One-pot synthesis of glutathione by a two-enzyme cascade using a thermophilic

ATP regeneration system

Xing Zhang ^{a, 1}, Hui Wu ^{a,1}, Bing Huang ^a, Zhimin Li ^{a, b*}, Qin Ye ^a

^a State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

^b Shanghai Collaborative Innovation Center for Biomanufacturing Technology, 130 Meilong Road, Shanghai 200237, China

* Corresponding author: Zhimin Li

Telephone: +86-21-64252095

Fax: +86-21-64252250

E-mail: lizm@ecust.edu.cn

¹ These authors contributed equally to this work.

Abstract

In vitro cascade catalysis using enzyme-based system is becoming a promising biomanufacturing platform for biofuels and biochemicals production. Glutathione is a pivotal non-protein thiol compound and widely applied in food and pharmaceutical industries. In this study, glutathione was synthesized by a bifunctional glutathione synthetase together with a thermophilic ATP regeneration system through a two-enzyme cascade in vitro. Four bifunctional glutathione synthetases from *Streptococcus sanguinis*, *S. gordonii*, *S. uberis* and *Bacillus cereus* were applied for glutathione synthesis. The bifunctional glutathione synthetase from *S. sanguinis* was selected and coupled with the polyphosphate kinase from *Thermosynechococcus elongatus* BP-1 for regenerating ATP to produce glutathione in one pot. In the optimized system, 28.5 mM glutathione was produced within 5 h due to efficient ATP regeneration from low-cost polyphosphate. The yield based on added L-cysteine reached 81.4 % and the productivity of glutathione achieved 5.7 mM/h. The one-pot system indicated a potential biotransformation platform for industrial production of glutathione.

Keywords: glutathione; bifunctional glutathione synthetase; ATP regeneration; polyphosphate; polyphosphate kinase;

1. Introduction

In vitro biocatalytic cascades, based on purified enzymes, crude cell extracts, or lyophilized cells, have attracted significant attentions for multistep reactions in one pot (Muschiol et al., 2015). Synthetic biosystem using purified enzymes shows many advantages, such as high product yield, low processing cost, fast reaction rate, and easy target product separation, which make it more suitable than traditional whole-cell catalysis for desired chemicals productions (Billerbeck et al., 2013; Zhang et al., 2010; Zhang, 2015). This kind of environmentally friendly platform has been successfully applied for the production of biofuels and biochemicals. For example, hydrogen was produced from cellulose, xylose and sucrose by synthetic enzymatic pathways, respectively at nearly theoretical yields, respectively (Martín del Campo et al., 2013a; Myung et al., 2014; Ye et al., 2009); glucose was directly converted to *n*-butanol with a molar yield of 82 % (Krutsakorn et al., 2013); 1,3- propanediol was efficiently produced from glycerol in an established cell-free system (Rieckenberg et al., 2014); These successfully constructed in vitro synthetic pathways have demonstrated their potential of industrial applications.

In many cases, costly cofactors are required for the construction of in vitro biosynthetic systems (Rollin et al., 2013; Welch and Scopes, 1985; Ye et al., 2012). One of the important cofactors, adenosine triphosphate (ATP), is involved in many energy-demanding biocatalytic reactions as a source of energy supply (Hara and Kondo, 2015). Direct addition of ATP to the system is cost-inefficient and leads to the accumulation of byproducts, such as adenosine diphosphate (ADP) or adenosine

monophosphate (AMP) (Iwamoto et al., 2007), which further complicates the downstream separation process. A cheap and efficient ATP regeneration system is crucial for ATP-dependent biosynthetic reactions, which can decrease the direct ATP usage and enhance the economics of industrial process significantly. Several enzymatic reactions have been employed for ATP regeneration in ATP-dependent systems, such as phosphoenolpyruvate (PEP) with pyruvate kinase, creatine phosphate with creatine kinase, acetyl phosphate with acetate kinase and polyphosphate (polyP) with polyphosphate kinase (PPK) (Andexer and Richter, 2015). However, the issues of product inhibition, high cost or low stability of PEP-, acetyl phosphate- and creatine phosphate- based systems might limit applications of these technologies in large-scale processes (Caschera and Noireaux, 2015; Martín del Campo et al., 2013b; Resnick and Zehnder, 2000). Inorganic polyP, a linear chain of many phosphate units, exists abundantly in volcanic condensates and deep-oceanic steam vents (Liao et al., 2012). Coupled with PPK, use of commercial grade polyP can significantly reduce the cost (Iwamoto et al., 2007). Due to the attractive advantages of low cost and high stability, the PPK-based ATP regeneration system shows more feasible for ATP-dependent reactions (Restiawaty et al., 2011).

