Continuous-exchange cell-free protein synthesis using PCR-generated DNA and an RNase E-deficient extract

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*Though the use of PCR-generated DNA (i.e., linear template) as template DNA is desirable because of its simple preparation, the linear template has not been routinely used in a conventional continuous-exchange cell-free (CECF) protein synthesis system due to the instability of the linear template and/or its transcript in the relatively long operation period. To overcome this problem and enhance soluble protein yield, an RNase E-deficient and molecular chaperone-enriched extract was used: (*i*) for compensating for the decrease in messenger RNA (mRNA) levels transcribed from the unstable linear template with improvement of mRNA stability by depletion of RNase E activity; and (*ii*) for enhancement of the soluble protein portion by assisting of the molecular chaperones. As a result, soluble erythropoietin production from a linear template was significantly enhanced in this modified CECF system using the RNase E-deficient and molecular chaperone-enriched extract, and the amount of soluble erythropoietin was estimated to be roughly 70% of that from a circular plasmid. We can conclude that the use of RNase E-deficient and molecular chaperone-enriched S30 extract mixture is effective in the enhancement of soluble protein expression from a linear template in the CECF system.*

INTRODUCTION

In vitro expression technologies offer significant time savings over cellular approaches and continue to rapidly expand. Moreover, cell-free protein synthesis has become a powerful alternative to cell-based methods in producing proteins on a preparative scale (1,2). Continuous-exchange cell-free (CECF) protein synthesis has been used for the preparative-scale production of proteins from a circular plasmid. In this method, incubation of the translation mixture in a simple dialysis bag was proposed for replenishing substrates and removing low molecular weight byproducts by diffusion exchange across the membrane during protein synthesis. The CECF system based on *Escherichia coli* extracts has recently been launched into the market by Roche Diagnostics GmbH (RTS 500, RTS 9000, RTS ProteoMaster; Mannheim, Germany). Although this RTS ProteoMaster instrument is favorable for obtaining a high yield of protein through controlled shaking and temperature control, the instrument is relatively expensive.

PCR-generated DNA (i.e., linear template) has been routinely used in the cell-free protein synthesis system in a high-throughput format as template DNA because of its simple preparation. However, it still has not been used for preparative-scale protein production due to the significantly lower expression level from it compared with a circular plasmid.

In this article, for the purpose of promoting rapid soluble protein production with a high yield from PCR-generated DNA, a simple CECF system was constructed using an RNase E-deficient and molecular chaperone-enriched extract.

MATERIALS AND METHODS

Preparation of S30 Extracts from BL21 (DE3) and Its Derivative Strains

A normal S30 extract and a molecular chaperone-enriched S30 extract were prepared from *E. coli* BL21 (DE3) and its derivative strains, which have genes of molecular chaperone or disulfide isomerase, as described previously (3). A mixture of the molecular chaperone-enriched S30 extracts was prepared by simple mixing of these extracts. The extracts used for the preparation of the mixture of the molecular chaperone-enriched S30 extract are presented in Table 1. The volumetric mixing ratio of the mixture is as follows:

S30BL:S30BL/Dna:S30BL/GroE:S30BL/DsbC = 67:82:17:1

The volumetric mixing ratio for the preparation of the mixture of a molecular chaperone-enriched extract was determined experimentally to maximize the activity of erythropoietin (EPO) translated as described previously (3).

Preparation of S30 Extracts from BL21 Star (DE3) and Its Derivative Strains

S30 extracts derived from *E. coli* BL21 Star (DE3) [F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm rne131* (DE3)] (Invitrogen, Carlsbad, CA, USA) and its derivative strains, which also have genes for molecular chaperone or disulfide isomerase, were prepared for use as an RNase E-deficient S30 extract and an RNase E-deficient and molecular chaperone-enriched S30 extracts as described previously (3). It was reported that the use of extract derived from BL21 Star (DE3) was favorable in obtaining a

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high yield of protein from a linear template in the batch-type cell-free system (4). A mixture of RNase E-deficient and molecular chaperone-enriched S30 extracts was prepared by simple mixing of the RNase E-deficient S30 extract and RNase E-deficient and molecular chaperone-enriched S30 extracts. The extracts used for the preparation of the mixture of RNase E-deficient and molecular chaperone-enriched S30 extracts are presented in Table 1. The same volumetric mixing ratio for the preparation of the mixture of an RNase E-deficient and molecular chaperone-enriched extract was applied. The volumetric mixing ratio of the mixture is as follows:

