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Communication

Extracellular Production of Glutathione by Recombinant *Escherichia coli* K-12

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Abstract: The goal of this study was to produce a sufficient amount of glutathione in the fermentation medium without the addition of cysteine. This would simplify and reduce the cost of its purification. In addition to reducing the cost of cysteine, it also avoids the inhibition of bacterial growth by cysteine. The *gshA*, *gshB*, and *cysE* genes of *Escherichia coli* were cloned under the control of the strong T5 promoter of the pQE-80L plasmid and introduced into an *E. coli* strain knocked out for the genes encoding γ-glutamyltranspeptidase and the GsiABCD glutathione transporter, which are responsible for the recycling of excreted glutathione. The overexpression of the *gshA* and *gshB* genes, genes for γ-glutamylcysteine synthetase and glutathione synthetase, and the $cysE^{V95R\ D96P}$ gene, a gene for serine acetyltransferase with the V95R D96P mutation that makes it insensitive to cysteine, were effective on glutathione production. $Na_2S_2O_3$ was a good sulfur source for glutathione production, while the addition of Na_2SO_4 did not affect the glutathione production. With the addition of 50 mM glutamic acid and 75 mM glycine, but without the addition of cysteine, to the simplified SM1 medium, 4.6 mM and 0.56 mM of the reduced and oxidized glutathione, respectively, were accumulated in the extracellular space after 36 h of batch culture. This can eliminate the need to extract glutathione from the bacterial cells for purification.

Keywords: antioxidant; kokumi; desensitization; transporter; T5 promoter; excretion

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1. Introduction

Glutathione (γ-Glu-Cys-Gly) is synthesized by various types of living cells, and the reduced form of glutathione serves as an antioxidant. Glutathione is synthesized by the sequential reactions of γ-glutamylcysteine synthetase (GshA; EC 6.3.2.2) and glutathione synthetase (GshB; EC 6.3.2.3). The exception is that certain bacterial strains synthesize glutathione by a bifunctional glutathione synthetase with both a γ-glutamylcysteine synthetase domain and a glutathione synthetase domain in one protein [1–3]. Glutathione (in its reduced form) is known for its hepatoprotective properties [4] and is approved as an ethical pharmaceutical ingredient in Japan. On the other hand, glutathione, one of the γ-glutamyl compounds, is recognized as one of the kokumi substances [5]. Kokumi substances have been defined as compounds that have a weak taste by themselves, but when added to dishes, even in small amounts, they enhance their flavor characteristics such as continuity, mouthfulness, and thickness [6]. The oxidized form of glutathione has been reported to be used as an enhancer to increase the seeds and biomass of plants [7]. The use of glutathione improves the tolerance of plants to stress, such as low temperature [8]. A number of other functions of glutathione and methods invented for microbial glutathione production have been reviewed [9].

Yeast, such as *Saccharomyces cerevisiae*, produces a high concentration of glutathione, but intracellularly. Therefore, it should be extracted prior to use. In the case of *Escherichia coli* K-12, a certain amount of glutathione is secreted from the cells during the exponential phase, but then the extracellular glutathione is degraded by γ-glutamyltranspeptidase

(GGT; EC 2.3.2.2) [10] or taken up and utilized by the cells [11]. We have proposed that this glutathione cycle is a salvage system for cysteine [12].

One method was to develop yeast strains containing high levels of glutathione. Many attempts have been made to achieve this, including optimization of the culture conditions, stress application, and metabolic engineering, and these were reviewed recently [13]. Another method was to couple an *E. coli* strain overexpressing GshA and GshB to ATP-regeneration systems [14]. A further method is to use the transpeptidation reaction of GGT with glutamine and *S*-benzyl-cysteinylglycine methyl ester as substrates [15]. Previously, we reported that deletions of both the *ggt* gene encoding GGT and the *gsiABCD* genes encoding a glutathione uptake transporter on the genome dramatically increased glutathione levels in the culture medium [11]. In this case, we transformed the mutant strain with the plasmid pSH1391, containing the *gshA* and *gshB* genes on pBR322 under the control of their own promoters. However, we did not assess the impacts of a potent promoter, different growth conditions, replacement of the less efficient UUG initiation codon of *gshA* with the standard AUG codon, the mutation that makes GshA insensitive to glutathione's feedback inhibition, and the mutation that renders CysE insensitive to cysteine's feedback inhibition. Therefore, we address these aspects in detail within this study.

