Use of L-Buthionine Sulfoximine for the Efficient Expression of Disulfide-containing Proteins in Cell-free Extracts of *Escherichia coli*

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Abstract We have developed a technique to improve the formation of correct disulfide bonds within cell-free synthesized proteins. Via the use of a metabolic inhibitor of glutamate-cysteine ligase, the accumulation of glutathione was effectively prevented in cell-free extracts, thereby enabling the stable maintenance of redox potential for extended reaction periods. As a result, in a reaction in which a model protein contatining 9 disulfide bonds was synthesized under cell-free conditions, the final amount of active protein products was increased by 50%. The method presented in this study will provide a rapid and robust route to the high-throughput expression and screening of proteins which require multiple disulfide bonds for their activity. © KSBB

Keywords: cell-free protein synthesis system, disulfide bond, glutathione, L-buthionine sulfoximine, recombinant plasminogen activator, sulfhydryl redox potential, S30 extract

INTRODUCTION

Owing to its excellent speed and flexibility, cell-free protein synthesis is becoming the focus of renewed attention as an alternative method to overcome the limits of conventional *in vivo* gene expression technology [1-6]. In particular, recent advances in our understanding of the *in vitro* metabolism of the key substrates in cell-free extracts have allowed for the development of more efficient and robust protocols for cell-free protein synthesis.

However, the proper folding of the synthesized protein remains one of the principal hurdles to overcome before the practical use of this technique can be seriously considered. In this context, the expression of eukaryotic proteins with multiple disulfide bonds represents a particular challenge, owing principally to the complications involved in the optimization of the reaction conditions for the proper formation of disulfide bonds, as well as the efficient translation of protein molecules.

As described previously [7], the redox potential of reaction mixtures during cell-free protein synthesis cannot be controlled properly by the simple use of redox buffer compounds. Although the initial redox potential of reaction mixtures can be adjusted via the addition of reduced and oxidized glutathione at the appropriate ratio, due to the presence of disulfide-reducing enzymes within the cell-free extracts, the disulfide bonds in the oxidized glutathione (GSSG) as well as in the expressed proteins are easily reduced to free sulfhydryl groups. In a previous report [8], we showed that the reducing activity of the cell-free extracts could be 'exhausted' via the pretreatment of the extract with excess quantities of oxidized glutathione prior to its use for protein synthesis. As the sulfhydryl groups of the active sites are oxidized during the reduction of GSSG, the reducing enzymes became impotent following the pretreatment procedures. As a result, when the pretreated extract was utilized for the cell-free synthesis of recombinant tissue-type plasminogen activator (rPA) in a reaction mix-

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ture buffered with fresh GSH and GSSG, the change in the redox potential was repressed to a marked degree, and active rPA was generated.

However, in a subsequent study in which the change of redox potential was monitored for extended reaction periods, we determined that the GSH concentration continued to increase even in a reaction mixture employing the pre-treated extract. Although the rate of accumulation was substantially lower than that observed in the normal extracts, the increase in GSH concentration was sufficient to affect the formation of disulfide bonds within the cell-free synthesized rPA. Interestingly, it was determined that the increase in the glutathione concentration was not the result of the reduction of GSSG, but rather to a cysteine metabolism catalyzed by glutamate-cysteine ligase present in the cell-free extract. For the better control of redox potential during cell-free reaction, we attempted to prevent the enzymatic conversion of cysteine into glutathione. L-Buthionine sulfoximine (L-BS) was employed as an inhibitor of glutamate-cysteine ligase to block the first step of glutathione generation from cysteine. Upon the addition of an optimal concentration of L-BS, the redox potential of the reaction mixture was maintained at a constant level throughout the reaction periods, which resulted in a substantial augmentation of active rPA production.

