Kor. J. Appl. Microbiol. Bioeng.
Vol. 16, No. 2, 92—97 (1988)

Purification and Properties of a Cysteinylglycinase from *Proteus mirabilis*

Choi, Shin-Yang[1*], Ju-Hyun Yu[1], Hidehiko Kumagai[2] and Tatsrokuro Tochikura[2]

[1]*Department of Food Engineering, Yonsei University, Seoul 120-749, Korea*
[2]*Department of Food Science and Technology, Kyoto University, Japan*

*Proteus mirabilis*가 生産하는 Cysteinylglycinase의 精製 및 性質

崔信良[1*]·柳洲鉉[1]·熊谷 英彦[2]·栃倉 辰六郎[2]

[1]延世大学校 食品工学科, [2]日本 京都大学 食品工学科

Cysteinylglycinase was partially purified from *Proteus mirabilis* by consecutive procedure. The specific activity was increased about 16-fold to that of cell-free extract. The enzyme was found rather unstable on ammonium sulfate precipitation and the precipitated enzyme protein became partially insoluble during dialysis. The precipitated enzyme was found to be solubilized by treatment of 4% Triton X-100 effectiviely. The optimum temperature and pH of the enzyme activity were 35°C and 7.3, respectively. After heat treatment of the enzyme at 50°C for 30 min, it lost the activity to 70%. The enzyme was stable at pH 7.0-8.0. The molecular weight of the cysteinylglycinase was found to be about 190,000 by Sephadex G-150 gel filtration. The enzyme was activated by the addition of Mn^{2+} and Mg^{2+} ions. The maximal activation was obtained in preincubation with Mg^{2+} ion for 30 min. The enzyme catalyzed the hydrolysis of various dipeptides and tripeptides. The Km and Vmax values for cysteinylglycine were 1.60 mM and 0.24 m unit/mg, respectively.

Glutathione, a tripeptide having a structure of γ-glutamylcysteinylglycine, occurs in animal cells, also in most plants and microorganisms. Glutathione participates in many important biological functions and these functions were investigated mainly in animals. Details of the physiological roles of glutathione in microorganisms have remained obscure, though glutathione was first discovered in yeast(1), its distribution(2) was wide, and the enzyme involved in its metabolism was also reported in microorganisms(3,4). Glutathione is the major nonprotein sulfhydryl compound in some Gram negative bacteria(5) and was appeared to be a cysteine source during the morphogenesis of the *B. cereus* spore coat(6). Hydrolysis and γ-glutamyl transfer of glutathione by intact cells and cell-free extracts of *Proteus vulgaris* were initially reported by Talalay(7). As a part of the hydrolysis of glutathione, it became necessary to study the behavior of the enzyme responsible for the hydrolysis of cysteinylglycine.

In this study, cysteinylglycinase, which participates in hydrolysis of cysteinylglycine, was partially purified and its properties were investigated as a preliminary search for the function of the peptidase in *Proteus mirabilis*.

Materials and Methods

Chemicals and reagents

Glutathione and γ-glutamyl-*p*-nitroanilide were obtained from Sigma Chem. Co. DEAE-Cellulofine was obtained from Chisso Chem. Co. Ltd. and Sephadex G-150 was purchased from Pharmacia Fine Chem. Co. The other chemicals were analytical grade reagent from commercial sources.

Key words: Cysteinylglycinase, *Proteus mirabilis*, glutathione
* Corresponding author

Enzyme production

A strain of *P. mirabilis* from the stock culture of Department of Food Science & Technology, Kyoto University was used in this study since it had been high activities of glutathione metabolizing enzymes. The medium for seed cultures was as follows: 1% peptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose at pH 7.0. The medium for jar cultures was consisted of 7g citric acid, 10g peptone, 10g glucose, 25mg L-threonine, 50mg L-leucine, 25mg L-proline, 50mg L-arginine, 10mg L-histidine, 1 mg thiamine-HCl, 10g K_2HPO_4, 10g $NaNH_4HPO_4 \cdot 4H_2O$, and 0.2g $MgSO_4 \cdot 7H_2O$ in 1 liter tap water. Cultivation was carried out at 30 °C for 24 hr with aeration (0.7 vvm) and agitation (150 rpm). The grown cells were harvested with a continuous-flow centrifuge and stored at-80 °C until preparation of the enzyme.

