(S7-C) MOLECULAR BREEDING OF GLUTATHIONE PRODUCING BACTERIAL STRAINS

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ABSTRACT: In order to increase the production of glutathione by maximizing the expression of recombinant *gsh* plasmids, two genes responsible for the biosynthesis of glutathione were cloned. A *gsh*I gene was cloned onto pBR322 plasmid as 3.6Kb *Pst*I DNA fragment from *E.coli* K-12 chromosomal DNA. Also *gsh*II gene was cloned onto pUC13 plasmid as 2.2Kb *Pst*I-*Bam*HI DNA fragment. In order to improve the glutathione producing activity more efficiently, various recombinant plasmids containing tandem repeated *gsh*I genes or both genes in various copy number onto the same vector were constructed. *E.coli* cells harboring pGH501 plasmid (pUC8-*gsh*I·I·II) showed the highest glutathione synthesizing activity. The conditions for glutathione production with an ATP-generating system such as acetate kinase reaction of *E.coli* cells or glycolytic pathway of yeast cells were examined using the *E.coli* cells harboring the pGH501 plasmid. When the acetate kinase reaction of *E.coli* cells was used as an ATP generating system, 20mM of L-cysteine was converted into glutathione with a yield of 100%.

INTRODUCTION

Traditional attempts to achieve strain improvement by random mutagenesis and screening for amino acid overproducing strains have recently been complemented by recombinant DNA techniques. Gene amplification by cloning with muticopy vectors frequently causes increased production of the protein encoded by the cloned gene. It seems reasonable to assume that amplification of genes in the rate limiting step of the biosynthetic pathway will improve yield of the end product. However, despite the application of recombinant DNA techniques to the microbial production of valuable metabolites, the production of these metabolites by microbial strains constructed by recombinant DNA techniques has not always met the critical of practical feasibility so far as the fermentation is concerned. The main impediment preventing the development of fermentation seems to concern the difficulty of achieving the efficient utilization of the increased enzyme in the transformed cells. In constrast to the fermentation, the enzymatic method

can be applied to the production of useful compounds.

Glutathione (r-glutamylcysteinylglycine) is a major free thiol in most living cells, and has many biological and phamacological properties (Meister and Anderson, 1983). Glutathione is synthesized intracellularly by the concecutive actions of r-glutamylcysteine synthetase (GSH-I) and glutathione synthetase (GSH-II). Glutathione has been produced by extracting yeast cells in industries. Yet the yeast cells have low contents of glutathione and the process of extraction is tedious and intricate. Therefore, a new method for glutathione production is being studied. Gushima *et al.*(1983a) reported glutathione producing strains of *E.coli* B constructed by recombinant DNA techniques. Various recombinant plasmids carrying the genes for GSH-I and/or GSH-II were constructed and the effects of these recombinant plasmids on glutathione production by *E.coli* cells were examined in a bioreactor. Murata *et al.*(1980a;b) introduced an ATP regenerating system instesd of direct addition of expensive ATP for glutathione production. However, commercial production cannot be realized since the yield is low. One of reasons for low yield seems to be caused by insufficient amounts of glutathione synthesizing enzymes.

In this study, the molecular breeding of glutathione producing bacterial strains has been attempted. Thus strains expressing high glutathione synthesizing activity were constructed by recombinant DNA techniques. The strains enriched in its contents of GSH-I and GSH-II. Enzymatic production of glutathione coupled with an ATP generating system was examined with glutathione producing *E.coli* strains constructed by recombinant DNA techniques.

MATERIALS AND METHODS

E.coli K-12 W3110 was used as a source of chromosomal DNA for the cloning of *gsh*I gene and primary strain for the construction of mutants. *E.coli* K-12 HB101 strain was used for most transformation experiments and propagation of plasmids. Most DNA manipulation techniques were carried out as described by Maniatis *et al.*(1982). The activity of GSH-I was determined by the method of Jackson (1969) with a slight modification. The activity of GSH-II was measured according to the method of Gushima *et al.*(1983b). One unit of enzyme activities is defined as the amount of enzymes that catalyzed the synthesis of 1 umole of r-glutamylcysteine or glutathione per hour. Specific activity is expressed in terms of units per miligram of protein. The amount

of glutathione was determined by the method of Tietze (1969) with glutathione reductase. The standard reaction mixture for glutathione production contained 60mM glutamate, 20mM cysteine, 20mM glycine, 20mM $MgCl_2$, 1mM ATP, 10mM acetylphosphate, 50mM potassium phosphate buffer, pH 7.5, and toluene treated *E.coli* cells(100mg wet wt./ml) or /and acetone treated yeast cells (600mg wet wt./ml) in 2ml. The optimal concentration of reactants for glutathione production was examined by changing of concentration of reactants.

