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Amplification of Glutathione Production in *E. coli* Cells Using Recombinant DNA Techniques

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Conditions for glutathione production in *E. coli* cells which possess pGH501 (2 *gsh*I+*gsh*II) were studied. In terms of ATP supply for the glutathione synthesis, two different systems have been constructed and compared. When the acetate kinase reaction of *E. coli* was used for ATP generation, 20 mM of L-cysteine was completely converted to glutathione by toluene-treated *E. coli* cells (100 mg/ml) harboring pGH501 within 2 h at 37°C. However, considering the economical aspects, the glycolytic pathway of yeast was chosen as a better system for ATP generation. The optimal concentrations of reactants for glutathione production were determined to be as follows; 80 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 50 mM potassium phosphate buffer (pH 7.5), 400 mM glucose, polyoxyethylene stearylamine (5 μl/ml), toluene-treated *E. coli* HB101/pGH501 (100 mg/ml), and dried yeast cells (400 mg/ml). The conversion ratio of L-cysteine to glutathione was 80% (about 5 mg/ml) under optimal condition within 6 h at 37°C.

Glutathione is a tripeptide of γ-glutamylcysteinylglycine and an important nonprotein thiol in living systems. Glutathione is known as a detoxifying substance, and has many pharmacological properties. For example, it is utilized for the treatment of hepatic disease and also reported to repress liver tumors (3).

Glutathione is synthesized through ATP-requiring reactions catalyzed by two enzymes, i.e. γ-glutamylcysteine synthetase (GSHI) and glutathione synthetase (GSHII) (6, 7). Because numerous biochemical reactions require the participation of various coenzymes and/or a supply of energy in the form of ATP (15), the construction of regeneration systems for such coenzymes and ATP is indispensable for the economical production of many useful compounds. The production of glutathione coupled with various ATP generation systems, however, has been investigated mainly by Murata *et al.* (8-12). But, the mass production of this tripeptide cannot be attempted commercially due to its low level of production. One of reasons for its low productivity seems to be caused by insufficient amounts of glutathione synthesizing enzymes available within cells. Thus, a mutant *E. coli* strain which cannot produce glutathione has been raised and subsequently two genes, *gsh*I and *gsh*II, have been cloned and characterized. Moreover, a *E. coli* strain to be able to express high level of glutathione synthesizing activity was raised using various recombinant DNA techniques (manuscripts in preparation). This strain was rich in its contents of γ-glutamylcysteine synthetase and glutathione synthetase. Although ATP required for glutathione biosynthesis is one of the cheapest cofactors, it is still too expensive for the production in large scale. Therefore, the construction of an ATP generation system seems to be very important for the production of glutathione. Langer *et al.* (4, 5) reported that it is advantageous to use whole cells containing an appropriate enzyme system as a catalyst for regeneration of ATP. Although the use of whole cells shows rather lower level of specific activity and the eventual decrease in selectivity, it is often balanced by the lower price and a simplified process in mass production. ATP necessary for the glutathione biosynthesis, therefore, can be supplied by whole cell systems such as the acetate kinase reaction in *E. coli* cells, ATP-producing strains like *Brevibacterium ammoniagenes*, or the glycolytic pathway of yeast cells. A feasible ATP generation system for glutathione pro-

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duction must be chosen by judging from merits and demerits of each ATP generation system.

To improve the productivity of glutathione using genetically manipulated *E. coli* cells, the production of glutathione coupled with ATP generation systems was studied.

MATERIALS AND METHODS

Strains and Cultivation

E. coli strain HB101 harboring the recombinant plasmid, pGH501, which contains two tandem copies of *gshI* gene region encoding γ-glutamylcysteine synthetase and one *gshII* gene region encoding glutathione synthetase in pUC8 vector (Fig. 1), was used as a source of glutathione synthesis enzymes. These transformed *E. coli* cells were grown in LB medium in the presence of ampicillin (50 μg/ml) at 37℃ for 16 hr with reciprocal shaking. The harvested cells were frozen immediately and kept at −20℃ until use. Dried baker's yeast was purchased from Choheung Chemical Industrial Co., LTD., Seoul, Korea. It was roughly ground with a pestle and kept at room temperature until use. *Brevibacterium ammoniagenes* ATCC 21190 was the stock culture obtained from the Laboratory of Enzyme Biotechnology, Department of Agricultural Chemistry at Korea University.

