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ORIGINAL PAPER

Enhancement of glutathione production with a tripeptidase-deficient recombinant *Escherichia coli*

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Abstract Glutathione (GSH) degradation exists in the enzymatic synthesis of GSH by *Escherichia coli*, however, its degradation pathway is not very clear. This paper examines the key enzymes responding to GSH degradation in *E. coli* with the purpose of improving GSH production. The enzymes that are probably associated with GSH degradation were investigated by disrupting their genes. The results suggested that γ-glutamyltranspeptidase (GGT) and tripeptidase (PepT) were the key enzymes of GSH degradation, and GGT contributed more to GSH degradation than PepT. Furthermore, GGT activity was affected greatly by culture temperature, and the effect of GGT on GSH degradation could be eliminated by shortening the culture time at 30°C and extending the induction time at 42°C. However, the effect of PepT on GSH degradation could be eliminated only by disrupting the PepT gene. Finally, GSH degradation was not observed in GSH biosynthesis by *E. coli* JW1113 (*pepT*$^-$, pBV03), which was cultured at 30°C for 3 h and 42°C for 5 h. GSH concentration reached 15.60 mM, which was 2.19-fold of the control. To the best of our knowledge, this is the first report of prohibiting GSH degradation with PepT-deficient recombinant *E. coli*. The results are helpful to investigate the GSH metabolism pathway and construct a GSH biosynthesis system.

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Introduction

Glutathione (γ-glutamyl-L-cysteinylglycine, GSH) is the most abundant free thiol compound in cells [1]. GSH is found in high concentrations in almost all prokaryotic and eukaryotic cells. GSH acts as the principal redox buffer, plays an important role in oxidative stress response and in the detoxification of metals and xenobiotics, and influences several essential processes, such as gene expression, cell proliferation, and apoptosis [1–3]. Therefore, nowadays GSH finds wide applications in the pharmaceutical, food and cosmetic industries [4–6], and the commercial demand for GSH is expanding.

The synthesis pathway of GSH is known and well investigated. GSH is synthesized by the consecutive action of γ-glutamylcysteine synthetase (EC 6.3.2.2, γ-GCS) and GSH synthetase (EC 6.3.2.3, GS). Compared with the fermentative method, the enzymatic production of GSH can achieve a higher concentration and can be more beneficial to its final purification. However, to date, the enzymatic production of GSH has not been commercialized. One of the key issues was that the degradation of GSH in its enzymatic synthesis greatly reduced its final production. Compared with its well-investigated synthetic pathway, there is only little research on the degradation pathway of GSH. Nakayama et al. reported that the degradation of GSH was the action of γ-glutamyltranspeptidase (EC 2.3.2.2, GGT) [7], and they tried to prohibit GSH degradation with the addition of a L-serine and borate mixture; however, no satisfactory results were obtained.

This work aimed to determine the key enzymes related to GSH degradation and then improved GSH production by

reducing its degradation. The main enzymes involved in GSH degradation were analyzed using their mutants, which were developed by the knockout of their genes. The results suggested that GSH degradation in the biosynthetic process was mainly due to the action of GGT and tripeptidase (EC 3.4.11.4, PepT). GSH production increased significantly after eliminating the action of both GGT and PepT. The demonstration of the key GSH degradation enzymes in the biosynthesis of GSH is helpful to guide the construction of GSH biosynthesis system.

Materials and methods

Chemicals

Adenosine triphosphate (ATP), GSH reductase (GR), GSH, and nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB), L-glutamic acid γ-(*p*-nitroanilide) hydrochloride (L-γ-Glu-*p*-NA), glycylglycine (Gly-Gly), and leucylglycylglycine (Leu-Gly-Gly) were purchased from Sigma-Aldrich (Shanghai, China). All other chemicals were of reagent grade.

