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Purification and Properties of γ-Glutamyltranspeptidase from *Proteus mirabilis*

REIKO NAKAYAMA, HIDEHIKO KUMAGAI,* AND TATSUROKURO TOCHIKURA

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

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γ-Glutamyltranspeptidase was purified ca. 15,200-fold from cell-free extracts of *Proteus mirabilis* to electrophoretic homogeneity and then crystallized. The enzyme has an estimated molecular weight of 80,000 and consists of two different subunits with molecular weights of ca. 47,000 and 28,000. The purified enzyme catalyzed hydrolysis and transpeptidation of various γ-glutamyl compounds, including the oxidized and reduced forms of glutathione, γ-glutamyl compounds of L-phenylalanine, L-tyrosine, L-histidine, L-α-aminobutyrate, L-leucine, and *p*-nitroaniline. Glycylglycine, L-phenylalanine, L-methionine, L-histidine, L-tryptophan, and L-isoleucine were good acceptors of the γ-glutamyl moiety in the transpeptidation reaction. K_m values for γ-glutamyl compounds were on the order of 10^{-4} to 10^{-5} M, and those for acceptor peptides and amino acids were on the order of 10^{-2} to 10^{-3} M. The enzyme was inhibited by L-serine plus borate and 6-diazo-5-oxo-L-norleucine, which are inhibitors of γ-glutamyltranspeptidases isolated from mammals. Various amino acids alone were found to inhibit the transpeptidation competitively with a γ-glutamyl donor. Kinetic analysis suggested that the reaction sequence of substrate binding and product release proceeds according to a ping pong bi bi mechanism.

Glutathione (GSH) is the major nonprotein sulfhydryl compound in some gram-negative bacteria (4), and its concentration in cells ranges between 3.5 and 6.6 mM (7). γ-Glutamyl transfer from GSH to amino acids, peptides, or water has been studied extensively with enzymes of animal origin. Data on the γ-glutamyl transfer in bacteria are rather scarce. Hydrolysis and γ-glutamyl transfer of GSH by intact cells and cell-free extracts of *Proteus vulgaris* were reported for the first time by Talalay (20). Milbauer and Grossowitz (13) reported the wide distribution of the γ-glutamyl transfer reaction in different bacterial species. They investigated the specificity for γ-glutamyl acceptors and kinetics and properties of the γ-glutamyl transfer reaction with cell-free extracts of *Proteus morganii*. Although these studies indicated that bacterial cells are good models for investigating the basic functions of glutathione and γ-glutamyltranspeptidase (γ-GT) in microorganisms, no detailed investigation has been reported thus far.

All of the enzymes involved in the γ-glutamyl cycle (10) were reported to exist in yeast cells (14). Many investigations have been performed to elucidate the role of γ-GT in amino acid uptake in *Saccharomyces cerevisiae*. Osuji (18) obtained results supporting the role of the enzyme and the γ-glutamyl cycle in an amino acid transport system. On the other hand, Robins and Davies (19) provided data opposing a role of γ-GT in amino acid transport.

In the course of investigating GSH production by bacteria, we found that *Proteus mirabilis* has high activities of γ-glutamylcysteine synthetase (EC 6.3.2.2) (8), glutathione synthetase (EC 6.3.2.3), and γ-GT (EC 2.3.2.2). γ-GT activity was detectable with intact cells of the bacterium, and inhibition of the enzyme during growth resulted in leakage of GSH into the medium (16). In this study, γ-GT was purified for the first time from bacterial cells, and its properties were investigated to obtain information on the metabolic functions of GSH and γ-GT in bacterial cells.

* Corresponding author.

MATERIALS AND METHODS

Reagents. Reduced and oxidized GSH, γ-glutamyl-L-phenylalanine, γ-glutamyl-L-tyrosine, γ-glutamyl-L-histidine, γ-glutamyl-L-leucine, DL-γ-glutamyl-aniline, γ-glutamyl-α-naphthylamide, γ-glutamyl-L-alanine, 6-diazo-5-oxo-norleucine, and fast garnet GBC salt were purchased from Sigma Chemical Co. Hydroxylapatite was prepared by the method of Tiselius et al. (23). γ-Glutamyl-L-α-aminobutyrate was synthesized with γ-glutamylcysteine synthetase (15). Other chemicals were the best grade reagents available from commercial sources.