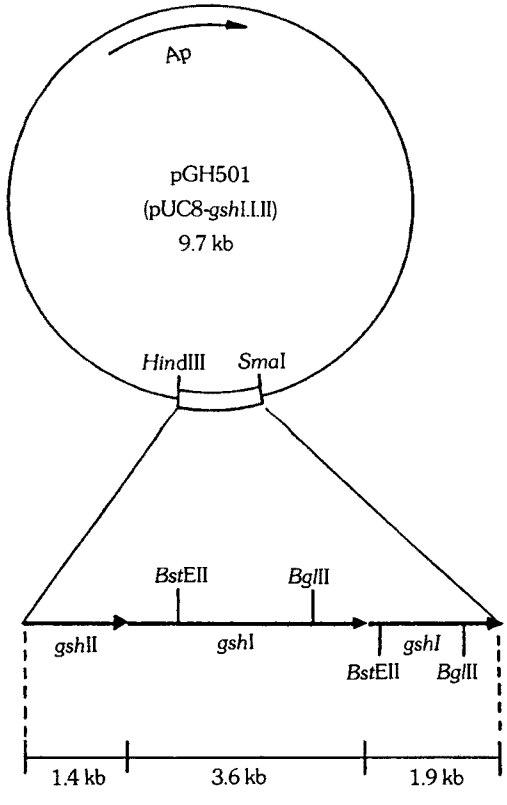

Fig. 1. Schematic diagram of pGH501 plasmid.

Treatment of Cells with Toluene or Acetone

In order to improve the transport of substrates and/or products, cell pellets were treated with either toluene or acetone as follows. Ten grams (wet wt.) of intact *E. coli* HB101/pGH501 cells were resuspended with 100 ml of 5.0 mM potassium phosphate buffer, pH 7.0, containing 10% toluene and 0.5 mM L-cysteine, and followed by incubation at 37℃ for 30 min with gentle shaking. The resulting toluene-treated *E. coli* cells were harvested and applied for the glutathione synthesis and the acetate kinase reaction. The dried yeast cells were suspended with 10 ml of 0.85% NaCl solution. Ninty milliliters of chilled acetone were added to it and the solution was mixed well with stirring for 5 min. The yeast cells were then recovered and washed twice with 5.0 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM cysteine. The *B. ammoniagenes* cells were freeze-dried after the culture and used directly. The acetone treated yeast cells or lyophilized *B. ammoniagenes* cells were used as the source for the ATP generation system.

Production for Glutathione

Unless otherwise noted, the standard reaction mixture for the synthesis of glutathione contained 60 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 1 mM ATP, 10 mM acetylphosphate, 50 mM potassium phosphate buffer, pH 7.5, and toluene-treated *E. coli* HB101/pGH501 cells (100 mg wet wt./ml) or/and acetone-treated yeast cells (600 mg wet wt./ml) in 2 ml. The reaction was carried out by incubating at 37℃ with shaking and was terminated by immersing the reaction tube in boiling water for 3 min. The optimal concentration of reactants for the production of glutathione was determined.

Assay of Glutathione

The amount of glutathione synthesized was measured by the method of Tietze (16) using glutathione reductase. The sum of reduced and oxidized forms of glutathione was determined using a kinetic assay. The assay solution contained 0.2 μmol of NADPH, 1.6 mmol of DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], 0.12 units of glutathione reductase, and a sample with a final volume of 1.0 ml. The rate of reaction was usually expressed as the change in absorbance at 412 nm per 1 min.

RESULTS AND DISCUSSION

Production of Glutathione Coupled with an ATP Regeneration System by Acetate Kinase in *E. coli*

To examine the glutathione producing activity without

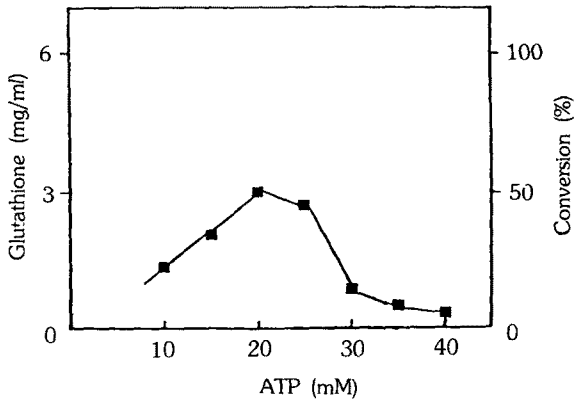

Fig. 2. Effect of ATP concentration on glutathione production by toluene treated *E. coli* cells containing pGH501 plasmid.
Glutathione producing reaction was carried out 2 ml of mixture containing 60 mM L-glutamic acid, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, various concentration of ATP, 50 mM potassium phosphate buffer, pH 7.5, and toluene treated *E. coli* cells (200 mg) at 37℃ with shaking for 2 hours.