2. Materials and Methods

2.1. Strains and Plasmids Used in This Study

The strains, plasmids, and primers used in this study are listed in Table 1.

Table 1. The strains, plasmids, and primers used in this study.

Strain		**Source or Reference**
DH5α	F$^-$ Φ80(Δ*lacZ*ΔM15) Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44* λ$^-$ *thi*-1 *gyrA96 relA1*	Toyobo
SH1391	pSH1391/DH5α	[11]
SI97	*rph-1* Δ*ggt-2 htp*$^+$-Tn*10* Δ*gshA* Δ*gsiAB*	[11]
TN4	pTN1/SI97	
TN9	pTN5/SI97	
TN10	pTN6/SI97	
TN13	pTN16/SI97	
TN31	pTN29/SI97	
TN37	pTN36/SI97	
TN40	*rph-1* Δ*ggt-2 htp*$^+$-Tn*10* Δ*gshA* Δ*gsiAB* Δ*cysE*::*kan*^{r}-FRT	
TN48	pTN36/TN40	
Plasmid	Genotype	
pQE-80L	ColE1 *amp*^{r} *lacI*^{q}	Qiagen
pSH1391	ColEI replicon *rop*$^+$ *amp*^{s}::*gshA*$^+$ *gshB*$^+$ *kan*^{r} *tet*^{s}	[11]
pSH1564	ColEI replicon *rop*$^+$ *amp*^{s}::*gshA*$^{L1M\ W100L}$ *gshB*$^+$ *kan*^{r} *tet*^{s}	Laboratory stock
pSH1778	ColE1 replicon *amp*^{r} T5p-*cysE*$^+$	
pTN35	ColE1 replicon *amp*^{r} T5p-*cysE*$^{V95R\ D96P}$	
pTO23	ColE1 replicon *amp*^{r} *lacI*^{q} T5p-ATG-His_6-*gshA*	

Table 1. *Cont.*

Strain		Source or Reference
pTN1	ColE1 replicon *amp*^{r} *lacI*^{q} T5p-ATG-His$_6$-*gshA*-*gshB*	
pTN5	ColE1 replicon *amp*^{r} *lacI*^{q} T5p-ATG-His$_6$-*gshA*^{W100L}-*gshB*	
pTN6	ColE1 replicon *amp*^{r} *lacI*^{q} T5p-ATG-His$_6$-*gshA*^{A494G}-*gshB*	
pTN16	ColE1 replicon *amp*^{r} *lacI*^{q} T5p-ATG-His$_6$-*gshA*$^{W100L\ A494G}$-*gshB*	
pTN29	ColE1 replicon *amp*^{r} *lacI*^{q} T5p-ATG-His$_6$-*gshA*-*gshB*-*cysE*	
pTN36	ColE1 replicon *amp*^{r} *lacI*^{q} T5p-ATG-His$_6$-*gshA*-*gshB*-*cysE*$^{V95R\ D96P}$	
pYU4	ColEI replicon *rop*$^{+}$ *amp*^{s}::*gshA*$^{L1M\ A494G}$ *gshB*$^{+}$ *kan*^{r} *tet*^{s}	Laboratory stock
Primer	Nucleotide sequence	
BamHI_gshA_F	CCCggatccATCCCGGACGTATCACAGGCG	
PstI_gshA_R	CCCctgcagTCAGGCGTGTTTTTCCAGCC	
PstI_gshB_F	CCCctgcagGAGGAGAAATTAACTATGATCAA GCTCGGCATCGTGATG	
HindIII_gshB_R	CCCaagcttTTACTGCTGCTGTAAACGTG	
EcoRI_SD_cysE_N	CCCgaattcATTAAAGAGGAGAAATTAACTATGTCG TGTGAAGAACTGGAAATTG	
HindIII_cysE_C	CCCaagcttTTAGATCCCATCCCCATACTC	
HindIII_SD_cysE_F	CCCaagcttGAGGAGAAATTAACTATGTCG	
V95R D96P	GTGCGTACCCGCGACCCGGCACGCCCGAAA TACTCAACCCCGTTGTTATACC	
V95R D96P comp	GGTATAACAACGGGGTTGAGTATTTCGGGCGTGCC GGGTCGCGGGTACGCAC	

Strains and plasmids for which no literature or source information is provided were prepared for this work as described below. Lowercase characters indicate the restriction endonuclease recognition sequences. Underlined characters indicate the Shine Dalgarno sequence. Green characters (ATC) indicate the second codon of the *gshA* gene. The red and blue characters indicate the initiation and stop codons, respectively. Double underlined characters indicate the introduced mutations.