Bacterial strain and culture. *P. mirabilis* was obtained from stock cultures of the Laboratory of Industrial Microbiology, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, Japan. The bacterial strain was cultured in a medium described previously (8). Cells were picked from an agar slant, inoculated into a test tube containing 5 ml of the medium, and cultured at 28°C for 36 h with reciprocal shaking (114 rpm). The culture was then transferred to a 2-liter flask containing 1 liter of the medium and cultured at 28°C for 36 h with shaking. For a large-scale culture, two of these subcultures were, in turn, inoculated into a 30-liter fermentor containing 25 liters of the medium. Cultivation was carried out at 30°C for 24 h with aeration (0.7 liter per liter of medium per min) and agitation (150 rpm). The grown cells were harvested with a continuous-flow centrifuge and washed with 0.85% saline. Approximately 38 g of cells (wet weight) was obtained per liter of medium. The packed cells were stored at −80°C until preparation of the enzyme.

Enzyme assay of γ-GT activity. Two different methods were used depending on the purpose of the assay.

(i) Spectrophotometric method. Enzyme assay by the spectrophotometric method was carried out as described previously (16).

(ii) Amino acid analysis. The standard reaction mixture contained 5.0 μmol of γ-glutamyl donor GSH, 120 μmol of glycylglycine (GlyGly), 100 μmol of Tris-hydrochloride (pH

8.0), 150 μmol of NaCl, and enzyme in a final volume of 2.0 ml. After incubation at 37°C for 20 min, the amounts of glutamate liberated and γ-glutamylglycylglycine produced were measured with an automatic amino acid analyzer as described previously (15). Hydrolytic activity of γ-glutamyl compounds was measured without a γ-glutamyl acceptor in 0.05 M acetate-sodium buffer, pH 6.0. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of a product per min. Specific activity is expressed in terms of units per milligram of protein.

Protein determination. The protein concentration was determined by the method of Lowry et al. (9), using ovalbumin as a standard, or spectrophotometrically by measuring the absorbance at 280 nm. An absorption coefficient of $E = 9.76$ (1% concentration and 1-cm light path) was obtained by absorbance and dry weight determination of the purified enzyme and used throughout.

Electrophoresis. Disc acrylamide gel electrophoresis was performed by the method of Davis (3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn (24). γ-GT activity staining was carried out by the method of Albert et al. (1). After electrophoresis was carried out at 4°C, gels were incubated in the substrate solution, which contained 5 μmol of γ-glutamyl-α-naphthylamide, 40 μmol of GlyGly, 80 μmol of potassium phosphate buffer (pH 7.0), 462 μmol of NaCl, and 2.5 mg of fast garnet GBC salt in a final volume of 5.0 ml at 37°C for 5 to 60 min.

Estimation of molecular weight. Molecular weight was estimated by gel filtration on a Sephadex G-150 column (2.0 by 100 cm). Five standard proteins, cytochrome *c* (molecular weight, 12,500), chymotrypsinogen A (25,000), ovalbumin (67,000), aldolase (158,000), and catalase (240,000), were used and eluted with 0.05 M Tris-hydrochloride buffer (pH 7.5) at a flow rate of 50 ml/h; 2-ml fractions were collected. The void volume of the column was determined with blue dextran. The subunit molecular weight was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Purification of γ-GT from *P. mirabilis*. All operations were carried out at 0 to 5°C. The pH of the enzyme solution was adjusted to 7.0 with 2.5% ammonium hydroxide solution after each addition of ammonium sulfate.

Purification steps. (i) Preparation of cell extracts. Frozen cell paste (wet weight, 5,300 g) was suspended in 26.5 liters of 0.05 M Tris-hydrochloride buffer, pH 7.4. The cells were disrupted with a Dyno-Mill (agitation rate, 3,000 rpm with glass beads, 0.25 to 0.50 mm in diameter) (Willy A. Bachofen, Maschinenfabrik, Basel, Switzerland), and the supernatant solution was obtained by centrifugation.