supplying any ATP generation system, various amounts of ATP were added to the reaction mixture containing toluene-treated *E. coli* cells. As shown in Fig. 2, the glutathione producing activity has been gradually elevated with the increasing concentration of ATP to the reaction mixture up to a 20 mM. However, it was decreased in the presence of more than 20 mM of ATP. It seems to be due to the inhibition of γ-glutamylcysteine synthetase by the accumulation of reaction product, namely, ADP. Therefore, it is imperative to employ an ATP regeneration system instead of the direct addition of ATP for the production of glutathione. Using the acetate kinase of *E. coli* cells as ATP generation system has a merit in that *E. coli* cells contain systems responsible for both glutathione synthesis and ATP generation. That is, the process for glutathione production is performed in a one cell system, i.e. *E. coli* with acetylphosphate as an energy donor. The glutathione producing activity coupled with the acetate kinase reaction of *E. coli* cells was investigated in the presence of various concentrations of acetylophosphate and L-glutamate. As shown in Fig. 3, when 40 mM of acetylphosphate was added to the reaction mixture, 20 mM of cysteine was converted into 18 mM of glutathione with a yield of about 90%. This result suggests that the acetate kinase reaction of *E. coli* cells as ATP generation system is feasible for the efficient synthesis of glutathione. Moreover, as shown in Fig. 4, when 140 mM of L-glutamate was added to the reaction mixture, 20 mM of L-cysteine was completely converted into glutathione. The high requirement

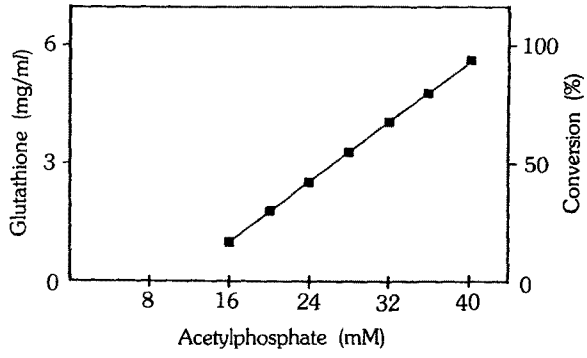

Fig. 3. Effect of acetyl phosphate concentration on glutathione production by toluene treated *E. coli* cells containing pGH501 plasmid.
Glutathione producing reaction was carried out 2 ml of mixture containing 60 mM L-glutamic acid, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 1 mM ATP, various concentrations of acetyl phosphate, 50 mM potassium phosphate buffer, pH 7.5, and toluene treated *E. coli* cells (200 mg) at 37℃ with shaking for 2 hours.

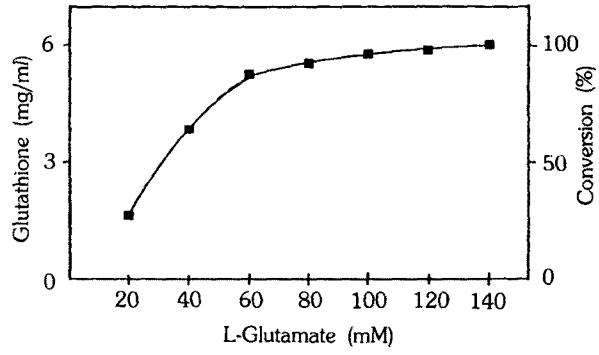

Fig. 4. Effect of L-glutamate concentration on glutathione production by toluene treated *E. coli* cells containing pGH501 plasmid.
Glutathione producing reaction was carried out 2 ml of mixture containing various concentrations of L-glutamic acid, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 1 mM ATP, 40 mM acetyl phosphate, 50 mM potassium phosphate buffer, pH 7.5, and toluene treated *E. coli* cells (200 mg) at 37℃ with shaking for 2 hours.

for L-glutamate is presumably due to the utilization of this particular amino acid in other reactions by the whole cell system having multi-enzyme reactions, because L-glutamate is a major intermediate in many biochemical reactions (2).