2.1.1. Introduction of Mutations into gshA Gene

Plasmid pTO23 has the DNA fragment consisting of the *gshA* gene inserted between the *Bam*HI and *Pst*I sites of pQE-80L, with a *Bam*HI site just before the second codon and a *Pst*I site just after the stop codon (Figure 1). The *gshB* was amplified by PCR using gshB_N and gshB_C as primers and pSH1391 [11] as a template. The amplified DNA fragment (984 bp) was cleaved with *Pst*I and *Hin*dIII and ligated to pTO23 cleaved with the same enzymes to obtain pTN1. The *gshA* gene fragments with W100L and A494G mutations were amplified by PCR using BamHI_gshA_F and PstI_gshA_R as primers and pSH1564 and pYU4 as templates, respectively. The amplified fragments (1572 bp) were cleaved with *Bam*HI and *Pst*I and ligated to pTN1 cleaved with the same enzymes to obtain pTN5 and pTN6. pTN5 was cleaved with *Bgl*II and *Hin*dIII and ligated to the *Bgl*II-*Hin*dIII fragment of pTN6 to obtain pTN16. Strain SI97 was transformed with pTN1, pTN5, pTN6, and pTN16, and TN4, TN9, TN10, and TN13 were obtained, respectively. These strains carrying *gshA*$^{+}$, *gshA*^{W100L}, *gshA*^{A493G}, and *gshA*$^{W100L\ A493G}$ were used for the purification of mutant GshA enzymes and the production of glutathione. This is because the desensitized mutant *gshA* gene has been reported to contain three amino acid substitutions compared to the wild-type GshA, i.e., W100L, A494G, and S495F [16], although we could not purify GshA containing S495F.

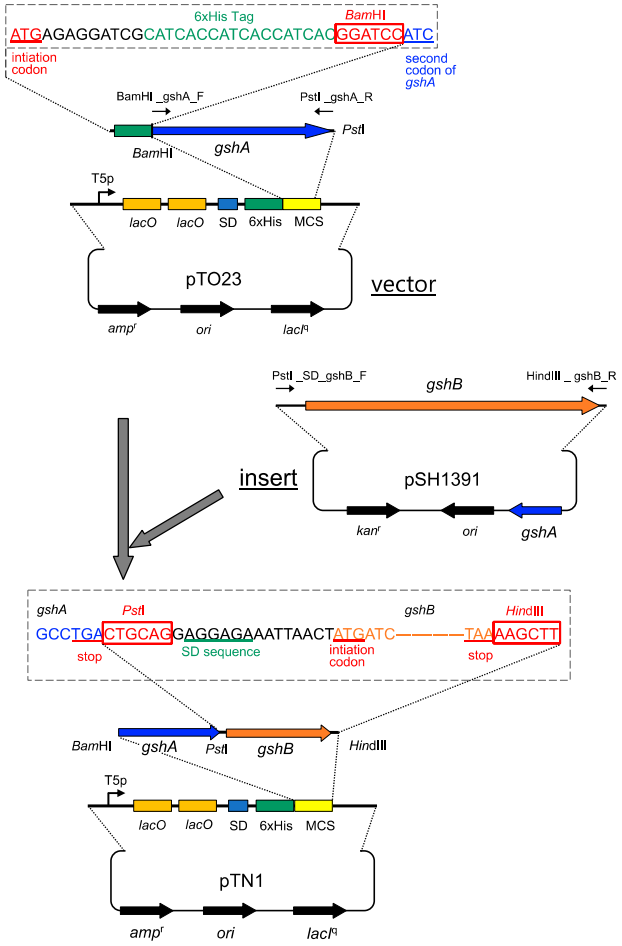

Figure 1. Construction of the plasmid pTN1 containing both *gshA* and *gshB* genes under the control of the T5 promoter.