(ii) Ammonium sulfate fractionation. Solid ammonium sulfate was added to 30 liters of the cell extract to 40% saturation. After being left to stand overnight, the supernatant solution was collected by centrifugation and brought to 70% saturation in the same manner. After being left to stand overnight, the precipitate was collected by centrifugation and dissolved in 10 liters of 0.05 M Tris-hydrochloride buffer (pH 7.4). This solution was dialyzed three times against 40 liters of the same buffer for 48 h.

(iii) Protamine sulfate treatment. A 10% protamine sulfate solution (1.2 liters) was added to 17 liters of the dialyzate, and the precipitate formed was removed by centrifugation. The supernatant solution was concentrated by the addition of solid ammonium sulfate to 70% saturation. The precipitate was centrifuged off, dissolved in 5.5 liters of the buffer, and then dialyzed for 48 h against three changes of 40 liters of the same buffer.

(iv) DEAE-cellulose column chromatography. The dialyzed enzyme solution was applied to a DEAE-cellulose column (21 by 70 cm) previously equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.4. The column was washed with the buffer and then the enzyme was eluted with 41 liters of 0.07 M NaCl in the buffer at a flow rate of 500 ml/h. The active fractions (13.4 liters) were combined and concentrated by the addition of ammonium sulfate to 80% saturation. After being left to stand for ca. 1 week to allow the complete precipitation of the protein, the supernatant solution was discarded roughly by decantation. The remaining precipitate was collected by centrifugation, dissolved in 420 ml of 0.05 M Tris-hydrochloride buffer (pH 7.4), and dialyzed against the same buffer.

(v) Second ammonium sulfate fractionation. Solid ammonium sulfate was added to the enzyme solution (630 ml) to 40% saturation. After the precipitate was removed by centrifugation, material precipitating between 45 and 65% saturation with respect to ammonium sulfate was dialyzed against 1 mM potassium phosphate buffer, pH 7.5.

(vi) Hydroxylapatite column chromatography. The dialyzed enzyme solution (250 ml) was placed on a hydroxylapatite column (5 by 20 cm) equilibrated with 1 mM potassium phosphate buffer, pH 7.5. After the column was washed with the buffer, elution was carried out sequentially with the following concentrations of the same buffer: 0.01, 0.03, 0.05, 0.07, and 0.1 M at a flow rate of 50 ml/h. The active fractions eluted with 0.05 to 0.07 M were combined and concentrated by the addition of ammonium sulfate to 70% saturation. The precipitate collected by centrifugation was dissolved in 10 mM potassium phosphate buffer, pH 7.5, and dialyzed against 0.8 M ammonium sulfate solution in 10 mM potassium phosphate buffer, pH 7.5.

(vii) Phenyl-Sepharose CL-4B column chromatography. The enzyme solution (40 ml) was applied to a phenyl-Sepharose CL-4B column (2 by 40 cm) equilibrated with 0.8 M ammonium sulfate in 10 mM potassium phosphate buffer, pH 7.5. After the column was washed with the same buffer, the enzyme was eluted by descending stepwise elution with the following buffers: 0.6 M ammonium sulfate containing 10% ethyleneglycol, 0.4 M ammonium sulfate containing 20% ethyleneglycol, and 0.2 M ammonium sulfate containing 30% ethyleneglycol in 10 mM potassium phosphate buffer (pH 7.5). The active fractions that eluted with 0.4 M ammonium sulfate containing 20% ethyleneglycol in 10 mM potas-

TABLE 1. Summary of purification of γ-GT from *P. mirabilis*[a]