MATERIALS AND METHODS

Reagents

Creatine phosphate, creatine kinase, *E. coli* total tRNA mixture, and restriction enzymes were obtained from Roche Applied Science (Indianapolis, IN, USA). L-[U-^{14}C] leucine was acquired from Amersham Biosciences (Little Chalfont, UK). All of the other reagents were obtained from Sigma (St. Louis, MO, USA). Plasmid pIVEX2.4d-rPA, which encodes for the sequence of the recombinant tissue-type plasminogen activator (rPA, Swissprot accession number P00750) was utilized as a template for cell-free protein synthesis [8]. rPA, a truncated version of the human tissue-type plasminogen activator (tPA,) lacks N-terminal amino acids and thus does not harbor the finger, EGF-like, and kringle 1 domains. The ORF of rPA was cloned between the T7 promoter and the T7 terminator of the pIVEX2.4d plasmid (Roche Applied Science) which was digested with NcoI/BamHI. Disulfide isomerase (DsbC) [9] with a C-terminal 6xHistidine tag was cloned from the genomic DNA of *E. coli* strain A19 into the pET28b vector (pET28b-DsbCHis), and purified from the cultures of the transformed cells. The cell-free extracts (hereafter referred to as the S30 extract) were prepared from the *E. coli* strain BL21-Star™(DE3) (Invitrogen, Carlsbad, CA, USA) as previously described [10]. The GroEL/ES-enriched extract was prepared from the BL21-Star™(DE3) strain harboring the T7-SL3 plasmid after inducing the overexpression of the T7 RNA polymerase and GroEL/ES using 1 mM of IPTG [11]. The standard rPA was a generous gift from Roche Applied Science.

Cell-free Protein Synthesis of rPA

The standard reaction mixture for cell-free protein synthesis consisted of the following components in a total volume of 15 μL: 57 mM Hepes-KOH (pH 7.5), 1.2 mM ATP, 0.85 mM each of CTP, GTP, and UTP, 0.17 mg/mL of an *E. coli* total tRNA mixture (from the MRE600 strain), 0.64 mM cAMP, 90 mM potassium glutamate, 80 mM ammonium acetate, 12 mM magnesium acetate, 34 μg/mL L-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 2 mM each of 20 amino acids, 2% PEG (8000), 67 mM creatine phosphate (CP), 3.2 μg/mL creatine kinase (CK), 10 μM L-U-^{14}C] leucine (11.3 GBq/mmol, Amersham Biosciences), 6.7 μg/mL of the plasmid pIVEX2.4d-rPA, and 27% (v/v) S30 extracts. The initial redox potential of the reaction mixture was adjusted via the addition of the mixture of 3 mM reduced glutathione (GSH) and 2 mM oxidized glutathione (GSSG). Depending on the experiments conducted, various concentrations of L-buthionine sulfoximine were utilized. The quantity of the synthesized protein was estimated from the TCA-insoluble radioactivities measured with a liquid scintillation counter (WALLAC 1410) as previously described [12].

Preparation of the Pre-treated S30 Extract

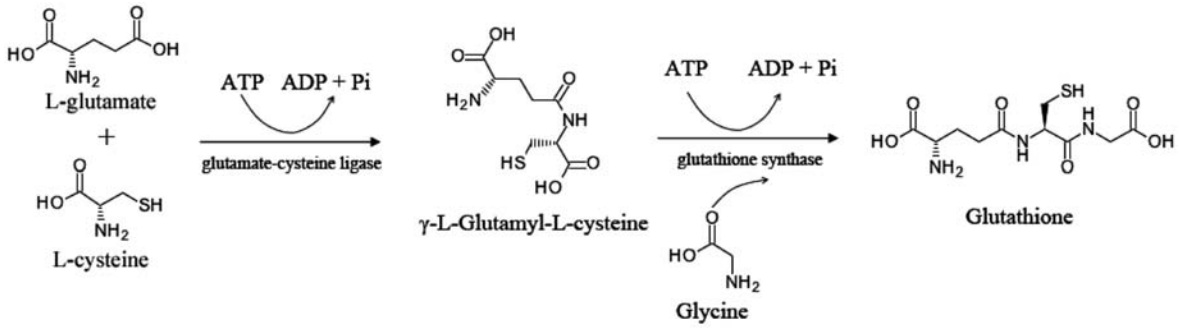
In order to exhaust its reducing activity, S30 extract was incubated with 10 mM oxidized glutathione for 2 h at 30°C. The residual glutathione molecules were removed via the dialysis of the treated extract against 200 volumes of S30 buffer (10 mM Tris-Cl, pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate) for 3 h at 4°C [8].

