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Studies on the Properties of *E. coli* γ-Glutamylcysteine Synthetase in Relation to the Enzymatic Synthesis of Glutathione

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Abstract : γ-Glutamylcysteine synthetase was purified from *E. coli* K-12 strain and its properties related to the *in vitro* synthesis of glutathione by enzymatic method were investigated. The activity of purified γ-glutamylcysteine synthetase was increased with increasing concentration of L-glutamate up to 60 mM, while it was decreased by about 50% and 40% under 60 mM of L-cysteine and 45 mM of glycine, respectively. The enzyme activity was reduced not only by ADP, one of the reaction products, but also by the reduced form of glutathione. Therefore, because the reduced glutathione as well as glycine which is the substrate for glutathione synthetase inhibit the activity of γ-glutamylcysteine synthetase, it is recommended to design a bioreactor system with two separate reactions for glutathione synthesis : one with γ-glutamylcysteine synthetase reaction and the other glutathione synthetase reaction. In addition since ADP, resulted from these reactions, reduces the activity of γ-glutamylcysteine synthetase, it is necessary to introduce an ATP regeneration system for glutathione synthesis.(Received September 10, 1997; accepted October 13, 1997)

Introduction

Since glutathione (γ-glutamylcysteinylglycine) plays many physiological roles in mammals such as detoxification of toxins from environment and protection of cells against reactive oxygen compounds and free radicals,[1] it has been utilized as one of the main components for hepatic medicine.[2] Although glutathione has been produced by extraction from yeast cells or by chemical synthesis, these methods have some disadvantages for the mass production of glutathione.[3] Therefore, development of new methods has been proceeded for the improvement of glutathione production.[4-7]

Glutathione is synthesized by the consecutive actions of γ-glutamylcysteine synthetase(E.C 6.3.2.2) and glutathione synthetase.[8] The γ-glutamylcysteine synthetase catalyzes the first reaction, known as the rate limiting step, of glutathione biosynthesis.[9,10] It has been reported that γ-glutamylcysteine synthetase is feedback inhibited by reduced glutathione[11,12] and the enzyme formation is not repressed by glutathione.[13] Glutathione synthetase, which catalyzes glutathione biosynthesis in the second step, is not inhibited by glutathione.[14] These results indicate that the synthesis of glutathione would be mainly controlled through feedback inhibition of γ-glutamylcysteine synthetase by reduced glutathione. Nam[15] reported that the enhancement of γ-glutamylcysteine synthetase activity would be crucial for the improvement of *in vitro* glutathione biosynthesis and the enzymatic method would be more suitable than fermentation for the glutathione production.

γ-Glutamylcysteine synthetase has been purified from several sources such as *Escherichia coli* B,[16] *Proteus mirabilis*[17,18] and rat liver.[19] Each of them showed differences in physical and catalytic properties. In this study, γ-glutamylcysteine synthetase was purified from *E. coli* HB101 strain containing a plasmid pGH500 (pUC 8-*gsh*I.I) and its properties, which relate to the *in vitro* synthesis of glutathione, were investigated.

Materials and Methods

Bacterial strain

E. coli HB101 strain harboring recombinant plasmid, pGH500, which contains two copies of γ-glutamylcysteine synthetase gene (*gsh*I) derived from *E. coli* K-12 wild type on pUC8 vector plasmid, was used for the purification of γ-glutamylcysteine synthetase.[20] *E. coli* HB101 cells harboring pGH500 were grown in LB medium with ampicillin (50 μg/m*l*) at 37℃ for 16 hr under

Key words : γ-glutamylcysteine synthetase, purification, enzymatic synthesis, glutathione
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reciprocal shaking condition.

Chemicals

ATP, acetylphosphate, amino acids, NADPH, NADH were purchased from Sigma (U.S.A). γ-Glutamylcysteine was purchased from Nakari Chemical LTD (Japan). Glutathione reductase, pyruvate kinase (type II), and lactate dehydrogenase (type II) were purchased from Sigma (U. S.A). DEAE-Trisacryl, Sephadex G-100 and G-150 were purchased from Phamacia Biotech (Sweden).