Glutathione (L- γ -glutamyl-L-cysteinyl glycine, GSH), a low-molecular-weight thiol, is widely found in living organisms (Meister and Anderson, 1983). Its significant functions in antioxidant defense, detoxification of xenobiotics and regulation of intracellular metabolism have attracted increasing interests in medicine, food additive, health care and cosmetic industries (Penninckx and Elskens, 1993; Wu

et al., 2004). GSH can be synthesized from L-glutamic acid, L-cysteine and glycine, and two moles of ATP are needed to form one mole of GSH. Traditionally, the reaction is catalyzed by two ATP-dependent enzymes in sequence: γ -glutamylcysteine (γ -GC) synthetase (γ -GCS, EC6.3.2.2, catalyzing L-glutamic acid and L-cysteine to form γ -GC) and GSH synthetase (GS, EC6.3.2.3, catalyzing γ -GC and glycine to form GSH).

The bifunctional GSH synthetase (GshF) possessing both γ -GCS and GS activities has been discovered in *Listeria monocytogenes*, *Streptococcus agalactiae* and *Pasteurella multocida*, respectively (Gopal et al., 2005; Janowiak and Griffith, 2005; Vergauwen et al., 2006). These GshFs consist of a γ -GCS-like N-terminal domain fused to an ATP-grasp domain on the C- terminal (Gopal et al., 2005; Vergauwen et al., 2006). It has been reported that most GshFs are less sensitive to feedback inhibition caused by GSH except those from *L. monocytogenes* and *P. multocida* (Gopal et al., 2005; Li et al., 2011; Vergauwen et al., 2006). Furthermore, many of them show higher specific activities than *E. coli* γ -GCS and GS (Yang et al., 2016). In our previous studies, a recombinant *E. coli* strain overexpressing GshF of *S. thermophilus* was constructed and production of GSH was investigated using the resting cells. Only 7 mM GSH was produced and the productivity achieved about 2 mM/h with *E. coli* endogenous ATP-regeneration system (Li et al., 2011). After optimization of the cultivation process on the level of dissolved oxygen, the amount of added precursor amino acids and glucose feeding, the GSH concentration achieved 15.21 g/L in the fermentation process lasted 19.5 h (Wang et al., 2015).

Compared to microbial fermentation, the enzymatic synthesis of GSH has the advantages of higher product concentration, shorter production period, less precursor and product degradation, and easier purification. In the present study, a one-pot reaction system was constructed for GSH biosynthesis through a two-enzyme cascade (Fig. 1). Four bifunctional GSH synthetases from *S. sanguinis*, *S. gordonii*, *S. uberis* and *Bacillus cereus* were overexpressed in *E. coli* Rosetta2 (DE3), respectively, and the capabilities for GSH biosynthesis were compared. The best GshF from *S. sanguinis* was selected and applied to in vitro GSH production coupled with the ATP regeneration enzyme, PPK from *Thermosynechococcus elongatus* BP-1 (Sato et al., 2007). The effect of polyP concentration and enzyme mass ratio were investigated, and GSH was efficiently produced even though no ATP was supplemented.

2. Materials and methods

2.1. Strains, plasmids, chemicals and culture media

E. coli TOP10 (Invitrogen) was used for plasmid amplification and preservation. *E. coli* Rosetta2 (DE3) (Novagen) and plasmid pET28a (+) (Novagen) were used for protein expression. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The polyP used in this study was sodium hexametaphosphate. Luria-Bertani (LB) medium contained tryptone (Oxoid, UK) 10 g/L, yeast extract (Oxoid, UK) 5 g/L, and NaCl 10 g/L. It was supplemented with 50 µg/mL of kanamycin for the cultivation of recombinant *E. coli* strains.

2.2. Construction of GshF and PPK expression system

Proteins with the potential GshF activity were searched in the NCBI database using GshF from *S. thermophilus* SIIM B218 (Li et al., 2011) as a template. Four proteins from *S. sanguinis* SK36, *S. gordonii*, *S. uberis* 0140J and *B. cereus* AH187 with high similarities were selected. The *gshF* genes of *S. sanguinis* (*gshFss*), *S. gordonii* (*gshFsg*), *S. uberis* (*gshFsu*) and *B. cereus* (*gshFbc*) were synthesized by GENEWIZ Co. Ltd. (Suzhou, China) and introduced to the *Bam*HI / *Hind*III restriction sites of pET28a (+). To enhance the gene expression level, the DNA sequences of *gshF* were codon-optimized with *E. coli* codon usage (Supplemental Information), yielding the plasmids of pET28a-*gshFss*, pET28a-*gshFsg*, pET28a-*gshFsu* and pET28a-*gshFbc*, respectively. The *ppk* gene of *T. elongatus* BP-1 was also synthesized by GENEWIZ Co. Ltd. (Suzhou, China) and introduced to the *Eco*RI / *Sal*I restriction sites of pET28a (+) to yield the plasmid of pET28a-*ppk*. Then, the recombinant plasmids were transformed to *E. coli* Rosetta2 (DE3) for GshF and PPK production, respectively.