Fraction no. and type	Total protein (mg)	Total activity (U)	Sp act (mU/mg)	Yield (%)
1. Extract	1,110,000	1,200	1.09	100
2. Ammonium sulfate 1	478,000	1,090	2.28	90
3. Protamine	186,000	1,080	5.80	89
4. DEAE-cellulose	27,000	1,060	39.3	88
5. Ammonium sulfate 2	8,050	1,020	127	84
6. Hydroxylapatite	2,050	642	313	53
7. Phenyl-Sepharose CL-4B	249	448	1,800	37
8. Sephadex G-150	92.2	335	3,630	28
9. DEAE-Sephadex	17.2	148	8,600	12
10. Chromatofocusing	8.6	142	16,500	11
11. Crystalline	5.1	84.6	16,600	7.0

[a] The enzyme activity was measured by a spectrophotometric method as described in the text. The protein amount in fractions 1, 2, and 3 were measured by the method of Lowry et al. (9), and that in fractions 4 through 11 was measured spectrophotometrically.

sium phosphate buffer, pH 7.5, were combined and concentrated by addition of ammonium sulfate to 70% saturation. The precipitate was dissolved in 0.05 M Tris-hydrochloride buffer, pH 7.4, and dialyzed against the same buffer.

(viii) Sephadex G-150 gel filtration. The enzyme solution (120 ml) was divided into three portions and each portion was applied to a Sephadex G-150 column (2 by 100 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.4, and eluted with the same buffer at a flow rate of 100 ml/h in 5-ml fractions. The active fractions were combined, and the protein precipitated by 70% saturated ammonium sulfate was dialyzed against the same buffer.

(ix) DEAE-Sephadex column chromatography. The dialyzed enzyme solution (25 ml) was placed on a DEAE-Sephadex column (1.5 by 44 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.4, and washed with the same buffer. Elution was carried out with 0.1 M NaCl in the buffer at a flow rate of 20 ml/h; 2-ml fractions were collected. The active fractions were combined and brought to 70% saturation with ammonium sulfate. The precipitate was dissolved in 0.025 M histidine-hydrochloride buffer, pH 6.2, and dialyzed against the same buffer.

(x) Chromatofocusing. After being washed with 5 ml of Polybuffer 74-hydrochloride (eightfold dilution [pH 4.0]), the dialyzed enzyme solution was applied on a PBE94 column (1.5 by 7 cm) equilibrated with 0.025 M histidine-hydrochloride buffer, pH 6.2, and eluted with 9 bed volumes of Polybuffer 74-hydrochloride (eightfold dilution [pH 4.0]) to form in the column a descending linear gradient of pH 6 to 4. The active fractions eluted at pH 4.2 to 3.9 were pooled and dialyzed against 0.05 M Tris-hydrochloride buffer, pH 7.4.

(xi) Crystallization. Solid fine-powdered ammonium sulfate was cautiously added to the purified enzyme solution until it became slightly turbid, and then the mixture was placed in a refrigerator. Crystallization of the enzyme as fine needles began after 2 days and was virtually complete within 3 weeks.

RESULTS

Enzyme purification. The successful purification scheme (Table 1), involving ammonium sulfate and protamine sulfate fractionations, six sequential column chromatographic fractionations, and chromatofocusing, gave a 15,200-fold purification with a recovery of 11%. The final step of chromatofocusing gave four protein peaks (Fig. 1), and the enzyme activity was found in the final peak eluted at pH 4.0. This peak was divided into two fractions as shown in Fig. 1, and both fractions were subjected to polyacrylamide gel electrophoresis (Fig. 2), in which the components were visualized by staining for γ-GT activity (data not shown) or for protein (lanes A and B). Fraction 1 showed one band on protein staining, and fraction 2 (which had the same specific activity as fraction 1) showed one major and one minor band. Every protein band of fraction 1 and fraction 2 showed γ-GT activity on activity staining and was stained red-brown by periodic acid-Schiff reagents, indicating that they were glycoproteins. The enzyme was crystallized by the addition of solid ammonium sulfate to the solution. The specific activity of the enzyme did not increase with the crystallization. The crystalline enzyme at this stage of purification was stable when stored at 4°C for at least 3 months.