γ-Glutamylcysteine synthetase assay

Two different methods were used for the assay of γ-glutamylcysteine synthetase as described by Kumagai.[17] The feedback inhibition of the enzyme by glutathione was determined by PK-LDH method and the usual enzyme activity was measured by DTNB method.

PK-LDH method : The standard reaction mixture is composed of 12.5 μmol L-glutamate, 12.5 μmol ATP, 125 μmol diethanolamine-HCl buffer (pH 9.15), 1.25 μmol $MgSO_4.7H_2O$, 125 μmol KCL, 0.75 mg bovine serum albumin, 0.25 μmol NADH, 0.125 μmol phosphoenolpyruvate, 7.4 units pyruvate kinase, 12.7 units lactate dehydrogenase, and the enzyme, to a final volume of 0.8 m*l*. The reaction was initiated in a 1 m*l* cuvette of 1 cm light path after the addition of 12.5 μmol cysteine at 37℃. The initial rate measurement was carried out by monitoring the change in absorbance at 340 nm for at least 2 min. For calculation of NADH consumed, an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ at 340 nm was used.

DTNB method : The activity of enzyme was measured by the method of Jackson[21] with a slight modification. The standard reaction mixture contained 30 μmol L-glutamate, L-cysteine, and ATP, respectively, and 20 μmol $MgSO_4.7H_2O$, 200 μmol of KCl, 200 μmol of diethanolamine-HCl buffer (pH 9.15), and the cell free extract to a final volume of 2.0 m*l*. It was incubated at 37℃ for 15 min. This reaction was terminated by adding 3.3 m*l* of ice cold 3.2% sulphosalicylic acid. The remaining cysteine in a reaction mixture was removed by the method of Ball.[22] After standing on ice for 20 min, the reaction mixture was centrifuged at 1,000 × g for 20 min. Then 1 m*l* of its supernatant was transferred to each of the tubes containing 4 m*l* of 1.0 M potassium phosphate buffer (pH 6.8), with 5 mM EDTA, 1.0 g/*l* ascrobic acid, and 3.0 g/*l* of glyoxylic acid monohydrate. These tubes were incubated at 60℃ for 5 min, cooled rapidly on ice, and then equilibrated at 25℃. 0.5 m*l* of 3.8 mM DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] in 1.0 M potassium phosphate buffer (pH 6.8) was added to each of tubes. The absorbance was read at 412 nm for 7 min after the addition of DTNB. One unit of γ-glutamylcysteine synthetase activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmol γ-glutamylcysteine per hour. Specific activity is expressed in terms of units per milligram of protein.

γ-Glutamylcysteine synthetase purification

Enzyme preparation procedures were carried out at 4℃, and centrifugation was at 17,000 × g for 20 min.

(1) Cell free extract

The harvested cells (60 g wet weight) were resuspended in 300 m*l* of 25 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM L-glutamate, 0.5 mM L-cysteine and 1.0 mM $MgSO_4.7H_2O$ (buffer I). Cells were lysed by sonication (80 KHz) for 20 min on ice and debris was removed by centrifugation. The resultant supernatant solution was dialyzed for overnight against 10 liters of buffer I.

(2) First ammonium sulfate fractionation

The supernatant (340 m*l*) was treated with solid $(NH_4)_2SO_4$. The precipitate obtained at a 30 to 60% ammonium sulfate saturation was dissolved in 240 m*l* of buffer I. This suspension was dialyzed twice for 48 hr against 20 liters of buffer I.

(3) Second ammonium sulfate fractionation

Solid $(NH_4)_2SO_4$ was added to 240 m*l* of the enzyme solution to obtain the precipitate at 35 to 60% saturation. The precipitate was dissolved in 45 m*l* of buffer I and dialyzed twice for 48 hr against 10 liters of buffer I.