2.3. Protein overexpression and purification

The recombinant cells were incubated in 3 mL LB medium at 37 °C and 220 rpm for 7 h. Then 2 mL of the cultures were transferred to 100 mL of newly prepared LB medium in 500 mL flasks and cultivated at 37 °C. The overexpression of GshF was induced by addition of appropriate concentration of isopropyl- β -D-thiogalactopyranoside (IPTG) when the optical density at 600 nm of the culture reached 0.6-0.8, and then incubated at 18 °C and 220 rpm for 16-18 h (Table 1). After

induction, the cells were harvested by centrifugation at 6000 rpm and 4 °C for 20 min, and washed once with 20 mM Tris-HCl buffer (pH 8.0). Cells were suspended in the same buffer and lysed by high-pressure homogenizer supplied by Shanghai Litu Mechanical Equipment Engineering Co. Ltd (Shanghai, China). The lysate was centrifuged at 8000 rpm for 20 min. The GshF in the supernatant was purified by Ni-chelating affinity chromatography. The purity of GshF was examined by SDS-PAGE, and the protein was quantified by the BCA Protein Assay Kit from Tiangen (Beijing, China). For PPK production, cells were grown at 37 °C to reach the optical density of 0.7-0.8 at 600 nm, and induced by adding 0.3 mM IPTG at 18 °C and 220 rpm for 16 h. After centrifugation and washing once with 20 mM Tris-HCl buffer (pH 8.0), the cells were suspended in the same buffer and followed by heating for 30 min at 70 °C. The heat-treated cells were collected and added directly into the coupled system for ATP regeneration.

2.4. GshF activity assay

The activity of GshF was assayed in 100 mM Tris-HCl (pH 8.0) containing 10 mM MgCl₂, 20 mM glycine, 20 mM L-glutamic acid, 20 mM L-cysteine and 20 mM ATP. The diluted purified GshF (10 µL), which contained 40 µg GshFSS, 61 µg GshFSG, 58 µg GshFSU or 1.2 mg GshFBC was added into 190 µL assay mixture and incubated for 10 min, respectively. The reaction was stopped by adding trichloroacetic acid to a final concentration of 10% (w/v), and centrifuged at 12,000 rpm for 20 min at 4 °C. One unit of enzyme activity was defined as the amount of enzyme that produces 1

mM GSH per hour (Yang et al., 2016). All the measurements were performed in triplicates.

The effect of pH on GshF activity was determined in buffers with different pH values from 6.0 to 10.0 at 37 °C by using the following buffers: citrate-phosphate buffer (pH 6.0-7.0), Tris-HCl (pH 8.0), boric-NaOH buffer (pH 9.0-10.0). The highest activities of GshFSS, GshFSG, GshFSU and GshFBC in the various pH conditions were set as 100 % of relative activity, respectively. The effect of temperature was carried out in 100 mM Tris-HCl buffer (pH 8.0) at different temperatures from 37 to 55 °C. The highest activities of GshFSS, GshFSG, GshFSU and GshFBC at various temperatures were set as 100% of relative activity, respectively.

2.5. One-pot GSH synthesis with ATP regeneration

GSH synthesis was carried out in 1 mL 100 mM Tris-HCl (pH 8.0) containing 5.2 mg purified GshFSS, 20 mM glycine, 20 mM L-glutamic acid, 20 mM L-cysteine, 20 mM MgCl₂, 2 mM ADP, 4 mM dithiothreitol and 12 mM polyP. PolyP was replaced with 12 mM ATP or not added as control. 53 mg wet cells of induced *E. coli*/pET28a-*ppk* were suspended with 100 µL 20 mM Tris-HCl (pH 8.0). After heat treatment, the cells were mixed with 900 µL reaction buffer. The synthesis was performed at 45 °C and initialized with the addition of the precursor amino acids. Samples (80 µL each) were withdrawn at different times to monitor the reaction process. To terminate reaction, 10 % (w/v) cold trichloroacetate (final concentration) was added into the samples on ice.

2.6. Optimization of enzyme amount

The amounts of PPK (induced cells harboring pET28a-*ppk*) and purified GshFSS used in the system were optimized at 45°C by changing their ratio while keeping total weight at 56 mg (Table S1). Samples were taken at different times to monitor the reaction.

2.7. Effect of initial polyP concentration on synthesis of GSH

The experiment was performed in 1 mL 100 mM Tris-HCl (pH 8.0) containing 22.6 mg purified GshFSS, 33.4 mg induced wet cells overexpressing *ppk*, 20 mM glycine, 20 mM L-glutamic acid, 20 mM L-cysteine, 4 mM dithiothreitol and 2 mM ADP together with 5, 10, 20 or 40 mM polyP at 45°C, respectively. Samples were taken at 4 h and 6 h to analyze the production of GSH.

2.8. Analytical methods

Cell density was measured by determination of the optical density of culture sample at 600 nm. The concentration of GSH was detected through high performance liquid chromatography (HPLC) equipped with a C18 column (4.6×150 mm, Wondasil, Shimadzu-GL). The mobile phase was composed of 10 mM sodium heptanesulfonate and 50 mM monopotassium phosphate (pH 3.0)-methanol (95:5, V/V) at a flow rate of 0.6 mL/min. The column temperature was maintained at 30°C and the detection wavelength was set at 210 nm.