Purification procedure

The following procedures were all done at 0 to 5 °C. Frozen cell paste was suspended in 50 mM Tris-HCl buffer at pH 8.0. The cells were disrupted with a Dyno-mill (agitation rate 3,000 rpm with glass beads, 0.25 to 0.5 mm in diameter; Willy A Bachofen Maschinenfabrik, Basel), and cell-free extracts was obtained by centrifugation. Ammonium sulfate was added to cell-free extracts to 40% saturation. After being left overnight, the supernatant was collected by centrifugation and brought to 80% saturation in the same manner. After being left overnight, the precipitate was collected by centrifugation and dissolved in 1 liter of 50 mM Tris-HCl buffer at pH 8.0. This solution was dialyzed three times against 40 liter of the same buffer for 72 hr. The pH of the enzyme solution was adjusted to 7.0 with 2.5% NH_4OH solution after each addition of ammonium sulfate. The dialyzed enzyme solution was applied to a DEAE-cellulofine column (21 × 70 cm) previously equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The colume was washed with the same buffer and the enzyme was eluted stepwise with the buffer containing 0.05, 0.07, 0.1, 0.15 and 0.2 M NaCl at a flow rate of 100 m*l*/hr. Cysteinylglycinase was eluted with the buffer containing 0.15 M NaCl (Fig. 1). The active fraction (4.61) were combined and concentrated by the addition of ammonium sulfate to 80% saturation. The precipitate was collected by centrifugation, dissolved in and dialyzed against the same buffer. The concentrated enzyme solution obtained by ultrafiltration was applied to a Sephadex G-150 column (1.4 × 100 cm) equilibrated with the same buffer and eluted with the same buffer at a flow rate of 100 m*l*/hr in 2 m*l* fractions. The active fractions were combined and was applied to a DEAE-cellulofine column (1.5 × 7 cm) equilibrated with 50 mM Tris-HCl buffer at pH 8.0. Elution was carried out with a linear gradient between 0.1 and 0.25 M NaCl in the same buffer at a flow rate of 80 m*l*/hr. The active fractions were combined and was used in the following experiments.

Enzyme assay

The γ-glutamyltranspeptidase activity was determined with L-γ-glutamyl-*p*-nitroanilide and glycylglycine(8). The assay solution contained 2.5 mM of γ-glutamyl-*p*-nitroanilide, 60 mM of glycylglycine (pH 8.0), 50 mM of Tris-HCl buffer (pH 8.0), 75 mM of NaCl, and enzyme solution in a final volume of 1.0 m*l*. The reaction was started by the addition of enzyme solution. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 2 m*l* of 3.5N acetic acid, and precipitate was removed by centrifugation. The absorbance of the supernatant was measured at 410 nm. A blank test

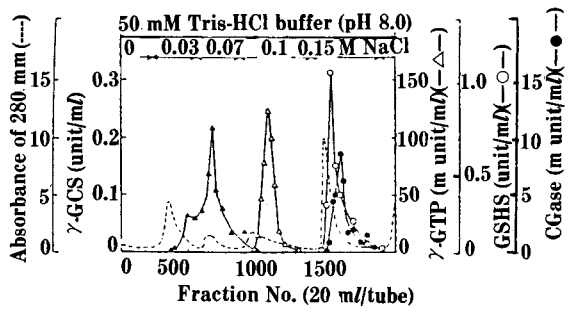

Fig. 1. DEAE-cellulofine column chromatography of glutathione related enzymes in *P. mirabilis*.

CGase, cysteinylglycinase; GSHS, glutathione synthetase; γ-GTP, γ-glutamyltranspeptidase; γ-GCS, γ-glutamylcysteine synthetase.

was carried out without glycylglycine.