(4) Heat treatment

The dialyzed enzyme solution was incubated at 45℃ for 5 min in a stirring condition. Then the precipitate was removed by centrifugation.

(5) DEAE-Trisacryl column chromatography

The enzyme solution (100 m*l*) was applied to a DEAE-Trisacryl column (3.4 × 22 cm) chromatography previously equilibrated with buffer I and then the enzyme was eluted with a linear gradient of 0 to 0.5 M KCl in buffer I at a flow rate of 80 m*l*/hr. The active fractions (60 m*l*) were pooled and dialyzed against buffer I. The dialyzed enzyme solution was concentrated approximately to 20 m*l* with cold PEG 6,000.

(6) Sephadex G-100 column chromatography

The enzyme solution (20 m*l*) was applied to a Sephadex G-100 column (1.6 × 80 cm) equilibrated with buffer I. The protein was eluted with buffer I at a flow rate of 10 m*l*/hr. Active fractions were pooled and concentrated approximately to 5 m*l* through filtration on

Centriprep-30 concentrator (Amicon).

(7) Sephadex G-150 column chromatography

The concentrated enzyme solution (5 m*l*) was applied to a Sephadex G-150 column (1.6 × 80 cm) equilibrated with the same buffer. The protein was eluted with buffer I at a flow rate of 6 m*l*/hr and 4 m*l* portion was collected. Active fractions were pooled and the enzyme solution was used throughout this study as a purified enzyme.

Assay of protein

The amount of protein was determined either with absorbance at 280 nm or using the method of Lowry.[23]

Gel electrophoresis

Proteins were analyzed on 10% SDS-polyacrylamide gels using the discontinuous buffer system as described by Laemmli.[24]

Results and Discussion

Purification of γ-glutamylcysteine synthetase from *E. coli* K-12

The purification of γ-glutamylcysteine synthetase was attempted after its overexpression of the cloned *gsh*I gene (pGH500) in *E. coli*. A homogeneous preparation of the γ-glutamylcysteine synthetase was obtained by ammonium sulfate fractionation, heat treatment, DEAE Trisacryl anion exchange column chromatography, Sephadex G-100 and Sephadex G-150 column chromatography.[16] The γ-glutamylcysteine synthetase obtained from each purification step was evaluated by SDS-PAGE (Fig 1). The purified enzyme showed a single band upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight of the purified enzyme was estimated to be 58,000 corresponding to the molecular weight from estimated amino acids sequences.[25] It agrees with that of γ-glutamylcysteine synthetase from *E. coli* B strain.[16] Specific activity of the purified enzyme was 468.1 U per mg of protein and a 10.1 fold purification was achieved. The yield was 7.8% (Table 1). Despite the low fold of purification for γ-glutamylcysteine synthetase, the homogeneous preparation of the enzyme was easily obtained due to the overexpression of the cloned *gsh*I gene in *E. coli* cells.

