiological role of GSH transporter we found. Bacterial cells seem to be very good research material for this purpose.

The catalytic nucleophile of EcGGT is the N-terminal Thr-391 in the small subunit, which is also the nucleophile in the processing reaction to become a mature enzyme from inactive precursor. This residue corresponds to Thr-380 in rat kidney and Thr-381 in human enzyme and is conserved among all GGTs for which the primary sequences are known to date. These results along with the three-dimensional structure of the catalytic site revealed that GGT belongs to the Ntn hydrolase superfamily.

Although much time and effort had been spent on crystal structure determination of EcGGT, analysis with high resolution was performed using Se-Met containing GGT. As the result, we were able to deepen our understanding of the enzymatic reaction mechanism and processing mechanism of GGT, which had not been studied so far on GGTs from any other organisms. The structures of the GGTs of the two bacteria, EcGGT and BsGGT, have the common topology, but there are different parts in their details, such as the existence of the lid-loop covering the active site groove and the C-terminus position of the large subunit. The relation between these partial differences of structure, and the catalytic activity and physiological significance is a subject that needs further investigation. The detailed analysis of the catalytic reaction at the active center of EcGGT clearly shows the commonality with human GGT, and is thought to have contributed to the structural analysis of human GGT later. The three-dimensional structure of GGT of H. pylori was determined, and it was clarified that it was almost the same as that of EcGGT. It was reported that HpGGT induces apoptosis of gastric epithelial cells and is predicted that it is brought about by the oxidant action of Cys-Gly, which generated by the degradation of GSH by GGT. Therefore, development of HpGGT specific inhibitor is desired. In such case, GGT inhibitors based on the reaction mechanism, which we used to probe the catalytic site of GGTs, are considered to be promising.

Enzymatic synthesis of various γ-glutamylpeptides with GGTs were examined and fairly good yields were shown but much more good productivity will be required on practical industrial production. Furthermore, in each case, gene recombination technology is used, and it is necessary to verify the safety before putting it into practical use, and it is also necessary to consider public acceptance in the application to the food industry in particular.

We hope that the research results introduced here will contribute to the further development of GGT research.

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Profile

Hideyuki Suzuki was born in Mie Prefecture in 1957 and grew up in Kyoto since he was 1 year old. He graduated from Kyoto University after majoring in Food Science and Technology in the Faculty of Agriculture in 1981, and he received his PhD in 1988. After receiving a research fellowship for young scientists from the Japan Society for the Promotion of Scientists for 1 year, he served in Kyoto University until 2007 and became a professor at Kyoto Institute of Technology. During his masters course, he had a chance to join a genetic study of *E. coli* RNA polymerase in Professor Carol Gross’s laboratory in the University of Wisconsin-Madison as an exchange student supported by the International Exchange Program of the Ministry of Education (1983–1984). He also had a chance to join a bacterial enhancer study in the laboratory of Professor Sydney Kustu in University of California, Berkeley (1994–1995). He started his study on bacterial \( \gamma \)-glutamyltranspeptidase under the guidance of Professor Hidehiko Kumagai when he entered the graduate program, and it has become his life work. He has been a front runner in both fundamental and applied study of bacterial \( \gamma \)-glutamyltranspeptidase. For his work, he received the Japan Society for Bioscience, Biotechnology, and Agrochemistry (JSBBA) Award for Young Scientists in 1997, 1st Applied Enzyme Symposium Award from Amano Enzyme in 2000, Academic Encouragement Award from the Mishima Kaiun Memorial Foundation in 2003, Topics Award from the JSBBA in 2017, and the JSBBA Award for Senior Scientists in 2020.

Profile

Keiichi Fukuyama was born in Kagawa Prefecture, Japan, in 1949 and graduated from the Faculty of Science, Osaka University, in 1971. He received his PhD degree in 1979 and worked as a postdoctoral fellow at the Department of Biological Sciences, Purdue University from 1980 to 1982. His main interest is the crystal structures and functions of proteins and spherical viruses. He started working at the Faculty of Engineering, Tottori University, in 1973, and then moved to the Department of Biological Sciences, Graduate School of Science, Osaka University in 1987. He became a Professor at Osaka University in 1995. His research includes iron-sulfur proteins of ferredoxins, flavin protein of flavodoxin, the heme protein of peroxidase, proteins that are involved in the biosynthesis of iron-sulfur clusters, DNA repair enzymes, heme catabolizing enzymes of heme oxygenase, \( \gamma \)-glutamyltranspeptidase, and ferredoxin dependent bilin reductases. The viruses include alfalfa mosaic virus, tobacco necrosis virus, and marine RNA virus. He developed a method for protein structure determination using anomalous scattering of atoms. He was awarded the Nishina Award in 1973, the Awards of CrSJ, Research Award, in 1994, and the award for education and research of Osaka University in 2008. He has also contributed in the committee of synchrotron radiation experiment at SPring-8 for many years. He was appointed as an emeritus member of Protein Science of Japan. He retired in 2013 from Osaka University and has continued to research in the field of structural biology.
Profile

Hidehiko Kumagai graduated from the Faculty of Agriculture, Kyoto University in 1964. He started his research career in 1964 under the guidance of Prof. Hideaki Yamada at the Institute for Food Science, Kyoto University, where he worked on microbial enzymes metabolizing amino acids and amines. He obtained his PhD degree in 1970. He was appointed as an Assistant Professor of the Institute for Food Science, Kyoto University in 1969. He engaged in research on tryptophan synthase as a visiting research fellow at NIH in the USA from 1971 to 1972. He moved to the Faculty of Agriculture, Kyoto University in 1977 as an Associate Professor at the Department of Food Technology and was promoted to Professor in 1991. He moved to the Graduate School of Biostudies, Kyoto University in 1999. After retirement from Kyoto University in 2004, he moved to Ishikawa Prefectural University as a Professor and was the President of the University from 2012 to 2018 and now he is a senior adviser at the university. He served as a President of the Japan Society of Bioscience, Biotechnology, and Agrochemistry between 2003 and 2005. He is an emeritus member of this society and The Vitamin Society of Japan. He received the Award of the Vitamin Society of Japan in 1994, the Japan Society of Bioscience, Biotechnology, and Agrochemistry Award in 2001, the Japan Academy Prize in 2012, and Ig Nobel Prize in the field of Chemistry in 2013. He received the Order of the Sacred Treasure, Gold and Silver Star in 2019.