2.1.2. Cloning of cysE on pQE-80L Plasmid and Introduction of V95R D96P Mutation

The *cysE* gene was amplified by PCR using EcoRI_SD_cysE_N and HindIII_cysE_C as primers and genomic DNA from *E. coli* K-12 as a template. The amplified fragment (861 bp) cleaved by *Eco*RI and *Hin*dIII was ligated to pQE-80L cleaved by the same enzymes to obtain pSH1778. The V95R D96P mutation [17] was introduced into pSH1778 by the Quik Change method using V95R D96P and V95R D96P comp as primers to obtain pTN35. The wild-type *cysE* gene and *cysE*$^{V95R\ D96P}$ gene were amplified by PCR using HindIII_SD_cysE_F and HindIII_cysE_C as primers and pSH1778 and pTN35 as templates. The amplified fragments (855 bp) cleaved with *Hin*dIII were ligated to pTN1 cleaved with the same enzyme to obtain pTN29 and pTN36, respectively. The PCR-amplified regions were sequenced to confirm the correctness of the DNA sequences.

2.2. Purification of GshA with Different Combinations of Mutations

2.2.1. Growth Conditions of Strains for Purification of GshA

E. coli K-12 strains were precultured in 5 mL of Difco LB broth Miller [18] (Becton Dickinson; Sparks, MD, USA) supplemented with 30 µg/mL kanamycin overnight at 37 °C with reciprocal shaking at 120 rpm. The preculture was then inoculated into 20 mL of the same medium in a 200 mL Erlenmeyer flask, resulting in an initial optical density at 600 nm (OD_{600}) of 0.1. Cultures were incubated at 37 °C with reciprocal shaking at 120 rpm until OD_{600} reached 0.4. IPTG was then added to the culture medium at a final concentration of 0.02 mM and shaken at 37 °C for another 8 h. The culture was centrifuged at 5000× *g* at 4 °C for 10 min to collect the cells. After the cells were washed with 10 mL of 20 mM Tris-HCl (pH 8), the cells were resuspended in 3 mL of the same buffer, followed by sonication for

5 min at 0 °C and 200 W with an ultrasonic insonator (model 201M; Kubota, Tokyo, Japan). The supernatant was obtained by centrifugation at 5000× *g* for 10 min at 4 °C.

2.2.2. Purification of GshA by Nickel Sepharose Column

The cell-free extracts obtained above were subjected to affinity column chromatography using 2 mL of nickel Sepharose 6 Fast Flow resin (GE Healthcare; Chicago, IL, USA) equilibrated with binding buffer (20 mM Tris, 0.5 M NaCl, and 20 mM imidazole–HCl, pH 7.9). After washing the column with 20 mL of the binding buffer, the His_6-GshA mutants were eluted with 12 mL of elution buffer (20 mM Tris, 0.5 M NaCl, and 500 mM imidazole-HCl, pH 7.9). The eluate was collected in 1 mL fractions. The fraction with the highest purity was identified by SDS-polyacrylamide gel electrophoresis as described [19], and the fraction was dialyzed against 20 mM Tris–HCl (pH 8).

2.2.3. Measurement of GshA Activity

When glutamate and cysteine are ligated by GshA, ADP is released from ATP. The amount of this ADP was measured as the reduction of NADH by the coupling reaction of pyruvate kinase and lactate dehydrogenase. This method was based on the previously described method [20]. In total, 1 mL of the reaction mixture consisted of 100 mM Tris-HCl (pH 8), 25 mM sodium glutamate, 10 mM cysteine, 5 mM sodium ATP, 5 mM $MgSO_4$, 10 mM KCl, 1 mM phosphoenolpyruvic acid, 0.14 mM NADH, 5 U/mL pyruvate kinase, 15 U/mL lactate dehydrogenase, and the appropriate amount of GshA. Various concentrations of glutathione were added to the reaction solutions to evaluate the extent to which the mutation introduced into GshA could desensitize the inhibition by glutathione. The rate of NADH consumption was monitored by the decreasing rate of absorbance at 340 nm with time at 37 °C using a spectrophotometer (UV-1600; Shimadzu; Kyoto, Japan).

2.3. Glutathione Production

2.3.1. Growth Conditions of Strains for Glutathione Production

Pre-cultured *E. coli* K-12 strains were grown in LB broth Miller supplemented with 30 µg/mL kanamycin at 37 °C with reciprocal shaking at 120 rpm. After the cells were collected by centrifugation at 6000× *g* for 5 min at 4 °C, they were washed with 1 × M9 buffer [18], and then suspended in M9 buffer and the OD_{562} was measured. A total of 1.2 g of dry heat-sterilized $CaCO_3$ was added to 60 mL of the simplified SM1 medium [21] supplemented with 30 µg/mL kanamycin (Table 2) in a 300 mL Erlenmeyer flask, and then the medium was inoculated with the precultured cells prepared as above to obtain an initial OD_{562} of 0.1. After 3 h of incubation at 37 °C with reciprocal shaking, IPTG was added to a final concentration of 0.04 mM. Cells were allowed to grow further to synthesize glutathione.