Activity Assay of the Cell-free Synthesized rPA

Fifteen μL samples were withdrawn during the incubation periods to determine the enzymatic activity of the synthesized proteins. After centrifugation (15000 RCF, 10 min), 10 μL of the supernatant was added to a microplate well containing 80 μL of assay buffer (53 mM NaCl/50 mM Tris-Cl, pH 8.4) and 10 μL of substrate solution (10 mM S-2288; Chromogenix, Italy). The rate of increase in absorbance was measured at a wavelength of 405 nm, and the quantity of active rPA was estimated in comparison with that of standard rPA (a kind gift from Roche Applied Science).

Analysis of Reduced Glutathione and Oxidized Concentration

The quantity of the total glutathione concentration (GSH + GSSG) was measured using a glutathione assay kit purchased from Sigma. Five μL samples of the reaction mixture were mixed with an equal volume of 10% 5-sulfosalicylic acid solution (SSA) to halt the enzymatic reduction reactions. Following centrifugation, 2 μL of the supernatant was diluted in 198 μL potassium phosphate buffer containing 1 mM EDTA (pH 7.0). Ten μL of diluted samples and 150 μL of a working solution (0.17 U/mL of glutathione reductase,



Scheme 1. Proposed pathway for the generation of GSH in the S30 extract

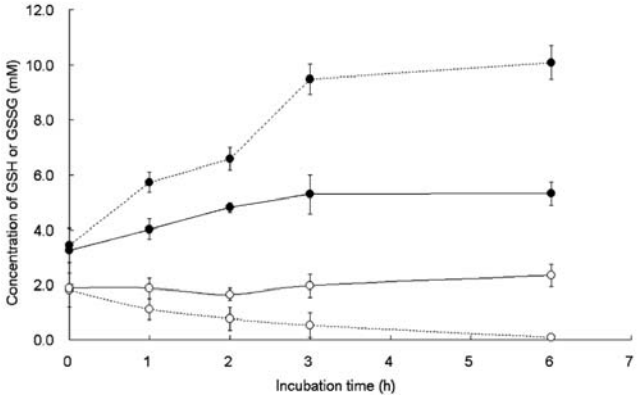


Fig. 1. Time-course analyses of GSH and GSSG concentrations during cell-free synthesis of rPA. Reaction mixture for cell-free rPA synthesis was prepared as described in the Materials and Methods section and incubated at 37°C. Filled circles, GSH; open circles, GSSG; solid lines, reactions using the S30 extract pre-treated with GSSG; dotted lines, reactions using the non-treated S30 extract.

43 μg/mL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 1 mM EDTA in 100 mM potassium phosphate buffer, pH 7.0) was added to 50 μL of 0.16 mg/mL of NADPH solution in a microtiter plate. The change in absorbance was determined at 412 nm and the total glutathione concentration was determined in comparison with a standard curve.

In order to determine the concentration of oxidized glutathione, the supernatant of the mixture of the reaction sample and SSA was treated with 40 μM 2-vinylpyridine to modify the GSH molecules. After 1 h of incubation at room temperature, the assay mixture was neutralized with 1/16 volume of triethanolamine incubated for 10 min at room temperature. The concentration of oxidized glutathione in the sample was determined in comparison with a standard curve of 2-vinylpyridine-treated oxidized glutathione [13].

RESULTS AND DISCUSSION

rPA was expressed in a cell-free protein synthesis system derived from *E. coli* via the incubation of the pIVEX2.4d-rPA plasmid in the reaction mixture, as described in the Materials and Methods section. In accordance with the previous results [8], it was verified that the pretreatment of the S30 extract with an excess quantity (10 mM) of GSSG substantially stabilized the concentrations of GSH and GSSG during protein synthesis. However, as is shown in Fig. 1, although not as dramatic as was seen in a normal extract without GSSG treatment, GSH concentration continued to increase even in the pretreated extract. After 6 h of incubation, the concentration of GSH in the reaction mixture was increased to approximately 5 from 3 mM at the beginning of incubation. We initially hypothesized that the increase in GSH was the result of the reduction of GSSG by reducing enzymes that survived GSSG treatment. If this was the case, the quantity of GSSG in the reaction mixture should have been reduced at half the rate of GSH accumulation. However, intriguingly, the residual GSSG concentration did not evidence significant changes during the incubation periods assessed (Fig. 1), thereby indicating that the increase in GSH concentration was unrelated to the reduction of GSSG.