3. Results and discussion

3.1 Screening, overexpression and purification of GshFs and PPK

To search for more efficient bifunctional enzymes for glutathione synthesis, the GshFST in our previous work (Li et al., 2011) was used as a reference with the BLAST tool of NCBI. Four potential bifunctional enzymes from *S. sanguinis* (GshFSS) (GenBank accession no. CP000387), *S. gordonii* (GshFSG) (GenBank accession no. CP000725), *S. uberis* (GshFSU) (GenBank accession no. AM946015.1) and *B. cereus* (GshFBC) (GenBank accession no. CP001179) were selected from the protein database. Multiple amino acid sequence alignments showed that GshFSS, GshFSG, GshFSU and GshFBC shared 73%, 72%, 63% and 49% of amino acid identity with GshFST, respectively (Fig. S1). The codon-optimized *gshFs* were synthesized and overexpressed in *E. coli* Rosetta2 (DE3) in LB medium. All the GshFs were successfully produced in soluble states (Fig. S2A). However, the expression levels of GshFSU and GshFBC were very low. The His-tagged recombinant proteins were purified through Ni-NTA affinity chromatography (Fig. S2B). SDS-PAGE analysis indicated that the four GshFs appeared with a molecular mass of approximately 88 kDa, which was close to the estimated value deduced from their amino acid sequences (Table 1).

The *ppk* gene of *T. elongatus* was synthesized and expressed in *E. coli* Rosetta2 (DE3). As shown in Figure S2C, PPK was expressed well after induction. The molecular weight of PPK is close to 85 kDa calculated from its amino acid sequence.

However, the protein was produced partly in insoluble form. It was reported that the PPKs of *Thermus thermophilus* HB27 and *E. coli* were outer membrane-bound proteins (Restiawaty et al., 2011; Akiyama et al., 1992). In this study, it was found that part of PPK was also associated with the membrane and remained in the precipitate (Fig. S2C). The membrane structure of *E. coli* cells can be partially or entirely disrupted at high temperatures, leading to the leakage of the thermophilic enzyme outside the cells to better access substrates (Ninh et al., 2013). Therefore, the induced *E. coli* cells harboring pET28a-*ppk* were heat-treated at 70 °C for 30 min, resulting in denaturation of indigenous mesophilic enzymes and elimination of membrane barrier. Due to the thermostability of PPK, PPK in the supernatant and pellets (Fig. S2C) could still retain the activity (Sato et al., 2007). The heat-treated mixture was directly collected for ATP regeneration.

3.2 Characterization of GshFs

Most GshFs were reported to be less sensitive to high concentration of GSH compared with the γ -GCS-GS system (Yang et al., 2016). To select a suitable GshF for GSH synthesis, the catalytic characteristics of the four GshFs were investigated. The effects of pH and temperature on these four GshFs are shown in Fig. 2. The optimal pH for the GshFs was around 8.0. The activities were completely lost when the pH reduced to 6.0 or surpassed 10.0. The optimum temperature range of GshFSS, GshFSG and GshFSU was 42 - 45°C. GshFSS retained its activity even at 55°C, but the activities of GshFSG and GshFSU dropped dramatically at the same temperature.

For GshFBC, the optimum temperature was 37 °C, and its activity declined sharply when the temperature increased. The specific activities of GshFSS, GshFSG, GshFSU and GshFBC are shown in Table 1. It was confirmed that GshFSS exhibited the highest specific activity (841 U/mg). Interestingly, although GshFSS was not derived from a thermophilic microorganism, its activity at 45°C was higher than that of GshFST (Yang et al., 2016). This result showed its potential application for GSH synthesis. Due to the wide temperature range and high specific activity, GshFSS was applied in the following experiments.

3.3 One-pot GSH synthesis with ATP regeneration from polyP

An ATP regeneration system is indispensable for the economical production of GSH. Hence, a two-enzyme system, consisting of GshFSS and PPK, was applied for GSH synthesis in vitro (Fig. 1). The PPK catalyzed the regeneration of ATP from polyP and ADP. As shown in Fig. 3, 7.6 mM GSH was produced within 4 h under the supplementation of 12 mM polyP and 2 mM ADP. The reaction continued to synthesize GSH and finally 10.2 mM GSH was obtained after 8 h with a yield of approximately 50% based on added L-cysteine. In comparison, when ATP was directly added, the GSH synthesis proceeded quickly but only 7 mM GSH was produced in 1 h. The reaction stopped because of ATP depletion in the reaction medium. When extra 8 mM ATP was added at 2 h, GSH was further synthesized (about 10 mM GSH in 6 h) (Fig. S3). The slow decrease of GSH titer later was possibly caused by the autoxidation of GSH to GSSG, which happens normally in

ordinary environment. In the reaction without polyP and ATP, slight GSH was formed. It might be due to the small amount of ATP present in the lysate of cells containing the overexpressed PPK.