Table 2. Composition of the simplified SM1 medium.

Ingredients	Volume Added
10× M9 buffer	6.0 mL
100 mM $CaCl_2$	0.06 mL
10% (weight/volume) glucose	3.0 mL
100 mM $Na_2S_2O_3$	3.0 mL
100 mM Na_2SO_4	3.0 mL
Lennox broth [22]	6.0 mL
1 M $MgSO_4$	0.6 mL
Distilled water	38.34 mL
Total	60 mL

2.3.2. Preparation of Supernatants from Extracellular Samples

The medium supplemented with $CaCO_3$ is cloudy and we cannot directly determine cell growth by OD. Therefore, after taking 1 mL of the sample from the culture, 20 µL of it was diluted 10-fold with 1 N HCl to dissolve $CaCO_3$ and then its OD_{562} was measured. The remainder of the sample was centrifuged at 4000 × *g* at 4 °C for 10 min, and the supernatant was stored at −20 °C until the glutathione assay. Immediately before the glutathione assay, the sample was thawed and mixed with 1/10 volume of 100% (*w*/*v*) trichloroacetic acid, and then passed through a Millex-LH filter (Millipore; Billerica, MA, USA).

2.3.3. Measurement of glutathione concentration

(1) Measurement by HPLC equipped with an Amino Na column

Glutamic acid and reduced and oxidized glutathione can be separated by an HPLC (model LC-10A; Shimadzu; Kyoto, Japan) equipped with a Shim-pack Amino Na column (Shimadzu) with gradient elution at 60 °C at a flow rate of 0.6 mL/min [23]. The mobile phase gradient was formed with buffer A (66.6 mM citrate, 1% perchloric acid, and 7% ethanol, pH 2.8) and buffer B (200 mM citrate, 200 mM boric acid, and 0.12 N NaOH, pH 10). The concentration of buffer B was kept at 0% until 9 min, then increased linearly to 7% from 9–13 min, to 8% from 13–17.2 min, and then to 11%. The amino group of amino acids and peptides was modified with *o*-phthalaldehyde by the post-column method, and fluorescence was detected with a fluorescence detector (model RF-$10A_{XL}$; Shimadzu) as the absorbance at 450 nm with excitation at 348 nm.

(2) Enzymatic method using glutathione reductase

While the HPLC method requires 45 min for one sample, the enzymatic method requires only 5 min. Therefore, we used the enzymatic method for Figures 2–4 and the HPLC method for Figure 5. Reduced glutathione in the sample was oxidized with $Na_2S_4O_6$, and glutathione reductase was used to determine the total amount of oxidized glutathione [24]. The results are expressed as the amount of reduced-form glutathione. In this case, the culture supernatant was used without mixing with trichloroacetic acid.

3. Results and Discussion

3.1. Evaluation of Desensitized Mutations of GshA

The initiation codon of *gshA* is a rare initiation codon, UUG. We have shown that the *gshA* gene is a member of the polyamine modulon. The translation of *gshA* mRNA is accelerated in the presence of polyamine, although UUG is an inefficient initiation codon [25]. In pTO23, AUG is used as the initiation codon, a His-tag is added to the N-terminus of GshA for rapid purification, and the original promoter is replaced by the strong T5 promoter for overexpression. The nucleotide sequences of the coding regions of *E. coli* B and K-12 *gshA* genes are identical. The DNA sequence of the desensitized GshA of *E. coli* B has been reported [16]. The nucleotide sequences of the mutant and the wild-type *gshA* genes were compared to find mutations that cause desensitization. There were three amino acid substitutions, i.e., W100L, A494G, and S495F. We made different combinations of the mutations and purified the mutant GshA enzymes. In each combination, derivatives of pTO23 plasmids with the S495F mutation could be made, but the mutant enzyme could not be purified. Therefore, the mutant GshA enzymes with either W100L or A494G and both W100L and A494G mutant enzymes were purified in addition to the wild-type enzyme and the effects of the mutations on desensitization were compared (Figure 2).