Strains, plasmid, and culture conditions

The bacterial strains used in this study were *E. coli* BW25113 and its mutants. *E. coli* JW3412 (*ggt*$^-$, encoding the γ-glutamyltranspeptidase), *E. coli* JW1627 (*gst*$^-$, encoding the GSH-S-transferase), and *E. coli* JW1113 (*pepT*$^-$, encoding the tripeptidase) were purchased from the coli genetic stock center (CGSC) at Yale University. They were constructed at Keio University, Japan, using the method of Datsenko and Wanner [8] by Baba et al. [9]. Plasmid pBV03 was constructed in our previous study [10]. It contained *gshI* (encoding the γ-glutamylcysteine synthetase) and *gshII* (encoding the GSH synthetase).

E. coli BW25113, JW3412, JW1113, and JW1627, used in the enzyme analysis of GSH degradation, were cultured in LB medium at 30ºC for 8 h. Media were sterilized at 121°C for 20 min. The plasmid pBV03 was introduced into *E. coli* BW25113, JW3412, and JW1113 to construct the recombinant strains for GSH production by electroporation. *E. coli* was grown in 50 ml LB (Luria-Bertani) media (1% Tryptone, 0.5% yeast extract, and 1% NaCl) with 20 mg/l kanamycin at 30°C to an OD_{600} of 0.6 and then made competent by concentrating 100-fold and washing three times with ice-cold 10% glycerol. Electroporation was performed by using a Bio-Rad Gene Pulser set at 1.8 kV, 25 mF, with a pulse controller of 200 Ω. Shocked cells were immediately added to 1 ml SOC medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), incubated for 1 h at 37ºC, and then 100 µl culture was spread onto LB agar medium to select the transformants with ampicillin resistance. *E. coli* BW25113 (pBV03), *E. coli* JW3412 (pBV03), and *E. coli* JW1113 (pBV03) were cultured in LB medium supplemented with ampicillin (100 mg/l) at 30 ºC, and then were induced at 42°C for the expression of *gsh I* and *gsh II*. The culture time was described in the text.

The cells were harvested by centrifuging the culture broth at 8,000×*g* and 4ºC for 5 min, and washed three times with 20 mM potassium phosphate buffer (pH 7.0). The washed cells were recollected by centrifugation at 8,000×*g* and 4ºC for 5 min, and stored at −80ºC for further use.

GSH enzymatic synthesis

The cells concentration was 200 g wet weight cells per liter. The reaction mixture contained 60 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 30 mM ATP, 30 mM $MgCl_2$, 0.5% (v/v) toluene, and 150 mM potassium phosphate buffer (pH 7.0). The mixture was incubated at 37°C with gentle shaking. Samples were taken and immediately heated in boiling water for 10 min; the cell debris was removed by centrifugation at 8,000×*g* for 5 min, and the supernatants were used for the analysis.

Analysis

GSH concentration was determined according to the method described by Cohn and Lyle [11]. GGT activity was assayed by the method of Payne and Payne [12] with some modifications. Cells permeabilized with 0.5% toluene (100 mg cells) were incubated in 1 ml of reaction mixture, containing 50 mM Tris-HCl (pH 9.0) and 3 mM L-γ-Glu-*p*-NA at 37°C for 30 min. The reaction was stopped by the addition of 100 µl 3.5 M acetic acid. Samples were centrifuged at 12,000×*g* for 5 min. The formation of *p*-nitroaniline was measured in supernatant spectrophotometrically at 410 nm ($M_{\in}$ 8,800 M^{-1} cm^{-1}). One unit of GGT activity was defined as the amount of the enzyme that produced 1 µmol of *p*-nitroanline per min under the assay conditions. Peptidase activity was determined by measuring the release of leucine (Leu) from the tripeptide leucyl-glycyl-glycine (Leu-Gly-Gly) [2 mM in 20 mM *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.0]. Permeabilized cells and substrate were incubated at 30°C for 10 min, and leucine release was measured with the modified cadmium–ninhydrin method as described by Doi et al. [13]. One unit of PepT activity was defined as the amount of enzyme that produced 1 µmol of Leu per min under the assay conditions.