Murata et al. (1980) established an acetate kinase-based ATP regeneration system with immobilized whole cells to produce GSH. However, compared to polyP, the instability and high cost of acetyl phosphate (Martín del Campo et al., 2013b; Resnick and Zehnder, 2000; Sato, M. et al., 2007) might limit the application for industrial enzymatic production of GSH. In this study, we developed an efficient system working at an elevated temperature for GSH production in one pot. Compared to traditional enzymatic GSH production using whole-cell biocatalysts (Li et al., 2011; Yoshida et al., 2011), this system had several advantages. First, the PPK-polyP system was inexpensive and efficient for ATP regeneration. A high titer of 10 mM GSH was achieved. Second, serious degradation of L-cysteine and GSH existing in whole-cell catalysis (Zhang et al., 2016) could be overcome. Although the cells overexpressing PPK were used, the heat-treatment could deactivate enzymes responsible for these degradation pathways and decrease the side reactions. Third, only a slight amount of ADP was required for turnover, suggesting a reduced cost of downstream separation. Fourth, both GshFSS and PPK showed higher enzymatic activities at 45°C, and a faster GSH production rate could be achieved at an elevated temperature (Fig. S4). In addition, lower viscosity and higher mixing ability of the reaction medium were allowed at 45°C. Hence, the two-enzyme system became more feasible for GSH production.

3.4 Effects of enzymes quantities

To improve the titer and yield of GSH by better coordinating the two enzymes,, the ratio of PPK to GshFSS in the reaction system was investigated. The GSH production at different mass ratios of PPK to GshFSS was compared by using the PPK expressed cells and purified GshFSS whose total weight was kept at 56 mg (Fig. 4). The GSH titer increased from 6.5 to 16.4 mM with the increasing amount of GshFSS from 2.2 to 22.6 mg. The yields of GSH on L-cysteine ranged from about 0.32 mol/mol to 0.82 mol/mol as the amounts changed. Notably, at the optimal concentrations of PPK and GshFSS (33.4 mg wet cells and 22.6 mg purified enzyme), the average rate of GSH generation reached 3.9 mM/h within 4 h. When the GshFSS concentration was further increased whereas PPK concentration was reduced, the GSH titer decreased, indicating the limitation of ATP supply by PPK. Using the optimal mass ratio of enzymes can facilitate the balances among enzymatic reactions, which is an important component in multi-enzyme systems (Zhang et al., 2014). In this two-enzyme system, appropriate enzyme ratio could well coordinate the reactions for GSH production and ATP generation. At the optimal enzyme ratio, the concentration of GSH reached 16.4 mM and the yield achieved 82 %, much higher than those at other ratios.

3.5. Effect of the initial polyP concentration

Since polyP was continuously consumed in the reaction system, the amount of supplied polyP could significantly affect the production of GSH. Therefore, the effect

of initial polyP concentration in the reaction system was investigated (Fig. 5). When the initial concentration of polyP was increased from 5 mM to 10 mM, the titer of GSH increased from 9.7 to 15.2 mM after 6 h. With the increasing concentration of polyP to 20 mM, the production of GSH did not improve after 6 h, indicating that 10 mM polyP was sufficient in this system. When 40 mM polyP was added, the concentration of GSH decreased dramatically and only 3.5 mM GSH was synthesized within 6 h. The higher initial concentration of polyP showed an inhibitory effect on GSH synthesis. This result was possibly due to the chelation of Mg^{2+} with polyP. When the concentration of Mg^{2+} was increased to 40 mM, more GSH was produced (14.9 mM in 6 h) compared with the system containing 20 mM Mg^{2+} (Fig. S5). However, high concentrations of Mg^{2+} and polyP could form a precipitate and polyP might bind to PPK and directly affect their activities (Kameda et al., 2001). The fed-batch addition with small amounts of polyP might be an alternative strategy for higher production of GSH.

3.6 GSH synthesis with the fed-batch mode

The titer and yield of GSH in the two-enzyme system were expected to be further enhanced by increasing the enzyme concentrations of PPK and GshFSS. The concentrations of enzymes were increased to 67 mg wet cells and 44 mg GshFSS, which were approximately two-fold higher than those at the experiment above (Fig. 4). It was reported that L-cysteine was easily oxidized by dissolved oxygen (Hogg, 1999). To avoid the inhibitory effect of polyP and oxidative loss of L-cysteine, the initial

concentrations of glycine, L-glutamic acid, L-cysteine and polyP were set at 20 mM, 20 mM, 15 mM and 10 mM, respectively. Afterwards, 10 mM polyP, 12 mM glycine, 12 mM L-glutamic acid and 10 mM L-cysteine were added into the 1 mL reaction mixture at 2 h and 4 h, respectively. As shown in Fig. 6, 28.5 mM GSH was efficiently synthesized in 5 h with a high yield of 81.4% based on added L-cysteine, and the average volumetric productivity was 5.7 mM/h. Within 3 h, 23.4 mM GSH was rapidly produced and a high yield of 94% was achieved. This indicated that nearly all the L-cysteine added to the reaction mixture was converted to GSH. The maximum production rate was 14.7 mM/h during the first hour. The pH was later reduced from 8.0 to 6.2 at 5 h, which might lead to a decrease of the GSH production rate according to the results shown in Fig. 2B. Since initially only 2 mM ADP was added to the mixture, ATP was regenerated approximately 28 times in the system during the 5 hours.