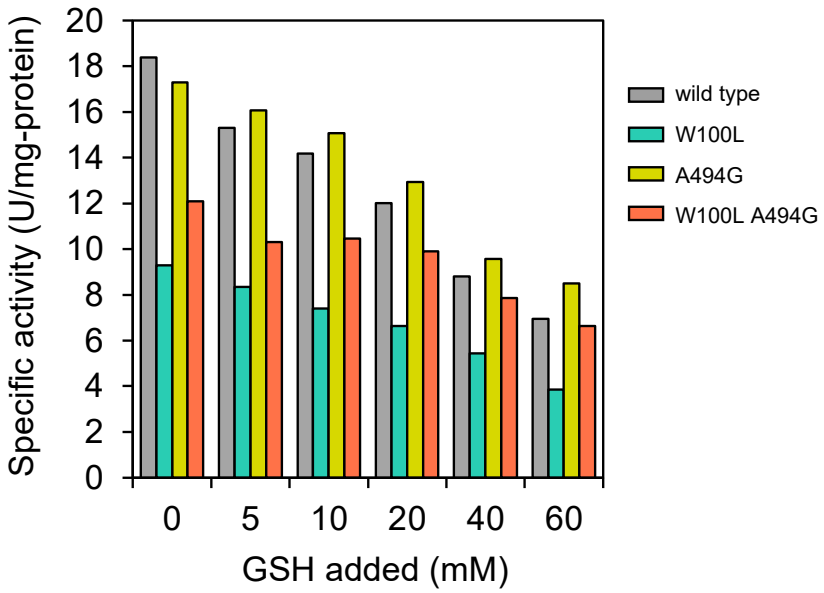

Figure 2. Residual enzymatic activities of desensitization mutants of GshA under different concentrations of glutathione (reduced form). Gray, wild type; bright teal, W100L; yellow-green, A494G; orange, W100L A494G. Each mutant enzyme was purified from strains TN4, TN9, TN10, and TN13, respectively.

The specific activity of the GshA^{W100L} mutant enzyme and that of the GshA$^{W100L\ A494G}$ mutant enzyme were much lower than that of the wild-type enzyme, while that of the GshA^{A494G} mutant enzyme was almost the same as that of the wild-type enzyme. This indicates that the W100L mutation decreases the specific activity of GshA. On the other hand, the A494G mutation has a slight desensitizing effect. When we compared the wild-type and A494G mutant enzyme on glutathione production, there was no difference. Therefore, we used the wild-type enzyme in the following experiments.

Richard and Meister [26] reported that the feedback inhibition of GshA is not allosteric but due to the binding of glutathione to the substrate binding site. Hibi et al. [27] determined the three-dimensional structure of *E. coli* GshA synthetase and reported that the catalytic domain is located at residues 18–387 and 442–518, but both residues W100L and A494G are not in the substrate binding sites. Unfortunately, they did not report a crystal structure of GshA binding to glutathione, so we cannot explain our results from its structure.

3.2. Effect of The Addition of Sodium Thiosulfate ($Na_2S_2O_3$) to M9 1% Glucose Medium Containing 1 mM $MgSO_4$

Glutathione contains cysteine, which has sulfur in its molecule. M9 1% glucose medium contains only 1 mM $MgSO_4$ as the sole source of sulfur. Since sulfur is required not only for glutathione production but also for protein synthesis, 1 mM $MgSO_4$ is not sufficient to produce a large amount of glutathione. Therefore, the effect of adding sulfur to the medium was compared. The addition of sodium thiosulfate was effective for glutathione production, but there was not much difference between the addition of 5 mM and 10 mM sodium thiosulfate (Figure 3). On the other hand, the addition of sodium sulfate showed no effect on glutathione production. We then added 5 mM sodium thiosulfate to increase glutathione production and 5 mM sodium sulfate for cell growth to the simplified SM1 medium which was used for high cysteine production.