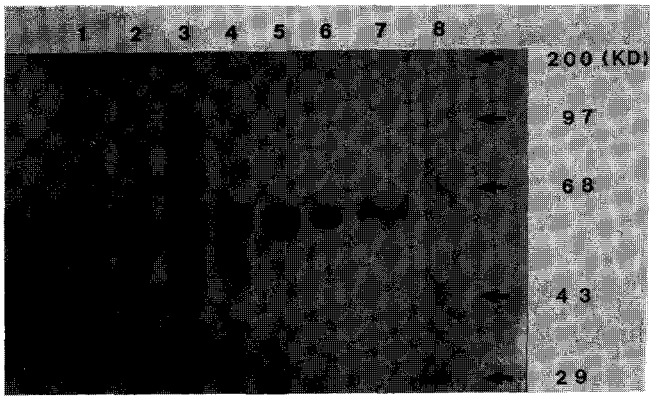

Fig. 1. SDS-PAGE pattern of γ-glutamylcysteine synthetase obtained from each successive purification step. The gel concentration was 10%. Standard proteins in gel (lane 8) were : myosin (H-chain) (MW = 200,000), phosphorylase B (MW = 97,400), bovine serum albumin (MW = 68,000), ovalbumin (MW = 43,000), carbonic anhydrase (MW = 29,000).
lane 1, Cell free extract; lane 2, 1st ammonium sulfate fraction; lane 3, 2nd ammonium sulfate fraction; lane 4, Heat treatment; lane 5, DEAE-trisacryl column chromatography; lane 6, Sephadex G-100 column chromatography; lane 7, Sephadex G-150 column chromatography; lane 8, Molecular size marker.

Properties of γ-glutamylcysteine synthetase in relation to the synthesis of glutathione.

It was reported that the amount of glutathione synthesized was not increased by increasing enzyme activity for glutathione biosynthesis in *E. coli* cell.[26] This suggests that an enzymatic bioreactor system would be advantageous for the production of the glutathione. For setting up bioreactor system, we have studied properties of γ-glutamylcysteine synthetase of *E. coli*.

The enzyme activity was determined at various pHs from 6.5 to 9.5 in several buffer systems (Fig 2). The highest enzyme activity was obtained at pH 7.5 in 0.1 M potassium phosphate buffer. The heat stability pro-

Table 1. Purification of γ-glutamylcysteine synthetase from *E. coli* K-12

Step	Total protein (mg)	Total unit	Specific activivity (unit/mg protein)	Purification fold	Yield (%)
Crude extract	11500	535370	46.5	1.00	100.0
1st Ammonium sulfate	7200	439450	61.0	1.31	82.0
2st Ammonium sulfate	3800	248325	65.3	1.40	46.4
Heat treatment	2000	203000	101.5	2.18	37.9
DEAE-Trisacryl	800	88550	110.7	2.38	16.5
1st Sephadex G100	270	67820	251.2	5.40	12.7
2st Sephadex G150	90	42130	468.1	10.10	7.8

Enzyme activity was determined by the DTNB method described in Materials and Methods.

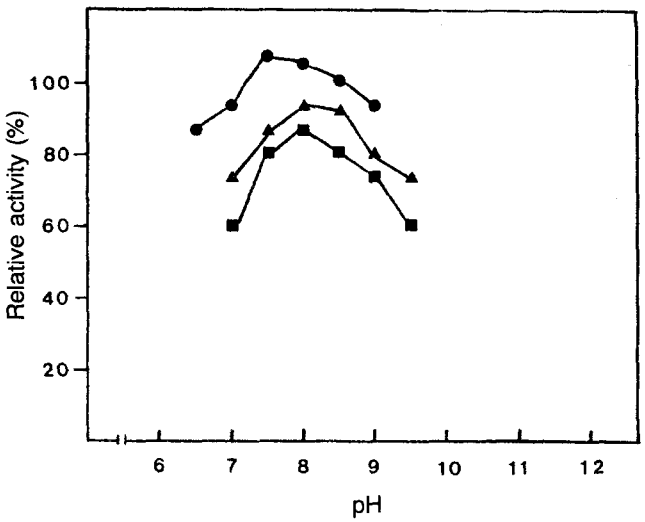

Fig. 2. Effect of pH on the purified γ-glutamylcysteine synthetase activity. The reaction was carried out in standard reaction mixture except changing buffers of various pH as described in Materials and Methods and the activity was determined by the DTNB method. Activity is expressed relative to that found in 0.1 M diethanolamine-HCl buffer. The buffers used were 0.1 M potassium phosphate (●—●), 0.1 M sodium phosphate (▲—▲), and 0.1 M Tris-HCl (■—■), respectively.

file of the enzyme activity is shown in Fig 3. When the enzyme activity was measured after preincubation at 45°C for 30 min, the enzyme retained its full activity. However, the enzyme activity was reduced to half in a reaction at 50°C for 15 min. The purified enzyme was stable during storage at 4°C for 10 months (Data not