Production of Glutathione Coupled with an ATP Generation System by the Enzymes Involved in the Glycolytic Pathway of Dried Yeast

The acetate kinase reaction system for ATP generation in *E. coli* is not sufficient for the glutathione biosynthesis in terms of economic value because of its high price

of acetylphosphate. Therefore, the glycolytic pathway of yeast seems to be adequate for the ATP generation system in the production of glutathione. Glucose, in fact, is relatively cheap and stable to generate ATP. Moreover it can be readily utilized by yeast. So, the generation of ATP using glucose by yeast system is much cheaper than that using acetylphosphate. This aspect can be applied for the mass production of glutathione considering the economics. For this reason, dried yeast cells were chosen to provide the glycolytic enzymes for supplying ATP into the glutathione biosynthesis system.

Nara *et al.* (13, 14) has described a practical method for the production of ATP by *B. ammoniagenes*. Evenmore, the glutathione production has been attempted in *E. coli* mixed with *B. ammoniagenes* for ATP supply (1). Therefore, lyophilized *B. ammoniagenes* cells were applied to the glutathione production system of *E. coli* to provide an ATP generating system. Fig. 5 shows the comparison of the glutathione production in the presence of *B. ammoniagenes* with that of dried yeast cells as an ATP-generating system. The gluthione production activity of the mixture of *E. coli* cells and dried yeast cells was higher than that of the mixture of *E. coli* and *B. ammoniagenes* cells. So, the yeast cells were chosen as the ATP generating system, because the ATP generated by yeast was expected to be more effectively utilized for the production of glutathione than those generated by *B. ammoniagenes* and yeast can be dealt with easily.

The generation of ATP and the synthesis of glutathione reactions were carried out by two different microbial cells. That is, most ATPs were generated by yeast cells, and then they were transferred to *E. coli* cells for glutathione biosynthesis. In the reactor system employed for the glutathione production, the ratio of yeast cells to *E. coli* may have been an important factor. When the 400 mg per ml of acetone-treated yeast cells were added to the reaction mixture containing 100 mg per ml of toluene-treated *E. coli* cells, the glutathione productivity was most efficient as shown in Fig. 6.

The effect of glutathione producing activity on the glutathione producing system coupled with yeast was examined in terms of different concentrations of substrates such as L-glutamate and glycine. The conversion rate of L-cysteine to glutathione was about 65% in the presence of 140 mM L-glutamate and was not further increased as the concentration of L-glutamate exceeded over 80 mM (data not shown). However, inspite of using the cells which had sufficient amounts of enzymes for the glutathione biosynthesis, the conversion rate was low as compared with that of using the acetate kinase reaction as a ATP generating system in the presence of high concentration of L-glutamate. Therefore, the conversion rate is presumably limited by the amount of ATP generated by yeast. Also the low conversion rate of L-cysteine to glutathione seemed to be inhibited by the ADP produced during the glutathione synthesis reaction (3).

Glutathione producing activity was little affected by the concentration of glycine (data not shown). It seemed that γ-glutamylcysteine was synthesized at a low level because of the limited amount of ATP available. That is, since the amount of γ-glutamylcysteine synthesized was not enough, the concentration of glycine did not affect the production of glutathione. Judgine from these

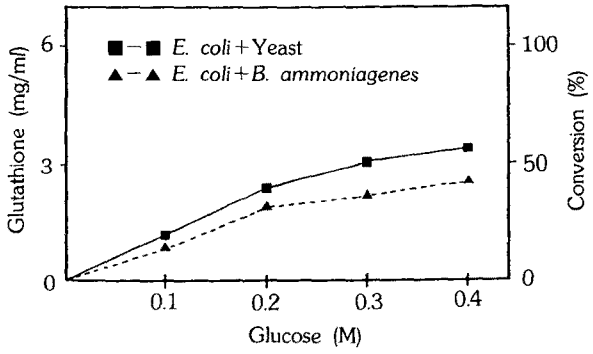

Fig. 5. Effect of utilization of *B. ammoniagenes* as ATP-generating system for glutathione production.
Glutathione producing reaction was carried out 2 ml of mixture containing 60 mM L-glutamic acid, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 50 mM potassium phosphate buffer, pH 7.5, various concentrations of glucose, POESA (10 μl), toluene treated *E. coli* cells containing pGH501 plasmid (200 mg) and yeast cells (600 mg) at 37℃ with shaking for 2 hours.