In their previous report, in order to explain the non-productive depletion of cysteine during cell-free protein synthesis, Kim and Choi hypothesized that cysteine could be metabolized by endogenous enzymes that were present in the S30 extracts [14]. Although experimental data were not presented, it was suggested that the high glutamate concentrations in the reaction mixture drove endogenous glutamate-cysteine ligase to combine cysteine and glutamate to form L-γ-glutamylcysteine (Scheme 1), which was then converted into glutathione via the activity of glutathione synthase.

The results shown in Fig. 2 are well-correlated with that hypothesis. When cysteine was omitted from a complete reaction mixture, the increase in glutathione concentration was not observed. By way of contrast, in the reaction mixtures containing cysteine, the accumulation rate of glutathione was in proportion to the initial cysteine concentration. As the increase in the concentration of GSH would interfere with the formation of disulfide bonds by disturbing the redox environment of the synthesized rPA, it was expected that the inactivation of the enzymes involved in cysteine metabolism would improve the efficiency of disulfide formation within the cell-free synthesized protein. The effects of L-buthionine sulfoximine (L-BS) were assessed, as it

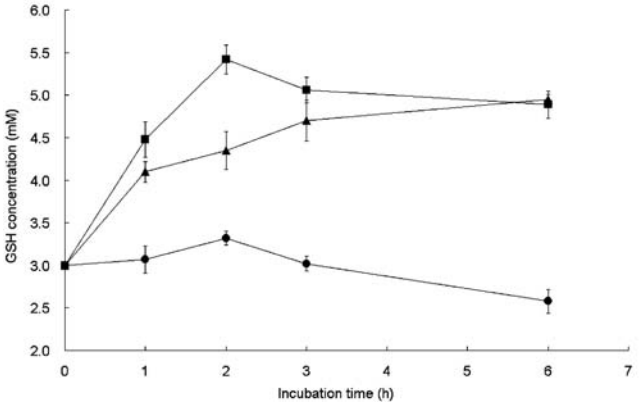


Fig. 2. Effect of initial cysteine concentration on the generation of reduced glutathione. Reaction mixtures for cell-free protein synthesis were prepared with varying initial cysteine concentrations. During the incubation of the reaction mixture at 37°C, aliquots of reaction samples were withdrawn and the residual GSH concentration was measured as described in the Materials and Methods section. Circles, 0 mM cysteine; triangles, 2 mM cysteine; squares, 5 mM cysteine.

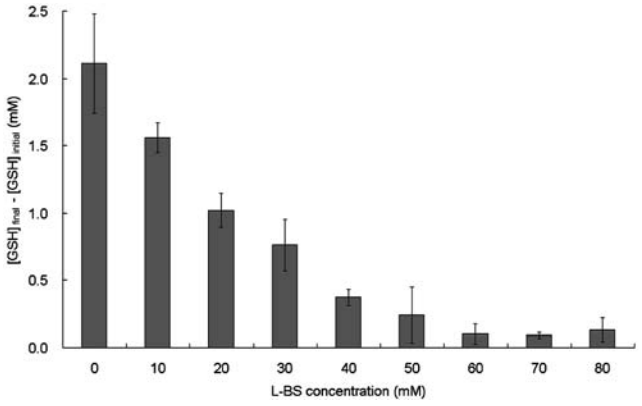


Fig. 3. Effect of L-BS on GSH concentration during the cell-free synthesis of rPA. A complete reaction mixture for cell-free rPA synthesis was incubated for 6 h in the presence of varying L-BS concentrations. After incubation, the residual GSH concentration was measured as described in the Materials and Methods section.