Cysteinylglycinase was assayed by the method of McCorquodale(9) with a slight modification. The assay solution contained 0.1 M diethylamine-HCl buffer (pH 9.0), 4 mM cysteinylglycine, 10 mM $MgSO_4 \cdot 7H_2O$, and enzyme solution in a final volume of 0.5 m*l*. After preincubation without the substrate at 30 °C for 30 min, the reaction started by the addition of 0.05 m*l* of 40 mM cysteinylglycine solution. The reaction was carried out at 30 °C for 15 min and terminated by the addition of 0.5 m*l* of 4% TCA. The precipitated protein was removed by centrifugation and an aliquot of the supernatant solution was subjected to cystein determination by acid-ninhydrin method according to Gaitonde(10). Pink color developed by the reaction was measured at 560 nm with a Hitachi 101 spectrophotometer and compared with cysteine standard curve.

One unit of γ-glutamyltranspeptidase was expressed as the number of 1 umole *p*-nitroaniline produced per min, and that of cysteinylglycinase was also expressed as 1 umole cysteine produced per min.

Protein determination

The protein concentration was determined by the method of Lowry *et al.*(11) using ovalbumin as a standard, or spectrophotometrically by measuring the absorbance at 280 nm.

Amino acid analysis

Identification of reaction products was performed with an automatic amino acid analyzer (Kyowa Seimitsu Co. Ltd.) with 0.2 N citrate buffer at pH 3.25.

Preparation of substrate

The substrate, cysteinylglycine, was prepared by enzymatic degradation of reduced glutathione (GSH) with partial purified γ-glutamyltranspeptidase. The reaction mixture contained 50 mM GSH (pH 6.0), 50 mM Na-acetate buffer (pH 6.0), 75 mM NaCl, and 0.125 unit of purified enzyme (pH 6.0) in total volume of 5 m*l*. After incubation at 30 °C for 16 hr, the reaction was terminared by the addition of 1 m*l* of 17.4 N acetic acid and filtrated on Toyo membrane filter TM-2 (pore size 0.45 um). The amount of cysteinylglycine liberated was estimated from the amount of glutamate liberated which was determined with an amino acid analyzer.

Molecular weight determination

The molecular weight of the enzyme was determined by Sephadex G-150 gel filtration with five standard proteins, cytochrome C(MW 12,500), chymotrypsinogen A(MW 25,000), bovine serum albumin(MW 67,000), aldolase(MW 158,000), and catalase(MW 232,000). One milliliter solution of the standard and enzyme proteins were applied on a Sephadex G-150 column (2.0 × 100 cm) separately and eluted with 0.05 M Tris-HCl buffer (pH 8.0) at a flow rate of 50 m*l*/hr, collecting 2 m*l*-fractions. The void volume of the column was determined with Blue-dextran before and after application of the standard and sample enzymes.

Results and Discussion

Purification of cysteinylglycinase

A summary of the purification prodecure is presented in Table 1. The purity of cysteinylglycinase from *P. mirabilis* gave a 16-fold purification with a recovery of 0.68%. The enzyme was decreased largely on the ammonium sulfate precipitation. Magnesium ion was found not only to activate the enzyme, but also to stabilize the enzyme against inactivation by ammonium sulfate treatment. It seems to be effective to purify the enzyme in the presence of magnesium ion to have better recovery. In addition to inactivation by ammonium sulfate treatment, the precipitated enzyme became partially insoluble during dialysis. The aggregated enzyme was found to be partially solubilized by treatment of 4% Triton X-100 (Table 2).

γ-Glutamyltranspeptidase, γ-glutamylcysteine synthetase and glutathione synthetase were purified simultaneously from the same cell-free extracts. γ-Glutamyltranspeptidase and γ-glutamylcysteine synthetase were separated from cysteinylglycinase

Table 1. Purification of cysteinylglycinase from *P. mirabilis*.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (m unit/mg)	Recovery (%)
Cell-free extract	219,000	339	1.55	100
40-80% Ammonium sulfate	127,000	100	0.79	30
1st DEAE-cellulofine	8,000	15.0	1.94	4.4
Sephadex G-150	3,000	12.0	4.10	3.5
2nd DEAE-cellulofine	140	2.3	16.5	0.68

Table 2. Solubilization of precipitated cysteinylglycinase by detergent.