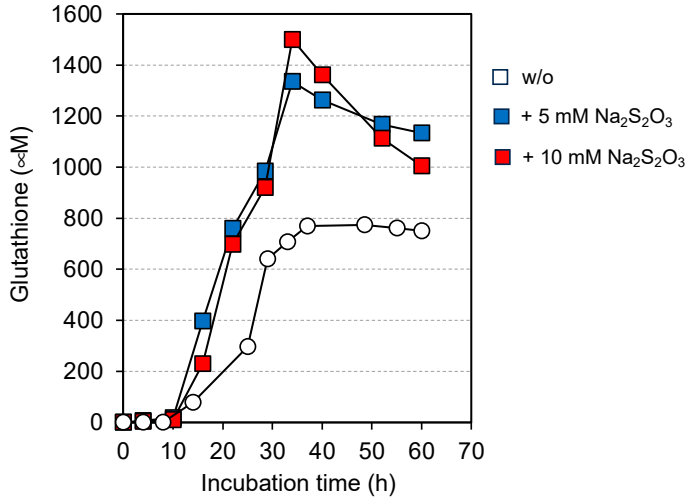

Figure 3. Effects of sulfur source on glutathione production by the strain TN4. The amounts of glutathione produced in M9 1% glucose medium containing only 1 mM $MgSO_4$ as the sole sulfur source with 120 μg/mL ampicillin (white circles) and in the medium supplemented with 5 mM $Na_2S_2O_3$ (blue squares) and 10 mM $Na_2S_2O_3$ (red squares) are shown. A total of 60 mL of each medium was used in a 500 mL Erlenmeyer flask. Glutathione was measured with glutathione reductase.

3.3. Effect of Wild-Type and Desensitized CysE on Glutathione Production

Cysteine is one of the components of glutathione, but a high concentration of cysteine is toxic to *E. coli* cells. Since the intracellular concentration of cysteine is regulated by the feedback inhibition of serine *O*-acetyltransferase (CysE) by cysteine, the reaction catalyzed by CysE is the rate-limiting step. $CysE^{V95R\ D96P}$ has been reported as a desensitizing mutation of CysE [17]. Therefore, we cloned the wild-type *cysE* gene and the $cysE^{V95R\ D96P}$ gene next to the *gshAB* genes on the plasmid to form an operon under the control of the T5 promoter, and their effect on glutathione production was compared. As shown in Figure 4, the V95R D96P mutation was obviously effective in increasing glutathione production.

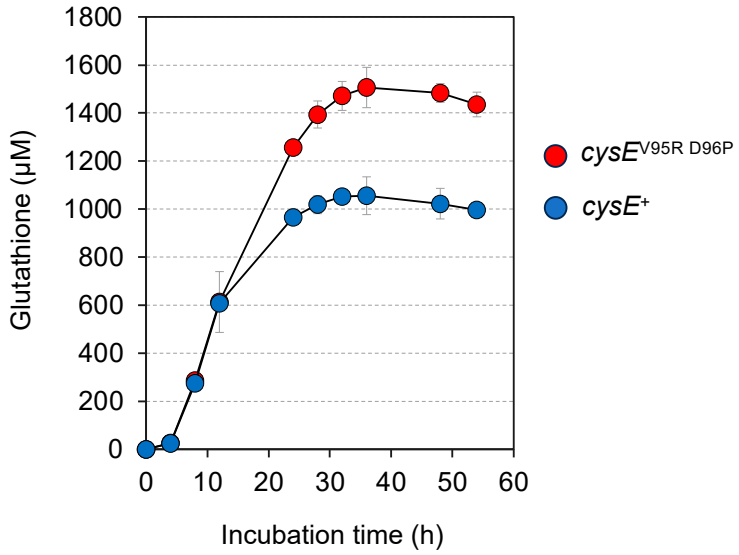

Figure 4. Effects of the CysE mutation on glutathione production in the simplified SM1 medium containing 5 mM $Na_2S_2O_3$, 5 mM Na_2SO_4, and 120 μg/mL ampicillin (60 mL of the medium was used in a 500 mL Erlenmeyer flask). TN31 (pQE-80L::T5p-ATG-His_6-$gshA^{wt}$-$gshB^{+}$-$cysE^{+}$/Δ*ggt* Δ*gshA* Δ*gsiAB*; blue circles), TN37 (pQE-80L::T5p-ATG-His_6-$gshA^{wt}$-$gshB^{+}$-$cysE^{V95R\ D96P}$/Δ*ggt* Δ*gshA* Δ*gsiAB*; red circles). Glutathione was measured with glutathione reductase. Values are expressed as the mean of three samples ± standard deviation.

3.4. Production of Reduced and Oxidized Glutathione by Addition of Sodium Glutamate and Glycine

In contrast to cysteine and compared to other amino acids, large amounts of glutamate and glycine are required for *E. coli* growth and glutathione production. In this study, we added glutamate and glycine to the medium to see if they were indeed the rate-limiting factors for glutathione production. In preliminary experiments, the optimal amounts of glutamic acid and glycine to be added to the simplified SM1 medium were 50 mM and 75 mM, respectively. Therefore, both were added simultaneously to produce glutathione, and after 36 h of incubation, 4.6 mM and 0.56 mM of the reduced and oxidized glutathione, respectively, were accumulated in the culture medium (Figure 5).