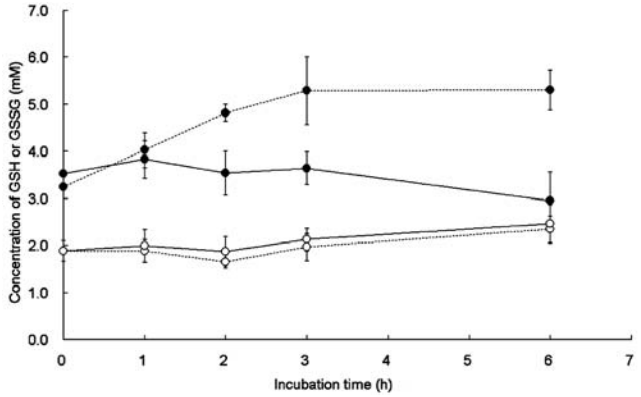


Fig. 4. Stable maintenance of redox potential by L-BS. The time-courses of the GSH (filled circles) and GSSG (open circles) concentrations were monitored during cell-free rPA synthesis in the presence (solid lines) or absence (dotted lines) of L-BS (60 mM). The S30 extracts were pretreated with GSSG prior to their use in the cell-free protein synthesis reactions.

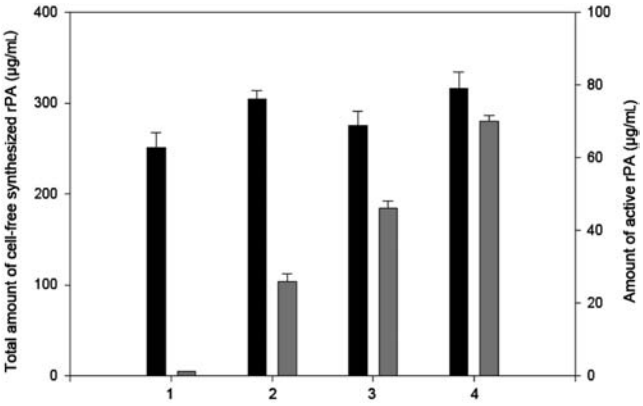


Fig. 5. Effect of L-BS on the active rPA yield. 1, Reaction without redox buffer and DsbC; 2, reaction with redox buffer (2 mM GSSG/3 mM GSH) and DsbC and non-treated extract; 3, reaction with redox buffer, DsbC, and GSSG-treated extract; 4, reaction with redox buffer, DsbC, GSSG-treated extract, and 60 mM L-BS. Black bars, total amount of cell-free synthesized rPA; gray bars, amount of active rPA.

has been identified as a potent inhibitor of glutamate-cysteine ligase [15-17]. As had been expected, the presence of L-BS clearly inhibited glutathione accumulation during cell-free protein synthesis reactions (Fig. 3). GSH accumulation was repressed in proportion to the L-BS concentration. When 60 mM or higher concentrations of L-BS were added to the reaction mixture, the change in glutathione concentration after 6 h of incubation became almost negligible. In addition, the time-course analysis of the reaction mixture showed that the initial ratio of GSSG and GSH was maintained at a stable level for at least 6 h (Fig. 4). Fortunately, although relatively high concentrations of L-BS were required for the complete repression of the generation of GSH, the presence of L-BS did not affect the efficiency of protein synthesis (black bars, Fig. 5). As a result, most likely via the stable maintenance of optimal redox potential, a substantially larger amount of active rPA (gray bars, Fig. 5) was generated in the presence of L-BS. The concentration of active rPA in the completed reaction mixture was approximately 50% higher than was observed in the control reaction without L-BS.

CONCLUSION

We have developed a simple and robust method for the stable maintenance of redox potential during cell-free protein synthesis in an *E. coli* S30 extract. In order to prevent the generation of reduced glutathione via the inhibition of glutamate-cysteine ligase, a cell-free protein synthesis reaction was carried out in the presence of L-BS. As the presence of L-BS effectively repressed the conversion of cysteine into glutathione without affecting the translation efficiency, most likely as the result of the stable maintenance of optimal redox conditions, a substantially higher proportion of protein products was determined to be active. The establishment of an optimal redox environment is crucial for the correct formation of disulfide bonds, and the method presented herein offers a rapid route to the efficient generation of complex eukaryotic proteins containing multiple disulfide bonds.

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