Detergent	Concentration (%)	Relative activity (%)
None		100
Triton X-100	1	19.1
	2	47.1
	4	92.4
Tween 20	0.1	7.6
	1	9.4
	2	10.2
SDS	0.1	10.2
	0.5	-*

* 0.5% SDS (sodium dodecyl sulfate) inhibited the assay.

but more than half of glutathione synthetase activity was associated with cysteinylglycinase.

Effect of pH and temperature on enzyme activity and stability

The enzyme was stable only in very narrow pH range pH 7.0-8.0 (Fig. 2b). The optimum pH of hydrolysis of cysteinylglycine was found around pH 7.3 (Fig. 2a). The enzyme was also rather unstable against heat treatment. It retained 90% activity after incubation for 30 min at 30 °C and lost 30% activity at 40 °C and 90% at 60 °C (Fig. 3b). The optimum temperature of cysteinylglycine hydrolysis was found to be 35 °C (Fig. 3a).

Olson and Binkley(12) purified cysteinylglycinase from the pig kidney and reported its optimal pH of 8-9.

Activation by metal ions

Effect of various cations on the enzyme activity

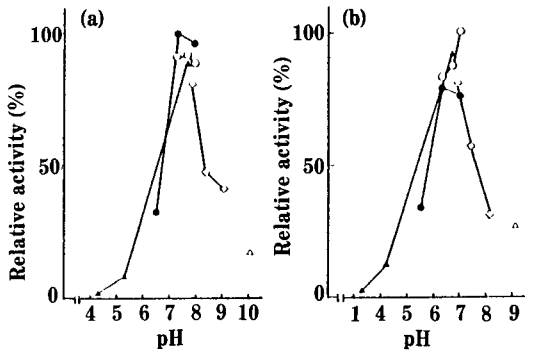

Fig. 2. Effect of pH on activity and stability of cysteinylglycinase.

Optimum pH (a): Activity is expressed relative to that found in 0.05 M potassium phosphate buffer, pH 7.3.
Stability (b): The enzyme was preincubated in various buffers containing 5 mM $MgCl_2$ at the indicated pH for 12 hr at 4 °C. Activity is expressed as the relative rate to that found in 0.05 M Tris-HCl buffer, pH 8.0.
Buffers used were acetate-sodium acetate (▲), potassium phosphate (●), Tris-HCl (○), diethanolamine-HCl (□) and sodium phosphate (△) at the concentration of 0.05 M.

was investigated by measuring the enzyme activity in the presence of 10 mM cations and summarized in Table 3. It is apparant that the activity was increased by the addition of manganese or magnesium ion. The highest activation was observed in the presence of more than 10 mM magnesium ion by the investigation of effect of magnesium ion concentration (Fig. 4). The enzyme required preincubation for more than 30 min to show the maximum activation in the presence of 5 mM $MgCl_2$ (Fig. 5).

Cysteinylglycinase from pig kidney(12) and *E. coli*(9) were reported to be activated by Mg^{2+}, Co^{2+}, and Fe^{2+} ions but not by Mn^{2+}.

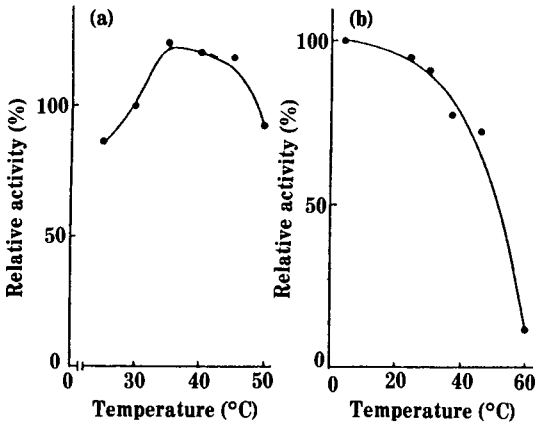

Fig. 3. Effect of temperature on activity and stability of cysteinylglycinase.

Optimum temperature (a): Activity is expressed relative to that found at 30 °C.