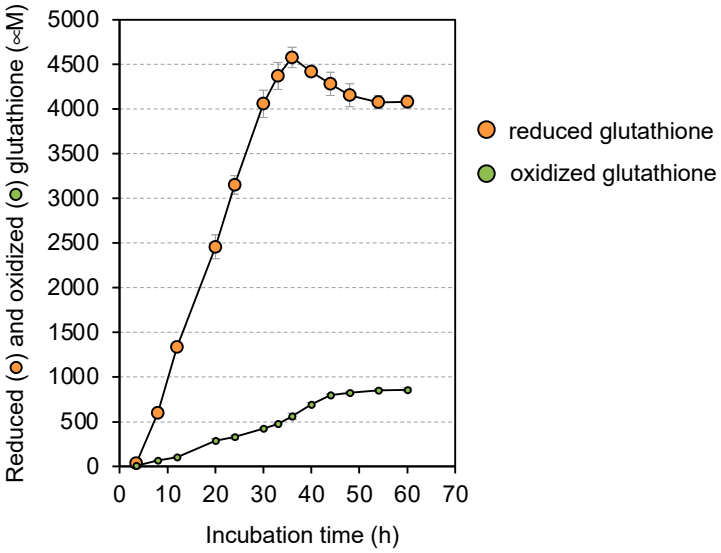

Figure 5. Production of reduced (orange circles) and oxidized (green circles) glutathione in the simplified SM1 medium containing 5 mM $Na_2S_2O_3$, 5 mM Na_2SO_4, and 120 μg/mL ampicillin with the addition of 50 mM sodium glutamate and 75 mM glycine by TN48 (pQE-80L::T5p-ATG-His_6-*gshA*^{wt}-*gshB*$^{+}$-*cysE*$^{V95R,\ D96P}$/*rph-1* Δ*ggt-2 htp*$^{+}$-Tn*10* Δ*gshA* Δ*gsiAB* Δ*cysE*::*kan*^{r}-FRT) (60 mL of the medium was used in a 500 mL Erlenmeyer flask). IPTG was added at a final concentration of 0.04 mM when OD_{562} was 3. Glutathione was measured with HPLC. Values are expressed as the mean of three samples ± standard deviation.

In the early days, bacterial glutathione fermentation was performed using *E. coli* with two genes, *gshA* and *gshB*, cloned from *E. coli* [28,29]. After the discovery of bifunctional glutathione synthetases (GshF) with both γ-glutamylcysteine synthetase and glutathione synthetase domains in one protein [1–3], *gshF* genes were cloned from several bacteria and used for glutathione fermentation by *E. coli* cells [30,31]. A large amount of glutathione was produced in these studies. However, in these studies, not only glutamate and glycine but also cysteine was added to the medium and glutathione was produced inside the cells [30,31]. Zhang et al. [32] cloned *gshF* on a pET vector and introduced it into a Δ*ggt* Δ*pepT* strain to avoid hydrolysis of the synthesized glutathione. They also added cysteine to the medium and synthesized glutathione inside the cells [32]. It is more enzymatic, but after whole *E. coli* cells expressing GshF were collected, they were frozen and thawed to become permeable. The cells were then suspended in the reaction mixtures containing glutamate, glycine, cysteine, and ATP or glucose to produce glutathione [3,32].

On the contrary, our strain makes cysteine by itself and excretes glutathione into the extracellular space.

4. Conclusions

The strengths of our strain are as follows: (1) It overexpresses *gshA*, *gshB*, and *cysE*$^{V95R\ D96P}$ from the strong T5 promoter on the multicopy plasmid to enhance the production ability of glutathione. (2) It excretes the produced glutathione into the extracellular space and extracellular glutathione is not taken up or cleaved because the strain lacks the GsiABCD glutathione transporter and GGT. As a result, we can collect glutathione from the medium without breaking the cells and this simplifies the purification step of glutathione. (3) Cysteine synthesis was enhanced by the V95R D96P mutation, and $Na_2S_2O_3$ was a good sulfur source. As a result, 5.7 mM glutathione in terms of reduced glutathione was produced in the extracellular space by the batch culture with the addition of glutamate and glycine to the medium.

In the future, our strain should be engineered to overproduce glutamate and glycine to reduce the cost of the medium. Another issue that needs to be addressed is the mechanism by which *E. coli* excretes glutathione from cells and to use this knowledge to accelerate glutathione excretion into the extracellular space.

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