Strategies to enhance the efficiency of GSH synthesis in the fermentative process have been studied in *Saccharomyces cerevisiae* and *E. coli*. Process controls, such as carbon source feeding based on respiratory quotient and optimized addition of the precursor amino acids, can be applied (Lorenz et al., 2015; Shang et al., 2008; Wang et al., 2016). However, despite of using complicated process controls, the improvement of the conversion of L-cysteine was limited and the economy of system was generally less concerned. While considering these factors, a high yield of 76 % based on added L-cysteine was achieved by manipulating pathways of L-cysteine degradation, energy supply and GSH degradation, but the GSH titer was only 19.1

mM in a process lasted 24 h (Zhang et al., 2016). When GSH synthesis was performed by using whole-cell catalysis in *S. cerevisiae*, intracellular ATP generation system was generally used. Due to the low efficiency of ATP generation and enzymatic activity of GSH synthetase, the titer and productivity of GSH were not satisfactory (Li et al., 2010). In the present in vitro two-enzyme system, the overall GSH production was improved under the optimal condition. Application of a simple fed-batch operation significantly reduced the inhibitory effect of polyP and enabled much more efficient utilization of substrates.

4. Conclusion

In this study, an efficient one-pot system for the synthesis of GSH was developed by utilizing an ATP regeneration system coupled with GshF-based catalysis. Among the four GshFs of *S. sanguinis*, *S. gordonii*, *S. uberis* and *B. cereus*, GshFSS was selected due to its best performances and combined with PPK from *T. elongatus* to construct an in vitro reaction system. It was shown that cost-efficient polyP could replace expensive ATP as energy donor for GSH production. After the optimization of enzyme ratio and initial polyP concentration, 28.5 mM GSH was produced within 5 hours with a high turnover of ATP regeneration (approximately 28 cycles). Moreover, a high GSH yield of 81.4% based on added L-cysteine was attained. The GSH production rate was higher than that in fermentation or synthesis using whole cells. In addition, degradation of substrate amino acid and GSH were effectively eliminated. This one-pot biosynthetic system showed the advantages of high yield, fast reaction

rate and low process cost, indicating its great potential as a promising platform for the production of GSH.

Author Contributions

Conceived and designed the experiments, supervision: HW ZL QY. Performed the experiments: XZ BH. Analyzed the data: XZ HW ZL QY. Contributed reagents, materials, and analysis tools: HW ZL QY. Prepared the paper: XZ HW ZL QY. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by the National Science Foundation for Young Scientist of China (Grant No. 21406065), the Fundamental Research Funds for the Central Universities (Grant No. 222201313007 & 22A201514042), and Shanghai Committee of Science and Technology (Grant No. 13DZ1930202). Partially supported by Open Funding Project of the State Key Laboratory of Bioreactor Engineering. Partially supported by the Foundation of Key Laboratory for Industrial Biocatalysis (Tsinghua University), Ministry of Education (Grant No. 2015101).

Competing interests

The authors declare that they have no competing interests.

References:

- Akiyama, M., Crooke, E., Kornberg, A., 1992. The polyphosphate kinase gene of *Escherichia coli*. Isolation and sequence of the *ppk* gene and membrane location of the protein. J. Biol. Chem. 267, 22556-22561.
- Andexer, J.N., Richter, M., 2015. Emerging enzymes for ATP regeneration in biocatalytic processes. Chembiochem 16, 380-386.
- Billerbeck, S., Härle, J., Panke, S., 2013. The good of two worlds: increasing complexity in cell-free systems. Curr. Opin. Biotechnol. 24, 1037-1043.
- Caschera, F., Noireaux, V., 2015. A cost-effective polyphosphate-based metabolism fuels an all *E. coli* cell-free expression system. Metab. Eng. 27, 29-37.
- Gopal, S., Borovok, I., Ofer, A., Yanku, M., Cohen, G., Goebel, W., Kreft, J., Aharonowitz, Y., 2005. A multidomain fusion protein in *Listeria monocytogenes* catalyzes the two primary activities for glutathione biosynthesis. J. Bacteriol. 187, 3839-3847.
- Hara, K.Y., Kondo, A., 2015. ATP regulation in bioproduction. Microb. Cell Fact. 14, 198.
- Hogg, N., 1999. The effect of cyst (e) ine on the auto-oxidation of homocysteine. Free Radic. Biol. Med. 27, 28-33.
- Iwamoto, S., Motomura, K., Shinoda, Y., Urata, M., Kato, J., Takiguchi, N., Ohtake, H., Hirota, R., Kuroda, A., 2007. Use of an *Escherichia coli* recombinant producing thermostable polyphosphate kinase as an ATP regenerator to produce fructose 1,6-diphosphate. Appl. Environ. Microbiol. 73, 5676-5678.