Stability (b): The enzyme was preincubated in 0.05 M diethanolamine-HCl buffer (pH 9.0) containing 5 mM $MgCl_2$ for 30 min at the indicated temperature. Activity is expressed relative to that found with the enzyme preincubated at 4 °C for 30 min.

Table 3. Effect of metal ions on cysteinylglycinase activity.

Metal ion (10 mM)	Relative activity (%)
None	100
$FeCl_3$	-*
$CaCl_2$	93
$CuCl_2$	-*
$MnCl_2$	161
$ZnCl_2$	-*
$BaCl_2$	58
$MgCl_2$	200
$CdCl_2$	23
$NiCl_2$	8
$CoCl_2$	-*

* Colour development in the assay was inhibited by the metal.

Substrate specificity

Substrate specificity of the enzyme was examined by replacing cysteinylglycine with various dipeptides and tripeptides. The enzyme attacked on all peptides examined and hydrolyzed triglycine much faster than diglycine (Table 4).

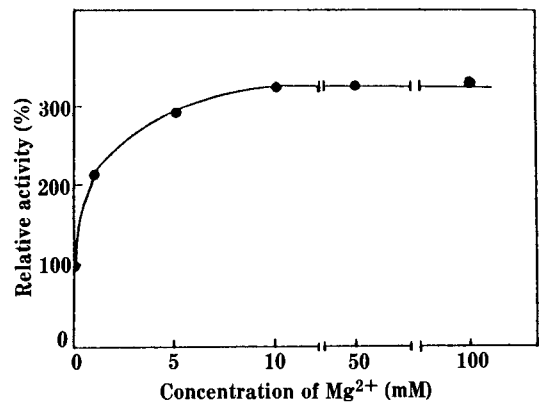

Fig. 4. Relationship of concentration of Mg^{2+} ion to cysteinylglycinase activity.

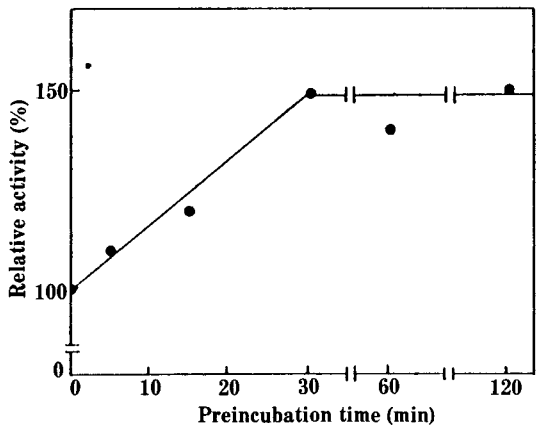

Fig. 5. Effect of preincubation with Mg^{2+} cysteinylglycinase activity.

Table 4. Substrate specificity of partially purified cysteinylglycinase from *P. mirabilis*.

Substrate	Relative activity (%)
L-Cys-Gly	100
L-Ser-L-Leu	79.1
L-Leu-Gly	83.6
Gly-Gly	205
L-Trp-L-Ala	96.3
L-Leu-L-Phe	63.4
L-Tyr-L-Val	78.4
L-Trp-Gly	80.6
$(L\text{-Cys})_2$-bis-Gly	166
L-Leu-Gly-Gly	153
Gly-Gly-Gly	349

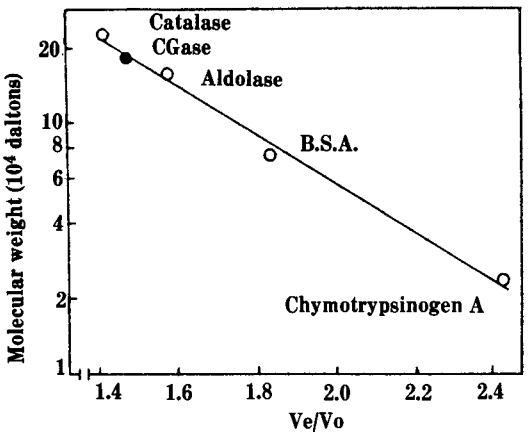

Fig. 6. Determination of the molecular weight of cysteinylglycinase by Sephadex G-150 gel filtration.