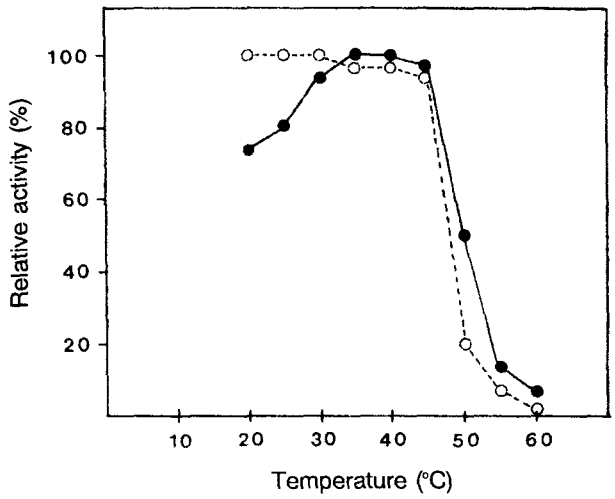

Fig. 3. Effect of temperature on the purified γ-glutamylcysteine synthetase activity. The reaction was carried out at the indicated temperature for 15 min instead of incubation at 37°C for 15 min (●—●). The enzyme for test of stability was preincubated in 25 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM L-Glu, 0.5 mM L-Cys, and 1.0 mM $MgSO_4.7H_2O$ at the indicated temperature for 30 min (○—○) and then its activity was determined by the DTNB method described in Materials and Methods. The activity is expressed relative to that found at 37°C.

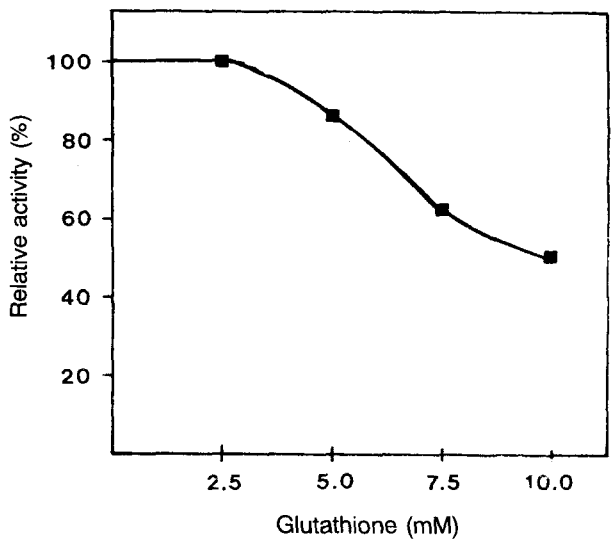

Fig. 4. Effect of reduced glutathione on the purified γ-glutamylcysteine synthetase activity. Enzyme activity was determined by PK-LDH method described in Materials and Methods. The activity is expressed relative to that determined in the absence of reduced glutathione (100%).

shown).

The changes in γ-glutamylcysteine synthetase activity were studied in order to examine the degree of feedback inhibition by the reduced form of glutathione in a range of 2.5 to 10 mM (Fig. 4). The activity was inhibited by 48% at 10 mM reduced glutathione. Inhibition of γ-glutamylcysteine synthetase by adenosine derivatives such as ADP, AMP, and adenosine, which are the reaction precursors or products of ATP in coupled reactions, was investigated with the cell free extract and the purified enzyme. ADP inhibited the activities of both the cell-free extract and the purified enzyme by 45% and 69%, respectively (Table 2). Adenosine inhibited both the non-purified and the purified enzymes, but the inhibition by AMP was not significant. These results indicate that the synthesis of γ-glutamylcysteine, the intermediate of glutathione synthesis, is strongly de-

Table 2. Inhibitory effect of adenosine derivatives on γ-glutamylcysteine synthetase activity.