Results

GSH degradation in its enzymatic synthesis by *E. coli* BW25113 (pBV03)

The GSH biosynthetic process was investigated using the cells of *E. coli* BW25113 (pBV03), which was cultured at 30ºC for 4 h and then induced at 42°C for 4 h. GSH production increased quickly within 1 h, and the maximal production reached 10.31 mM at 1 h, and then GSH production decreased gradually through the time (Fig. 1). GSH production was only 7.50 mM at 5 h, which was 69.2% of the maximal production.

The key enzymes responding to GSH degradation in *E. coli*

To analyze the cause of GSH degradation during its enzymatic synthesis, the enzymes related to GSH metabolism were studied. The GSH metabolism pathway in *E. coli* was described in Fig. 2. Three main enzymes maybe related to GSH degradation, which are glutathione S-transferase (GST), tripeptidase (PepT), and γ-glutamyltranspeptidase (GGT).

E. coli BW25113, JW3412 (*ggt*$^-$), JW1113 (*pepT*$^-$), and JW1627 (*gst*$^-$), used in the enzyme analysis of GSH degradation, were cultured in LB medium at 30ºC for 8 h. The cells of *E. coli* BW25113 or its mutants (*gst*$^-$, *pepT*$^-$, and *ggt*$^-$) were incubated with 10 mM added GSH at 37°C for 1 h, and the residual GSH was determined (Fig. 3). The highest concentration of residual GSH was 7.90 mM in *E. coli* JW3412 (*ggt*$^-$), which is 4.07-fold of the control (*E. coli* BW25113). Due to the significant decrease of GSH degradation after disrupting the *ggt* gene, GGT was determined as a key enzyme responding to GSH degradation. The reduction of GSH degradation was also remarkable in

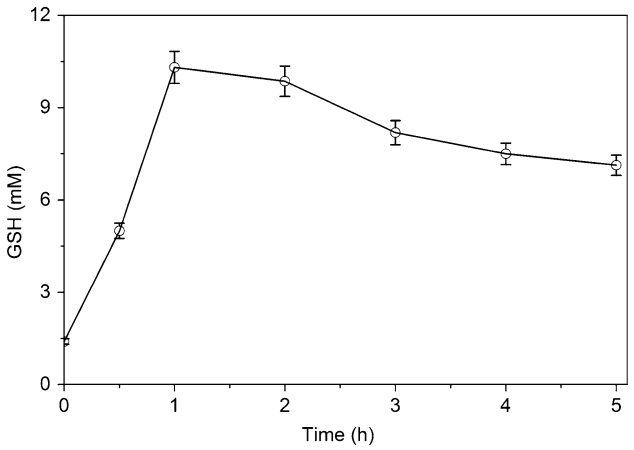

Fig. 1 Time course of GSH enzymatic synthesis by *E. coli* BW25113 (pBV03). The cells were cultured at 30°C for 4 h and then induced at 42°C for 4 h. The reaction mixture contained 60 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 30 mM ATP, 30 mM $MgCl_2$, and 150 mM potassium phosphate buffer (pH 7.0)

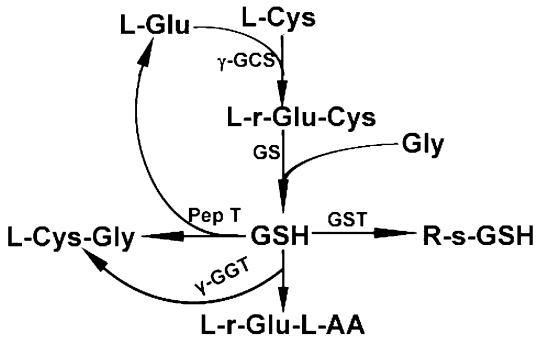

Fig. 2 Schematic diagram of GSH metabolism pathway in *E. coli* (referring to the GSH metabolism of KEGG 2009). γ-GCS, γ-glutamylcysteine synthetase; GS, GSH synthetase; GGT, γ-glutamyltranspeptidase; PepT, tripeptidase; GST, GSH S-transferase