- Janowiak, B.E., Griffith. O.W., 2005. Glutathione synthesis in *Streptococcus agalactiae* one protein accounts for gamma-glutamylcysteine synthetase and glutathione synthetase activities. J. Biol. Chem. 280, 11829-11839.
- Kameda, A., Shiba, T., Kawazoe, Y., Satoh, Y., Ihara, Y., Munekata, M., Ishige, K., Noguchi, T., 2001. A novel ATP regeneration system using polyphosphate-AMP phosphotransferase and polyphosphate kinase. J. Biosci. Bioeng. 91, 557-563.
- Krutsakorn, B., Honda, K., Ye, X., Imagawa, T., Bei, X., Okano, K., Ohtake, H., 2013. In vitro production of n-butanol from glucose. Metab. Eng. 20, 84-91.
- Liao, H., Myung, S., Zhang, Y.H.P., 2012. One-step purification and immobilization of thermophilic polyphosphate glucokinase from *Thermobifida fusca* YX: glucose-6-phosphate generation without ATP. Appl. Microbiol. Biotechnol. 93, 1109-1117.
- Li, W., Li, Z.M., Ye, Q., 2010. Enzymatic synthesis of glutathione using yeast cells in two-stage reaction. Bioprocess. Biosyst. Eng. 33, 675–682.
- Li, W., Li, Z., Yang, J., Ye, Q., 2011. Production of glutathione using a bifunctional enzyme encoded by gshF from *Streptococcus thermophilus* expressed in *Escherichia coli*. J. Biotechnol. 154, 261-268.
- Lorenz, E., Schmach, M., Stahl, U., Senz, M., 2015. Enhanced incorporation yield of cysteine for glutathione overproduction by fed-batch fermentation of *Saccharomyces cerevisiae*. J. Biotechnol. 216, 131-139.
- Martín del Campo, J.S., Rollin, J., Myung, S., Chun, Y., Chandrayan, S., Patiño, R., Adams, M.W., Zhang, Y.H.P., 2013a. High-yield production of dihydrogen from

- xylose by using a synthetic enzyme cascade in a cell-free system. *Angew. Chem. Int. Ed.* 52, 4587-90.
- Martin del Campo, J.S., Chun, Y., Kim, J.E., Patiño, R., Zhang, Y.H.P., 2013b. Discovery and characterization of a novel ATP/polyphosphate xylulokinase from a hyperthermophilic bacterium *Thermotogamaritima*. *J. Ind. Microbiol. Biotechnol.* 40, 661-669.
- Meister, A., Anderson, M.E., 1983. Glutathione. *Annu. Rev. Biochem.* 52, 711-760.
- Murata, K., Tani, K., Kato, J., Chibata, I., 1980. Glutathione production coupled with an ATP regeneration system. *Appl. Microbiol. Biotechnol.* 10, 11-21.
- Muschiol, J., Peters, C., Oberleitner, N., Oberleitner, N., Mihovilovic, M.D., Bornscheuer, U.T., Rudroff, F., 2015. Cascade catalysis—strategies and challenges en route to preparative synthetic biology. *Chem. Commun.* 51, 5798-5811.
- Myung, S., Rollin, J., You, C., Sun, F., Chandrayan, S., Adams, M.W., Zhang, Y.H.P., 2014. In vitro metabolic engineering of hydrogen production at theoretical yield from sucrose. *Metab. Eng.* 24, 70-77.
- Ninh, P.H, Honda, K., Yokohigashi, Y., Okano, K., Omasa, T., Ohtake, H., 2013. Development of a continuous bioconversion system using a thermophilic whole-cell biocatalyst. *Appl. Environ. Microbiol.* 79, 1996-2001.
- Penninckx, M.J., Elskens, M.T., 1993. Metabolism and functions of glutathione in microorganisms. *Adv. Microb. Physiol.* 34, 239-301.

- Resnick, S.M., Zehnder, A.J., 2000. In vitro ATP regeneration from polyphosphate and AMP by polyphosphate: AMP phosphotransferase and adenylate kinase from *Acinetobacter johnsonii* 210A. Appl. Environ. Microbiol. 66, 2045-2051.
- Restiawaty, E., Iwasa, Y., Maya, S., Honda, K., Omasa, T., Hirota, R., Kuroda, A., Ohtake, H., 2011. Feasibility of thermophilic adenosine triphosphate-regeneration system using *Thermus thermophilus* polyphosphate kinase. Process Biochem. 46, 1747-1752.
- Rieckenberg, F., Ardao, I., Rujananon, R., Zeng, A.P., 2014. Cell-free synthesis of 1, 3-propanediol from glycerol with a high yield. Eng. Life Sci. 14, 380-386.
- Rollin, J.A., Tam, T.K., Zhang, Y.H.P., 2013. New biotechnology paradigm: cell-free biosystems for biomanufacturing. Green Chem. 15, 1708-1719.
- Sato, M., Masuda, Y., Kirimura, K., Kino, K., 2007. Thermostable ATP regeneration system using polyphosphate kinase from *Thermosynechococcus elongatus* BP-1 for D-amino acid dipeptide synthesis. J. Biosci. Bioeng. 103, 179-184.
- Shang, F., Wang, Z., Tan, T.W., 2008. High-cell-density cultivation for co-production of ergosterol and reduced glutathione by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 77, 1233-1240.
- Vergauwen, B., Vos, D.D., Van Beeumen, J.J., 2006. Characterization of the bifunctional gamma-glutamate-cysteine ligase/glutathione synthetase (GshF) of *Pasteurellamultocida*. J. Biol. Chem. 281, 4380-4394.