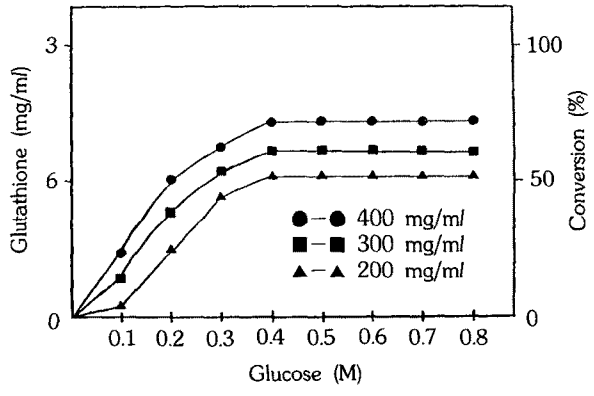

Fig. 6. Effect of concentration of acetone treated yeast cells on glutathione production.
Glutathione producing reaction was carried out 2 ml of mixture containing 60 mM L-glutamic acid, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 50 mM potassium phosphate buffer, pH 7.5, various concentrations of glucose, POESA (10 μl), toluene treated *E. coli* cells containing pGH501 plasmid (200 mg) and various concentrations of acetone treated yeast cells at 37℃ with shaking for 2 hours.

results, it is conceivable that the first step which catalizes the formation of γ-glutamylcysteine is a rate-limiting step for the glutathione biosynthesis.

The importance of membrane permeability for the efficient accumulation of metabolite has been noted in the field of industrial fermentation. The improvement of membrane permeability is considered to be the effective factor for increasing yield of glutathione, since cell membranes of bacteria and yeast are generally impermeable to nucleotide. Therefore, in order to improve the permeability of substrate and/or products, yeast cells were treated with toluene, acetone or polyoxyethylene stearylamine (POESA). When 1% of POESA was added to the reaction mixture, the highest activity of glutathione production has been observed as shown in Table 1. The results imply that ATP synthesized in yeast are not transferred to the glutathione synthesizing system of *E. coli* cells. Therefore, it is worth reducing the barrier of compartmentalization of cells for the efficient transportation of ATP or substrates to enhance the production of glutathione.

The optimal concentrations of reactants were determined to be as follows; 80 mM L-glutamate, 20 mM L-cysteine, 20 mM $MgCl_2$, 50 mM potassium phosphate buffer, pH 7.5, 400 mM glucose, POESA (5 μl/ml), toluene-treated *E. coli* cells (100 mg/ml) and dried yeast cells (400 mg/ml). The conversion rate of cysteine to glutathione was 80% (about 5.0 g/l) under the optimal conditions. However, to increase the productivity, an attempt was made to add more cysteine to the reaction mixture. When the concentration of cysteine was increased to 100 mM, the amount of glutathione rose to 19.6 g/l, but the conversion ratio was decreased to 64%. These results indicate that the optimal condition for the mass production of glutathione must be reexamined based on the concentration of reactants. Namely, it would be necessary to investigate the reaction conditions in temrs of the ratio of *E. coli* and yeast cells, and amounts of cells, glucose and substrates.

Table 1. Effect of treatment of cells with various agents on glutathione production

Cells		Glutathione (mg/ml)
E. coli	Yeast	
Toluene treated (10%)	None	N.D
Toluene treated (10%)	Toluene treated (10%)	2.6
Toluene treated (10%)	Acetone treated (90%)	3.6
Toluene treated (10%)	POESA added (1%)	4.5

Glutathione producing reactions were carried out in 2 ml of mixture containing 60 mM L-glutamate, 20 mM L-cysteine, 20 mM glycine, 20 mM $MgCl_2$, 50 mM potassium phosphate buffer, pH 7.5, 0.4 M glucose, toluene treated *E. coli* cells (200 mg) and various agents treated yeast cells (600 mg) at 37℃ with shaking for 6 hours. Figures in parentheses indicate the concentration of various agents.
N.D: not detected
POESA: Polyoxyethylene stearylamine

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