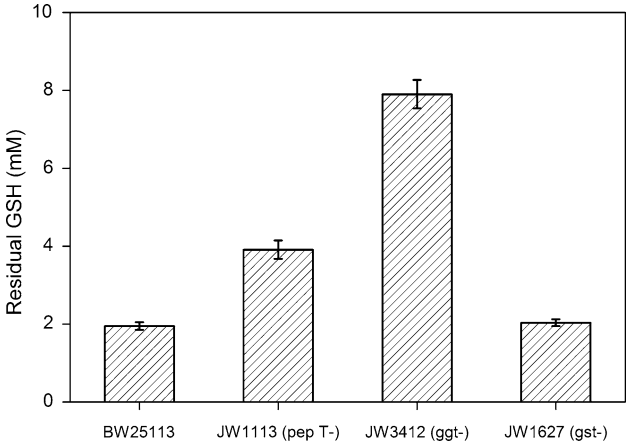

Fig. 3 GSH degradation by *E. coli* BW25113 and its mutants. The cell concentration was 100 g wet cells per liter, and the initial GSH was 10 mM. The mixture was incubated at 37°C with gentle shaking for 1 h. *E. coli* BW25113, GGT mutant, GST mutant, and PepT mutant used in this experiment were cultured in LB medium at 30ºC for 8 h

E. coli JW1113 (*pepT*$^-$), and the residual GSH concentration was 3.91 mM, which was 2.02-fold of *E. coli* BW25113. This result suggested that PepT is also a key enzyme of GSH degradation, but its effect on GSH degradation was inferior to GGT. The residual GSH concentration was only 2.03 mM in *E. coli* JW1627 (*gst*$^-$), which was almost the same as *E. coli* BW25113 (1.94 mM). Therefore, GST was not considered as a key enzyme in this study. GGT and PepT were further investigated as the key enzymes related to GSH degradation in *E. coli*.

The effect of culture condition on the activities of GGT and PepT

The activities of GGT and PepT in the cells of *E. coli* BW25113 (pBV03), used for GSH production under the culture condition of 30°C for 3 h and 42°C for 5 h, were investigated (Table 1). The results showed that GGT and PepT activities were 4.0 U and 66.6 U, respectively.

Table 1 Effect of culture conditions on GGT and PepT activities of recombinant *E. coli*

Strain	GGT activity (U)			PepT activity (U)		
	30°C for 8 h	30°C for 4 h, 42°C for 4 h	30°C for 3 h, 42°C for 5 h	30°C for 8 h	30°C for 4 h, 42°C for 4 h	30°C for 3 h, 42°C for 5 h
E. coli BW25113	44.8 ± 0.9	11.5 ± 0.5	4.5 ± 0.3	65.8 ± 0.5	65.5 ± 0.3	64.7 ± 0.5
E. coli BW25113 (pBV03)	44.5 ± 1.2	10.3 ± 0.5	4.0 ± 0.4	67.4 ± 0.3	63.5 ± 0.2	66.6 ± 0.5
E. coli JW3412 (*ggt*$^-$) (pBV03)	3.6 ± 0.3	3.3 ± 0.6	3.5 ± 0.5	66.3 ± 0.4	65.3 ± 0.5	64.5 ± 0.2
E. coli JW1113 (*pepT*$^-$) (pBV03)	45.1 ± 0.8	11.5 ± 0.4	4.3 ± 0.3	4.2 ± 0.2	3.5 ± 0.3	4.8 ± 0.2

The enzyme activity was determined as described in "Materials and methods." Cells were cultured in LB medium supplemented with ampicillin (100 mg/l) at 30℃, and then were induced at 42°C for the expression of *gsh I* and *gsh II*. Cells were permeabilized with 0.5% toluene. One unit of GGT activity was defined as the amount of the enzyme that produced 1 μmol of *p*-nitroanline per min under the assay conditions. One unit of PepT activity was defined as the amount of enzyme that produced 1 μmol of Leu per min under the assay conditions