Molecular weight and subunit structure. The molecular weight of *Proteus* γ-GT was determined to be ca. 80,000 by Sephadex G-150 gel filtration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified γ-GT gave two major components with apparent molecular weights of

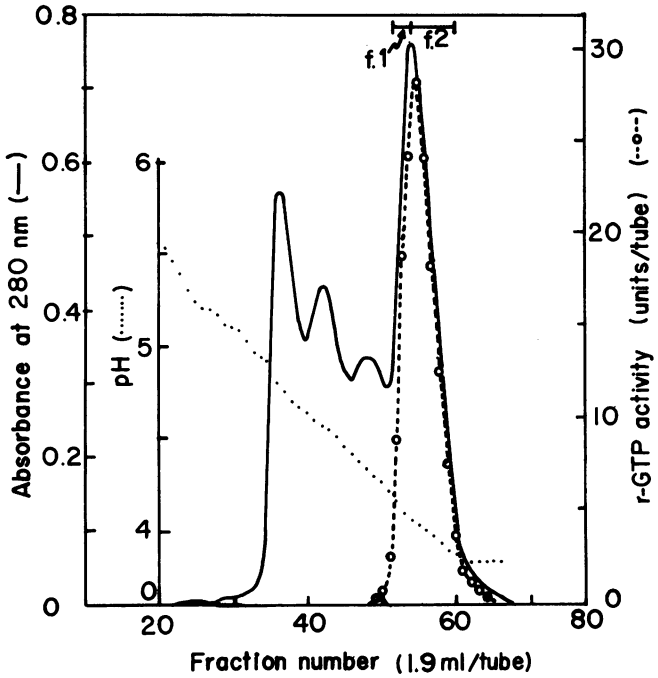

FIG. 1. Chromatofocusing pattern of *Proteus* γ-GT. Approximately 148 U of enzyme was applied on a PBE94 column (1.5 by 7 cm) equilibrated with 0.025 M histidine-hydrochloride buffer (pH 6.2) and eluted with Polybuffer 74-hydrochloride (pH 4.0) as described in the text.

47,000 and 28,000 (Fig. 2), indicating that the native enzyme contained one each of these two subunits.

Catalytic properties and substrate specificity. The purified enzyme catalyzed hydrolysis of γ-glutamyl compounds and transfer of the γ-glutamyl moiety of the compounds to an acceptor such as GlyGly or some amino acids (transpeptidation). The optimum pH of hydrolysis of GSH or γ-glutamyl-*p*-nitroanilide (γ-GpNA) was 6.0 and that of transpeptidation from GSH or γ-GpNA to GlyGly was 7.5 to 8.0. The same optimum temperature of 37°C was found for both reactions of hydrolysis and transpeptidation. Hydrolysis and transpeptidation to GlyGly of various γ-glutamyl compounds were measured at the optimum pH of each reaction (Table 2). The reaction rate of hydrolysis of GSH was 0.67 μmol/min per mg of protein at pH 6.0 and that of γ-glutamyltranspeptidation from GSH to GlyGly was 0.8 μmol/min per mg of protein under the standard reaction conditions (pH 8.0) described above. The enzyme used various amino acids as γ-glutamyl acceptors from γ-GpNA (Table 3). L-Glutamate and β-chloro-L-alanine were not used as acceptors. K_m values for GSH, γ-GpNA, and glutamine in the hydrolysis were 3.3×10^{-5}, 3.1×10^{-4}, and 1.0×10^{-2} M, respectively. In the transpeptidation, GSH and γ-GpNA showed K_m values of 1.8×10^{-4} and 4.0×10^{-4} M, respectively, as donors, and GlyGly and phenylalanine showed K_m values of 2.9×10^{-3} and 32×10^{-3} M, respectively, as acceptors.