RESULTS AND DISCUSSION

Cloning of genes for the biosynthesis of glutathione from *E.coli* K-12: In order to construct an efficient glutathione producing strain, a *gsh*I gene for GSH-I and a *gsh*II gene for GSH-II were cloned from *E.coli* K-12 W3110 wild type. To clone a *gsh*I gene, the GS903 strain, which is deficient in GSH-I activity, was developed. A *gsh*I gene was cloned onto pBR322 plasmid as 3.6Kb *Pst*I DNA fragment from *E.coli* K-12 chromosomal DNA. Also a *gsh*II gene was cloned onto pUC13 plasmid as 2.2Kb *Pst*I-*Bam*HI DNA fragment. In order to study the effects of plasmid copy number and passenger DNA size on the expression levels of *gsh* genes, various recombinant plasmids such as pGH100, pGH101, pGH200, pGH201 pLF4, pLF6 and pGH300 were constructed. The expression levels of *gsh* genes were increased approximately two times higher by using pUC series plasmids than that of pBR322 plasmid. Also the sizes of the passenger DNA containing *gsh* genes in the vector plasmid did not affect on the expression levels of *gsh* genes (Table 1).

Construction of recombinant plasmids for the enhancememt of glutathione producing activity in *E.coli*: It is ideal for glutathione biosynthesis that the amount of GSH-I should be three times that of GSH-II (Watanabe *et al.*,1986). Thus the recombinant plasmids containing tandem repeated *gsh*I genes were constructed (Fig. 1). The recombinant plasmids contained monomeric, dimeric and trimeric 3.6Kb *Pst*I DNA fragments on pBR325 vector plasmid, respectively, and all copies of *gsh*I genes were present with the same transcriptional direction. GSH-I activities of *E.coli* cells harboring the constructed recombinant plasmids were found to be proportional to the number of *gsh*I genes incorporated into the pBR325 vector plasmid. Also the recombinant plasmids containing both genes for glutathione biosynthesis were constructed and introduced into *E.coli* HB101 cells (Table 2). Glutathione

producing activity of the *E.coli* strains containing tandem repeated *gsh*I genes improved with an increase the GSH-I activities, and glutathione producing activities of strains harboring plasmids containing both genes were two fold higher than those of the strains harboring the plasmids containing the *gsh*I gene only. The highest glutathione producing activity was observed in the case of the cells harboring pGH501 plasmid (Table 3).

Table 1. Enzyme activities of *E.coli* HB101 strain harboring various recombinant plasmids

Strains	Properties	Glutathione contents in cells[a*]	Activities GSH-I	GSH-II
HB101		0.22	0.15	0.34
HB101/pGH100	pBR322-*gsh*I	0.65	2.15	0.34
HB101/pGH101	pBR322-*gsh*I	0.60	2.05	0.34
HB101/pGH200	pUC8-*gsh*I	0.82	4.60	0.32
HB101/pGH201	pUC8-*gsh*I	0.79	4.52	0.32
HB101/pLF4	pUC13-*gsh*II	0.28	0.15	3.10
HB101/pLF6	pUC13-*gsh*II	0.27	0.15	3.10
HB101/pGH300	pBR322-*gsh*I	0.26	0.15	2.40

a* : Glutathione content [mg/g(wet. wt cells)]

Table 2. GSH-I and GSH-II activities in *E.coli* HB101 strains harboring recombinant plasmids.

Strains	Properties	Glutathione contents in culture broth[a*]	Activities GSH-I	GSH-II
HB101		0.01	0.16	0.38
HB101/pGH325-101	pBR325-*gsh*I.II	0.08	2.40	2.70
HB101/pGH325-201	pBR325-*gsh*I.I.II	0.08	5.00	2.60
HB101/pGH325-301	pBR325-*gsh*I.I.I.II	0.08	6.30	2.50
HB101/pGH400	pUC13-*gsh*I.II	0.08	4.10	3.80
HB101/pGH501	pUC8-*gsh*I.I.II	0.08	8.00	3.90

a* : Glutathione content (mg/ml)

Table 3. Glutathione producing activities in *E.coli* HB101 strains harboring recombinant plasmids.

Strains	Properties	Glutathione producing activities (mg/ml)
HB101		0.05
HB101/pGH325-100	pBR325-*gsh*I	0.90
HB101/pGH325-101	pBR325-*gsh*I.II	1.50
HB101/pGH325-200	pBR325-*gsh*I.I	1.20
HB101/pGH325-201	pBR325-*gsh*I.I.II	2.10
HB101/pGH325-300	pBR325-gshI.I.I	1.30
HB101/pGH325-301	pBR325-*gsh*I.I.I.II	2.30
HB101/pGH400	pUC13-gshI.II	2.40
HB101/pGH501	pUC8-*gsh*I.I.II	2.80