Chemical	Concentration (mM)	ATP (mM)	Relative activity(%)	
			Cell free extract	Purified enzyme
None	-	15	100	100
ADP	10	15	55	31
AMP	10	15	95	83
adenosine	10	15	76	46

Enzyme activity was determined by the DTNB method described in Materials and Methods. Each of adenosine derivatives was added into standard reaction mixture at indicated concentration and the activity is expressed relative to that determined in the presence of 15 mM ATP (100%).

Table 3. Effect of various substrates on γ-glutamylcysteine synthetase activity.

Chemical	Concentration (mM)	Relative activity(%)	
		Cell free extract	Purified enzyme
L-Glutamate	15	100	100
	30	117	121
	45	120	124
	60	120	135
L-Cysteine	15	100	100
	30	106	100
	45	98	97
	60	48	51
	75	32	29
Glycine	0	100	100
	15	82	68
	30	74	63
	45	66	60

Enzyme activity was determined by the DTNB method described in Materials and Methods. Each substrate was added into standard reaction mixture at indicated concentration and the activity is expressed relative to that determined in standard reaction mixture (100%).

pendent to the method of ATP supply. Therefore, a coupling reaction system with an ATP regenerating system is recommended for optimal synthesis of the γ-glutamylcysteine intermediate, since the ATP regenerating system can maintain ADP in low concentration and consequently the enzyme inhibition by ADP would be minimized.

The effect of substrates such as L-glutamate, L-cysteine and glycine on the activity of purified γ-glutamylcysteine synthetase was investigated (Table 3). The activity of γ-glutamylcysteine synthetase was increased 35% with the elevation of concentration of L-glutamate up to 60 mM, while it was decreased by about 50% and 40% under 60 mM of L-cysteine and 45 mM of glycine, respectively. On the other hand, no γ-glutamylcysteine hydrolyzing activity was detected in the purified enzyme (Data not shown).

In summary, it is recommended that glutathione producing reactions should be separately conducted in two bioreactors : the first reaction proceeds in a mixture consisting of γ-glutamylcysteine synthetase, L-glutamate, L-cysteine and an ATP-regenerating system, and the second reaction proceeds in a mixture consisting of glutathione synthetase, the products of the first reaction, glycine and an ATP-regenerating system. This system can eliminate the feedback inhibition by the reduced glutathione and the inhibition by glycine on the γ-glutamylcysteine synthetase in the first reaction. Additionally the substrate inhibition of γ-glutamylcysteine synthetase by L-cysteine can be reduced by maintaining the substrate concentration low in the first reaction.

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글루타치온의 효소적 생합성에 관계되는 *E.coli* γ-Glutamylcysteine Synthetase의 특성 연구

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요 약 : *E. coli* K-12 균주에서 γ-Glutamylcysteine synthetase를 정제하고 효소적 방법에 의한 글루타치온 합성에 관련된 특성을 검사하였다. 정제한 효소의 활성은 L-glutamate의 농도가 60 mM 까지 증가와 더불어 증가하였으나, 60 mM L-cysteine 에서는 50% 그리고 45 mM glycine 에서는 40%의 효소활성이 감소되었다. 효소의 활성은 반응산물 중의 하나인 ADP 뿐만 아니라 환원형 글루타치온에 의해서 감소되었다. 그러므로 환원형 글루타치온 뿐만 아니라 glutathione synthetase의 기질인 glycine은 γ-glutamylcysteine synthetase 활성을 저해하므로 글루타치온 생산을 위해서는 γ-glutamylcysteine synthetase 반응과 glutathione synthetase의 두 분리된 반응으로 이루어진 생반응계를 고안하는 것이 바람직하다. 또한 글루타치온 합성반응으로 부터 생성되는 ADP는 γ-glutamylcysteine synthetase의 활성을 감소시키므로 글루타치온 합성을 위해서 ATP 재생계의 도입이 필요하다.

찾는말 : γ-glutamylcysteine synthetase, 정제, 효소적 합성, 글루타치온
*연락저자