Stability toward pH, temperature, and sulfhydryl reagents. The enzyme retained full activity after incubation in the pH range of 5.0 to 9.0 at 37°C for 60 min and lost 50% of its activity at pH 4.0 and 10.0 under the same conditions. The enzyme retained 90% activity after incubation for 10 min at 40°C and pH 7.0 and lost 37% activity at 45°C and 95% at 50°C. Sulfhydryl compounds, dithiothreitol, 2-mercaptoethanol, GSH, GSSG, cysteine, and cystine, and sulfhydryl

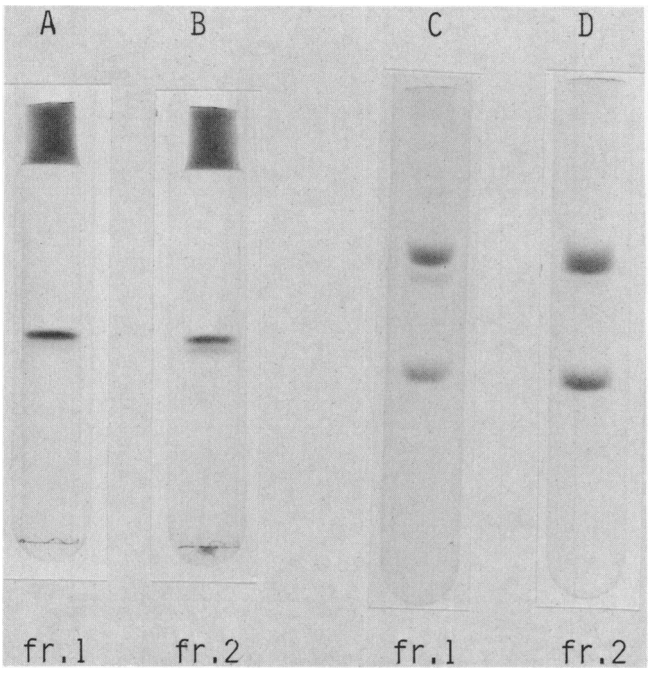

FIG. 2. Polyacrylamide gel electrophoresis of *Proteus* γ-GT. Approximately 40 μg of each enzyme (fraction 1 and fraction 2) was used, and the gels were run at 1.5 mA per gel for 2 h. Protein staining with amide black 10B (lanes A and B) was carried out as described in the text. Sodium dodecyl sulfate-acrylamide gel electrophoresis was performed in 10% gels at 8 mA per gel for 4 h. Enzyme protein (40 μg) was applied on each gel (lanes C and D). Polyacrylamide-disc gel electrophoresis was used in lanes A and B. SDS-polyacrylamide disc electrophoresis was used in lanes C and D.

reagents, pCMB and *N*-ethylmaleimide, did not have any significant effect on the enzyme activity on incubation with the enzyme for 10 min at pH 8.0 and 37°C at a concentration of 1 or 10 mM. Only iodoacetamide, iodoacetate, and $HgCl_2$ reduced the activity by 31, 32, and 34%, respectively, after a 10-min incubation at a 1 mM concentration.

TABLE 2. Substrate specificity of *Proteus* γ-GT for γ-glutamyl donors[a]

Substrate	Relative rate (%)	
	Transpeptidation[b]	Hydrolysis[c]
GSH	100	100
GSSG	100	88.9
γ-Glu-L-Phe	100	90.3
γ-Glu-L-Tyr	87.7	82.3
γ-Glu-L-His	77.3	78.1
γ-Glu-α-Abu[d]	66.3	104
γ-Glu-L-Leu	40.7	70.8
Gln	35.9	83.9
γGpNA	17.5	36.1
DL-γ-Glu-aniline	9.7	18.1
γ-Glu-α-naphthylamide	0.1	1.5
α-Glu-L-Ala	0	0

[a] γ-Glutamylglycylglycine (transpeptidation at pH 8.0) and glutamate (hydrolysis at pH 6.0) were measured with an amino acid analyzer as described in the text with the replacement of glutathione by its analogs at the same concentration. Activity is expressed relative to that found with glutathione (100%).