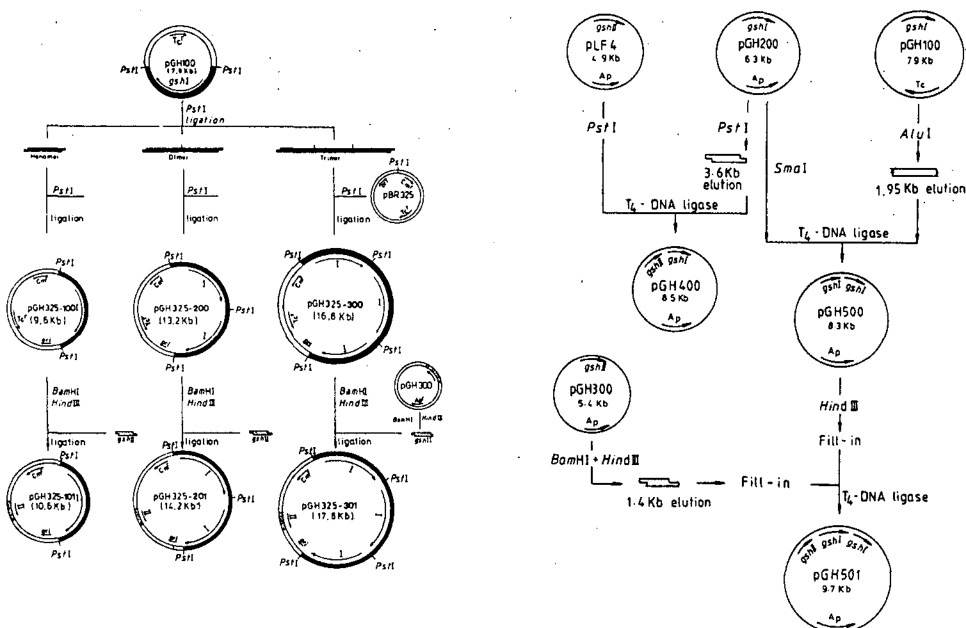

Fig. 1. Scheme for the construction of various recombinant plasmids

Purification and properties of GSH-I related to the biosynthesis of glutathione *in vitro*: To investigate some properties of GSH-I related to the biosynthesis of glutathione, GSH-I was purified from *E.coli* HB101 strain harboring recombinant plasmid, pGH500, which contains two copies of *gsh*I gene derived from *E.coli* W3110 wild type on pUC8 vector plasmid. The molecular weight of purified enzyme was 60,000 daltons and the enzyme was very stable. The purified enzyme was feedback inhibited by 48% at 10mM of reduced glutathione and also inhibited by ADP, adenine, cysteine and glycine. And the enzyme activity was increased with the elevation of L-glutamate concentration. Therefore, it is desirable that glutathione producing reaction is separately performed by two steps.

Production of glutathione: The conditions for glutathione production with an ATP generating system were studied using *E.coli* cells harboring pGH501 plasmid. When acetate kinase reaction of *E.coli* cells was used as an ATP-regenerating system, 20mM of L-cysteine was converted into glutathione with a yield of 100% by toluene treated *E.coli* cells (100mg/ml) harboring pGH501 plasmid within 2 h at 37°C (Fig. 2). However, considering economical aspects, the glycolytic pathway of yeast cells was chosen as an ATP generating system. The optimal concentrations of reactants for glutathione

production were determined and the conversion ratio of L-cysteine to glutathione was 80% (about 5mg/ml) under optimal condition within 6h at 37°C (Fig. 3).

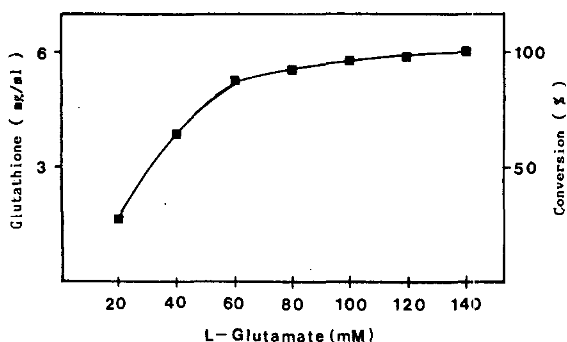

Fig.2. Effect of L-glutamate concen-tration on glutathione production by toluene-treated *E.coli* cells con-taining pGH501 plasmid

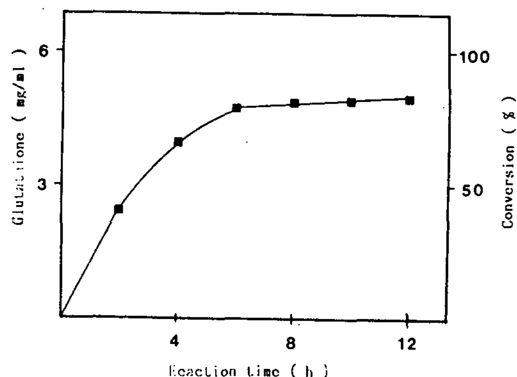

Fig.3. Time course of glutathio-ne production coupled with yea-st cells under optimal conditi-on

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