The PepT activity was close to that in *E. coli* BW25113 (cultured at 30°C for 8 h) (67.5 U). However, the GGT activity (4.0 U) in *E. coli* BW25113 (pBV03) was much lower than that in *E. coli* BW25113 (44.2 U), which was cultured at 30°C for 8 h. As shown in Table 1, GGT activity of *E. coli* BW25113 (with or without pBV03) varied greatly with the culture condition. GGT activity of the cells cultured at 30°C for 8 h was 11.1-fold of that cultured at 30°C for 3 h and 42°C for 5 h. Similarly, GGT activity in *E. coli* JW1113 (*pepT*$^-$) (pBV03) also decreased quickly by shortening the culture time at 30°C and extending the induction time at 42°C. Furthermore, GGT activity of the cells cultured at 30°C for 3 h and 42°C for 5 h was near to that of *E. coli* JW3412 (*ggt*$^-$). These results suggested that, instead of the knockout of *ggt*, GSH degradation caused by GGT could be inhibited by the optimization of culture condition. As shown in Fig. 4, GSH production of *E. coli* BW25113 (pBV03) cultured at 30°C for 3 h and 42°C for 5 h was 12.22 mM after 5 h, which was only 4.2% lower than that of *E. coli* JW3412 (*ggt*$^-$). However, unlike GGT, PepT activity was almost constant under different culture conditions (Table 1); thus, GSH degradation caused by PepT could be inhibited only by disrupting *pepT*.

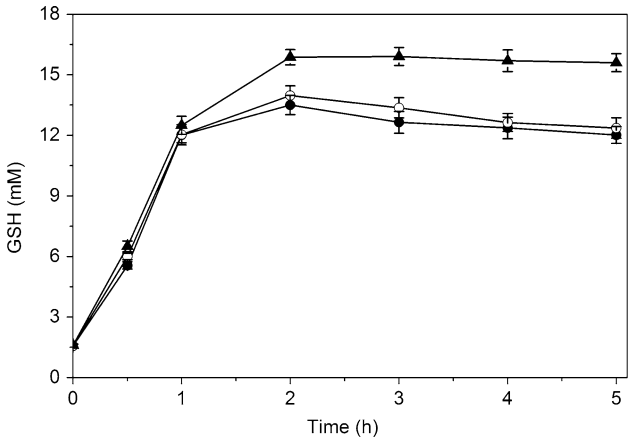

Fig. 4 Time course of GSH biosynthesis by *E. coli* BW25113 (pBV03), *E. coli* JW3412 (*ggt*$^-$) (pBV03), and *E. coli* JW1113 (*pepT*$^-$) (pBV03). (*Filled circle*) *E. coli* BW25113 (pBV03) cultured at 30°C for 3 h and induced at 42°C for 5 h. (*Open circle*) *E. coli* JW3412 (*ggt*$^-$) (pBV03) cultured at 30°C for 3 h and induced at 42°C for 5 h; (*filled triangle*) *E. coli* JW1113 (*pepT*$^-$) (pBV03) cultured at 30°C for 3 h and at 42°C for 5 h. The reaction mixture contained 60 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 30 mM ATP, 30 mM $MgCl_2$, and 150 mM potassium phosphate buffer (pH 7.0)

GSH production by *E. coli* JW1113 (*pepT*$^-$) (pBV03) under optimized culture condition

Compared with the enzymatic synthesis of GSH by the cells of *E. coli* BW25113 (pBV03) cultured at 30°C for 4 h and 42°C for 4 h (Fig. 1), GSH degradation decreased greatly in the enzymatic synthesis of GSH by *E. coli* BW25113 (pBV03) cultured at 30°C for 3 h and 42°C for 5 h (Fig. 4). Moreover, its ability for GSH production was similar to *E. coli* JW3412 (*ggt*$^-$) (pBV03). However, GSH degradation still existed in the enzymatic synthesis of GSH after eliminating the effect of GGT by gene knockout or culture optimization (Figs. 3 and 4). This result was due to the action of PepT. Therefore, GSH production was further studied in *E. coli* JW1113 (*pepT*$^-$) (pBV03) that was cultured at 30°C for 3 h and 42°C for 5 h. As shown in Fig. 4, GSH degradation was not observed in the enzymatic synthesis of GSH by *E. coli* JW1113 (*pepT*$^-$, pBV03) that was cultured at 30°C for 3 h and then induced at 42°C for 5 h. Finally, GSH production reached 15.60 mM, which was 27.7% higher than that by *E. coli* BW25113 (pBV03) cultured at 30°C for 3 h and induced at 42°C for 5 h. Under the same reaction conditions of GSH enzymatic synthesis, this production was 2.19-fold of that by *E. coli* BW25113 (pBV03) (7.50 mM, Fig. 1) cultured at 30°C for 4 h and induced at 42°C for 4 h.