[b] γ-Glutamylglycylglycine formation at pH 8.0

[c] Glutamate formation at pH 6.0

[d] γ-Glutamyl-α-aminobutyrate

TABLE 3. Substrate specificity of *Proteus* γ-GT for γ-glutamyl acceptors[a]

Substrate	Relative rate (%)
GlyGly	100
L-Phe	51.7
L-Met	46.7
L-His	36.9
L-Trp	36.9
L-Ileu	25.2
L-Arg	20.7
L-Leu	20.7
L-Asp	20.1
Gly	16.6
γ-Aminobutyrate	16.3
L-Lys	16.0
L-Cys[b]	15.7
L-Thr	15.6
L-Hyp	14.7
L-Pro	13.2
L-Asp	12.9
L-Val	12.6
L-Homoserine	6.7
L-Tyr[b]	6.6
L-Ser	6.2
L-α-Aminobutyrate	5.5
L-Ala	4.0
L-Gln	2.5
Aminoxyacetate	1.5
L-Cys	1.3
L-β-Chloroalanine	0
L-Glu	0

[a] The enzyme activity was measured by a spectrophotometric method as described in the text with the replacement of GlyGly by its analogs at the same concentration, except for L-cystine and L-tyrosine. Activity is expressed relative to that found with GlyGly (100%).

[b] The acceptors were used at a concentration of 60 mM, except that L-cysteine and L-tyrosine were used at 20 mM.

Inhibition by amino acids and their derivatives. The transpeptidation reaction catalyzed by the enzyme was inhibited by various free amino acids (Table 4). Inhibition was also observed when intact cells were used as the enzyme preparation. Kinetic investigation showed that the inhibition was competitive for γ-glutamyl donors and noncompetitive for the acceptors. K_i values of L-alanine and L-serine were calculated to be 2.9×10^{-5} M and 3.4×10^{-4} M. Serine-borate and 6-diazo-5-oxo-L-norleucine were shown to inhibit and inactivate hog kidney γ-GT by affinity labeling of the enzyme (6, 21, 22). These glutamate analogs also inhibited *Proteus* γ-GT competitively with γ-glutamyl donors. K_i values of serine-borate and 6-diazo-5-oxo-L-norleucine were calculated to be 3.1×10^{-4} and 3.8×10^{-6} M, respectively.

Sequence of substrate binding and product release during transpeptidation. Initial velocities of the γ-glutamyltranspeptidation from γ-GpNA to L-phenylalanine were measured by determining the amount of γ-glutamylphenylalanine with an amino acid analyzer. Parallel reciprocal plots were obtained when the concentration of L-phenylalanine was varied at several fixed concentrations of γ-GpNA. One product, *p*-nitroaniline, inhibited noncompetitively the formation of γ-glutamylphenylalanine. γ-Glutamylphenylalanine was found to inhibit phenylalanine noncompetitively and γ-GpNA competitively when the initial velocities were measured photometrically by determination of released *p*-nitroaniline. These results indicate that the γ-glutamyltranspeptidation reaction catalyzed by *Proteus* γ-GT proceeds through a ping pong bi bi mechanism (2).

TABLE 4. Inhibition of *Proteus* γ-GT by amino acids[a]

Amino acid	Amt (mM)	Inhibition (%) of: Transpeptidation	Hydrolysis
Purified enzyme			
None		0	0
L-Ala	10	97.3	78.7
L-β-Cl-Ala	10	96.7	66.9
L-Gln	10	94.0	91.3
L-Ser	10	86.4	60.9
L-Hse	10	85.0	56.5
L-Glu	10	84.2	70.9
L-α-Abu[b]	10	84.2	54.3
L-Cys	10	80.8	55.9
Gly	10	38.8	5.5
L-Asn	10	30.1	27.6
L-Asp	10	21.3	23.6
D-Ser	10	20.2	1.2
Aminoxyacetate	10	0	7.1
Intact cells			
None		0	
L-Ala	1	59.1	
	10	93.0	
L-Ser	1	34.0	
	10	83.5	

[a] The enzyme activity was determined by a spectrophotometric method as described in the text in the presence (10 mM) or absence of various free amino acids.

[b] L-α-Aminobutyrate.