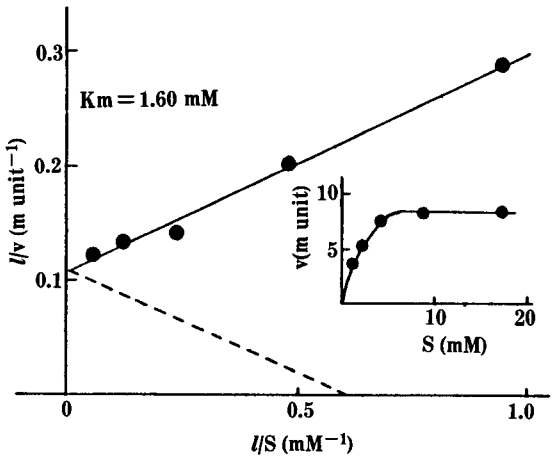

Fig. 7. Effect of substrate concentration on the reaction velocity.

Molecular weight of cysteinylglycinase

A molecular weight of 190,000 was obtained by a Sephadex G-150 gel filtration. Cysteinylglycinase was eluted at the position close to catalase (Fig. 6).

Effect of substrate concentration on enzyme activity

Effect of concentration of cysteinylglycine on the reaction rate was investigated (Fig. 7). The Michaelis constant for cysteinylglycine was calculated to be 1.60 mM from Lineweaver-Burk plot.

요 약

*Proteus mirabilis*로부터 glutathione 분해에 관여하는 cysteinylglycine 분해효소를 정제하고 그 성질을 검토하였다. 본 균이 생산하는 cysteinylglycinase의 정제는 무세포추출액에 비해 비활성이 16배 증가하였고 0.68%의 낮은 수율을 나타내었다. Cysteinylglycinase는 $(NH_4)_2SO_4$ 침전과정에서 활성을 크게 손실하는 등 정제과정에서 불안정하였으며 투석중에 형성되는 불용성 침전물은 4% Triton X-100 처리에 의해 효과적으로 용해되었다. 본 효소의 일반적 성질은 pH 7.3, 온도 35℃에서 최대 활성을 나타내었다. 열안정성은 50℃에서 30분간 열처리에 의해 30%의 활성손실을 보였으며 pH 7.0-8.0에서 안정하였다. 이 효소의 분자량은 190,000이었으며 Mn이온과 Mg이온에 의해 활성이 촉진되었고 반응액에 Mg이온을 첨가한 후 30분간 preincubation 하므로서 최대 활성을 보였다. Cysteinylglycine에 대한 Km값과 Vmax 값은 각기 1.60 mM과 0.24 munit/mg 이었다.

References

1. Hopkins, F.G.: *J. Biol. Chem.*, **84**, 269 (1929).
2. Fahey, R.C., W.C. Brown, W.B. Adams and M.B. Worsham: *J. Bacteriol.*, **133**, 1134 (1978).
3. Milbauer, R. and N. Grossowicz: *J. Gen. Microbiol.* **41**, 185 (1965).
4. Mooz, E.D. and L. Wigglesworth: *Biochem. Biophys. Res. Commun.*, **68**, 1066 (1976).
5. Fahey, R.C., W.C. Brown, W.B. Adams and M.B. Worsham: *J. Bacteriol.*, **113**, 1126 (1978).
6. Talalay, S.P.: *Nature*, **174**, 516 (1954).
7. Cheng, H.M., A.I. Aronson and S.C. Holt: *J. Bacteriol.*, **121**, 144 (1974).
8. Novogrodosky, A., S.S. Tate and A. Meister: *Proc. Natl. Acad. Sci.*, **73**, 2414 (1976).
9. McCorquodale, D.J.: *J. Biol. Chem.*, **238**, 3914 (1963).
10. Gaitonde, M.K.: *Biochem. J.*, **104**, 627 (1967).
11. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randel: *J. Biol. Chem.*, **193**, 265 (1951).
12. Olson, C.K. and F. Binkley: *J. Biol. Chem.*, **186**, 731 (1950).

(Receivd February 15, 1988)