- Wang, D., Wang, C., Wu, H., Li, Z., Ye, Q., 2016. Glutathione production by recombinant *Escherichia coli* expressing bifunctional glutathione synthetase. J. Ind. Microbiol. Biotechnol. 43, 45-53.
- Wang, C., Zhang, J., Wu, H., Li, Z., Ye, Q., 2015. Heterologous gshF gene expression in various vector systems in *Escherichia coli* for enhanced glutathione production. J. Biotechnol. 214, 63-68.
- Welch, P., Scopes, R.K., 1985. Studies on cell-free metabolism: Ethanol production by a yeast glycolytic system reconstituted from purified enzymes. J. Biotechnol. 2, 257-273.
- Wu, G., Fang, Y., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489-492.
- Yang, J., Li, W., Wang, D., Wu, H., Li, Z., Ye, Q., 2016. Characterization of bifunctional glutathione synthetases from *Actinobacillus pleuropneumoniae* and *Actinobacillus succinogenes* for efficient glutathione biosynthesis. Appl. Microbiol. Biotechnol. 100: 6279-6289.
- Ye, X., Honda, K., Sakai, T., Okano, K., Omasa, T., Hirota, R., Kuroda, A., Ohtake, H., 2012. Synthetic metabolic engineering-a novel, simple technology for designing a chimeric metabolic pathway. Microb. Cell Fact. 15, 120.
- Ye, X., Wang, Y., Hopkins, R.C., Adams, M.W., Evans, B.R., Mielenz, J.R., Zhang, Y.H.P., 2009. Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails. Chemsuschem 2, 149-52.

- Yoshida, H., Hara, K. Y., Kiriya, K., Nakayama, H., Okazaki, F., Matsuda, F., Ogino, C., Kondo, A., 2011. Enzymatic glutathione production using metabolically engineered *Saccharomyces cerevisiae* as a whole-cell biocatalyst. *Appl. Microbiol. Biotechnol.* 91, 1001-1006.
- Zhang, J., Quan, C., Wang, C., Wu, H., Li, Z., Ye, Q., 2016. Systematic manipulation of glutathione metabolism in *Escherichia coli* for improved glutathione production. *Microb. Cell Fact.* 15, 38.
- Zhang, Y.H.P., 2015. Production of biofuels and biochemicals by in vitro synthetic biosystems: opportunities and challenges. *Biotechnol. Adv.* 33, 1467-1483.
- Zhang, Y.H.P., Sun, J., Zhong, J.J., 2010. Biofuel production by in vitro synthetic enzymatic pathway biotransformation. *Curr. Opin. Biotechnol.* 21, 663-669.
- Zhang, Y., Lyu, F., Ge, J., Liu, Z., 2014. Ink-jet printing an optimal multi-enzyme system. *Chem. Commun.* 50, 12919-12922.

Figure captions:

- Fig. 1. Scheme of two-enzyme cascade for the synthesis of GSH with the ATP-regeneration system.
- Fig. 2. Effects of temperature (A) and pH (B) on the activities of GshFs. GshFSS(◆), GshFSG (■), GshFSU (▲), GshFBC (●). Results are means $\pm$ SD of three parallel replicates.
- Fig. 3. One-pot synthesis of GSH using GshFSS and PPK with 0 mM polyP (◆), 12 mM ATP (■) and 12 mM polyP (▲). Results are means $\pm$ SD of three parallel replicates.
- Fig. 4. Effect of PPK and GshFSS mass ratio on the synthesis of GSH. Wet cells harboring pET28a-*ppk* were changed from 19.5 to 53.8 mg parallel with purified GshFSS from 36.5 decreasing to 2.2 mg. Results are means $\pm$ SD of three parallel replicates.
- Fig. 5. Effect of initial polyP concentrations on GSH synthesis. The reactions were performed with varying concentrations of initial polyP from 5 to 40 mM. Results are means $\pm$ SD of three parallel replicates.
- Fig. 6. GSH synthesis with the fed-batch manner. Arrows indicate the time of polyP and amino acid addition. Results are means $\pm$ SD of three parallel replicates.

Table 1. Properties of GshFs used in this study

Enzyme	Plasmid	Induction conditions	MW (kDa)	Specific Activity (U/mg)
GshFSS	pET28a- <i>gshFss</i>	0.2 mM IPTG for 16 h	84.8	841 ± 26 (45 °C)
GshFSG	pET28a- <i>gshFsg</i>	0.2 mM IPTG for 16 h	84.8	558 ± 7 (45 °C)
GshFSU	pET28a- <i>gshFsu</i>	0.5 mM IPTG for 18 h	85.2	160 ± 16 (42 °C)
GshFBC	pET28a- <i>gshFbc</i>	0.5 mM IPTG for 18 h	86.8	10 ± 0.4 (37 °C)

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.