DISCUSSION

Highly purified γ-GT preparations have been obtained from mammalian tissues, mainly kidneys (11, 12), and their properties were investigated extensively. Although its occurrence has been reported in many bacteria (13, 20), the enzyme has not been purified from microbial sources, except for partial purification from a glutamate-producing bacteria, *Corynebacterium glutamicum* (5).

In this study, we obtained a homogeneous preparation of γ-GT from *P. mirabilis* which showed high glutathione-metabolizing activity compared to many other bacterial strains. The enzyme in mammalian tissues was reported to exist in membranes of cells that exhibit secretory or absorptive functions and to be solubilized by treatment with a detergent, organic solvent, or proteinases (11, 12). *Proteus* γ-GT could be easily solubilized by ultrasonic oscillation of the cells, and the enzyme in other bacteria was also reported to be solubilized by ultrasonic treatment (5, 20). A tremendously high magnitude of purification (15,200-fold) was necessary to obtain a homogeneous preparation of *Proteus* γ-GT. Because of this we used as a starting material completely disrupted cell homogenate obtained with a Dyno-Mill, and we purified γ-glutamylcysteine synthetase (14) and glutathione synthetase simultaneously from the same homogenate. Much more gentle ultrasonic (19 kilocycles, 2 min) treatment of cells was found to give a γ-GT solution having 7.6-times-higher specific activity, which is better as a starting solution for the purification of γ-GT only.

The purified enzyme preparation (fraction 1) was homogeneous as judged in disc gel electrophoresis, and γ-GT activity was associated with the protein band and was positive by periodic acid-Schiff staining. The other enzyme preparation (fraction 2) gave one minor band or disc gel electrophoresis in addition to the one major band which migrated on electrophoresis as fraction 1 did. Both the major and minor bands showed γ-GT activity and were positive to periodic acid-Schiff staining. The minor band seems to be an isozyme, although the possibility of modification by proteolysis cannot be excluded. Additional investigation is necessary to confirm this. Sialic acid was not found in the enzyme.

Proteus γ-GT has a smaller molecular size than the native enzyme from mammalian tissues and a molecular weight and subunit structure similar to the proteinase-solubilized rat kidney enzyme (68,000) that consists of two subunits (46,000 and 22,000) (11, 12).

Proteus γ-GT catalyzes both transpeptidation and hydrolysis of various γ-glutamyl peptides and uses many amino acids as acceptors of the γ-glutamyl residue as reported for hog kidney γ-GT (16). The ratio of the velocity of transpeptidation of an amino acid versus that of GlyGly was higher than that reported for the enzyme from hog kidney. In the transpeptidation, aromatic and basic amino acids were better substrates than cysteine, alanine, and serine, which were good substrates for γ-glutamyl amino acid synthesis by γ-glutamylcysteine synthetase from the same bacterium, *P. mirabilis* (8). Low substrate specificity for γ-glutamyl acceptors may indicate that this enzyme does not work in amino acid transport since the transport systems thus far reported have much higher specificity for amino acids. In the hydrolysis, GSH and γ-GpNA showed low K_m values, but glutamine showed a very high one; in the transpeptidation, acceptors showed very high K_m values in comparison with γ-glutamyl donors. High K_m values for γ-glutamyl acceptors also suggest that γ-GT does not function in amino acid transport since uptake of amino acids into bacterial cells was observed at lower concentrations than these K_m values. The autotranspeptidation rate by this *Proteus* γ-GT was very low (around 1%) under the reaction conditions we employed, and the reaction was neglected when the reaction mechanism was analyzed kinetically. Kinetic studies on the enzyme showed a ping pong mechanism as reported for kidney enzymes (11).

Various free amino acids strongly inhibited the transpeptidation catalyzed by *Proteus* γ-GT. This phenomenon has never been reported for γ-GT isolated from mammals and is interesting because the inhibition was competitive not for γ-glutamyl acceptors but for donors. If *Proteus* γ-GT functioned in an uptake system of amino acids into cells, amino acids should compete with the acceptor of the γ-glutamyl residue. This result and high K_m values of *Proteus* γ-GT for the acceptors may suggest that this enzyme does not function in a transport system of amino acids. Further investigation is needed to resolve this problem.

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