Discussion

Inhibiting the degradation of GSH is beneficial to improve its production and to realize the industrialization of

enzymatic synthesis of GSH. Compared with its well-investigated synthetic pathway, its degradation pathway is not very clear. Therefore, the key enzymes degrading GSH have to be investigated for the decrease of GSH degradation. To date, the complete genome of *E. coli* has been sequenced, and a simple and high efficiency of the gene-knockout method has been developed [8]. Based on these, the key enzymes of the GSH degradation pathway can be accurately and efficiently determined by investigating the effect of its gene knockout on GSH degradation.

GGT was the key enzyme of GSH degradation (Fig. 4), and the knockout of the *ggt* gene can greatly reduce the GGT activity (Table 1). Furthermore, the production of GSH improved significantly because of the decrease of GSH degradation caused by GGT (Figs. 1 and 4), which coincided with the results reported by Nakayama et al. [7]. However, Figs. 3 and 4 both showed that the knockout of the *ggt* gene could not completely inhibit the degradation of GSH. Therefore, besides GGT, there must be other enzymes responding to the GSH degradation.

As shown in Fig. 3, PepT was another key enzyme of GSH degradation. So far as we know, this is the first report to investigate the effect of PepT on the degradation of GSH in *E. coli*. PepT (EC 3.4.11.4) in the microorganism was first isolated from *E. coli* and *Salmonella typhimurium* [14, 15]. Since then, PepTs from lactic acid bacteria have been extensively studied [16, 17]. In this paper, the knockout of the *pepT* gene greatly decreased the degradation of GSH, so it was determined to be another key enzyme related to GSH degradation. As anticipated, the knockout of the *gst* gene has no effect on the reduction of GSH degradation (Fig. 3). Glutathione S-transferase (GST; EC 2.5.1.18) catalyzes the nucleophilic addition of the sulfur atom of GSH to the electrophilic groups of a large variety of hydrophobic molecules including organic halides, epoxides, arene oxides, α- and β-unsaturated carbonyls, organic nitrate esters, and organic thiocyanates [18–21]. There are no substrates that can be used to accept the sulfur atom of GSH in our biosynthetic system. Therefore, in this study GST is not the key enzyme related to GSH degradation.

Interestingly, GGT activity in recombinant strains was greatly affected by changing culture conditions (Table 1). The action of GGT on GSH degradation can be almost completely eliminated by the optimization of culture temperature. There is very little research related to the effect of the culture temperature on the activity of GGT. The best interpretation was that cells grown at a lower temperature produced a larger amount of GGT protein than those grown at a higher temperature [22]. Therefore, in this study, shortening the culture time at 30°C could reduce the activity of GGT.

In conclusion, this work demonstrated that GGT and PepT were the key enzymes of GSH degradation. The activity of GGT in the whole cell was affected greatly by the culture condition. Optimizing the culture condition can almost totally eliminate the action of GGT on GSH degradation. Therefore, the knockout of *ggt* gene was unnecessary in the biosynthesis system constructed in this study. However, the culture temperature had no effect on the activity of PepT; therefore, the knockout of PepT was essential to inhibit the degradation of GSH caused by PepT. GSH degradation was nearly completely inhibited in the biosynthetic system of GSH constructed with tripeptidase-deficient recombinant *E. coli* JW1113 (*pepT*$^-$, pBV03), which was cultured at 30°C for 3 h and 42°C for 5 h. Our results are helpful to determine the catabolism pathway of GSH and construct the optimal biosynthesis system of GSH.

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