S30Star:S30Star/Dna:S30Star/GroE:
S30Star/DsbC = 67:82:17:1

Preparation of Linear Templates for Cell-free Protein Synthesis

To generate the linear templates, two successive PCRs were performed. In the first round of gene-specific PCR, defined overlapping regions with the sequence in the DNA fragments required in the subsequent overlap extension PCR were added to the first-round PCR products using gene-specific primers. In a subsequent overlap extension PCR, the regulatory elements necessary for expression in a prokaryotic system based on T7 polymerase (e.g., the T7 promoter), the ribosomal binding site, and the T7 terminator were introduced into the second-round PCR products. Chloramphenicol acetyl transferase (CAT) and EPO were chosen as model proteins. Gene-specific primers containing both an overlapping region with the sequence in the DNA fragment and gene-specific priming sequence were designed for the first gene-specific PCR (Table 2). To exclude the possibility of expression from the template plasmid of the first gene-specific PCR, the cloning plasmid without any regulatory elements for protein expression or the plasmid derived from a eukaryotic

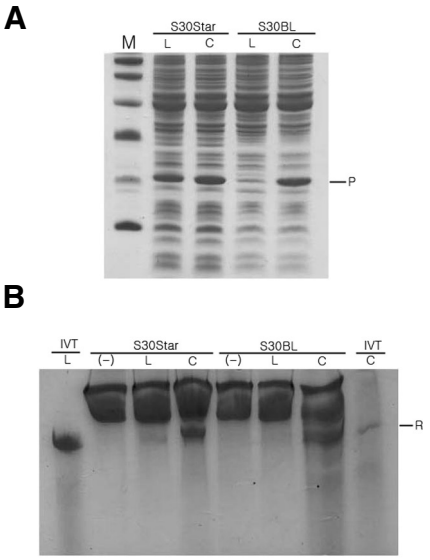

Figure 1. Analysis of the levels of protein yield and comparison of the level of messenger RNA (mRNA) transcript present in the translation mixture. (A) The effect of template type on the level of protein yield was examined in two different extract-utilizing continuous-exchange cell-free (CECF) systems. L and C indicate the linear template and the circular plasmid, respectively. Lane M contains molecular weight markers (14.3, 20.1, 30, 45, 66, and 97 kDa), and the uppercase letter P represents the band corresponding to chloramphenicol acetyl transferase (CAT). (B) Cell-free protein synthesis was performed by the CECF system. IVT indicates the reaction mixture of in vitro transcripts. (-), negative control reactions performed without template DNA; L, linear template; and C, circular plasmid. The uppercase letter R represents the band corresponding to CAT mRNA.

Table 1. Types of S30 Extracts

S30 Extract	Host Strain	Plasmid	Introduced Folding Effectors
S30BL	BL21(DE3)	—	—
S30BL/GroE	BL21(DE3)	T7-SL3	GroEL/ES
S30BL/Dna	BL21(DE3)	T7-KJE3	DnaK/J-GrpE
S30BL/DsbC	BL21(DE3)	pET-28b::DsbC	DsbC
S30Star	BL21 Star (DE3)	—	—
S30Star/GroE	BL21 Star (DE3)	T7-SL3	GroEL/ES
S30Star/Dna	BL21 Star (DE3)	T7-KJE3	DnaK/J-GrpE
S30Star/DsbC	BL21 Star (DE3)	pET-28b::DsbC	DsbC

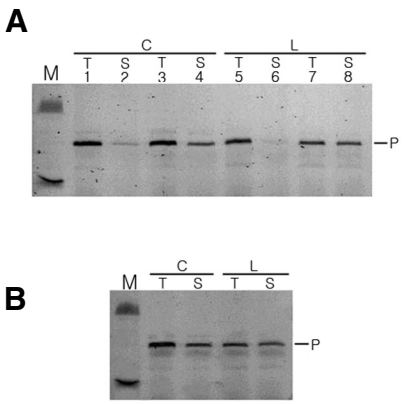

Figure 2. Analysis of soluble protein production in the continuous-exchange cell-free (CECF) systems by fluorescent gel scanning. Erythropoietin (EPO) is a eukaryotic protein that is produced in inclusion bodies when it is expressed in bacteria. (A) For lanes 1 and 2, S30BL; lanes 3 and 4, the molecular chaperone-enriched S30 extract mixture; lanes 5 and 6, S30Star; and lanes 7 and 8, the RNase E-deficient and molecular chaperone-enriched S30 extract mixture were used as an extract. (B) The RNase E-deficient and molecular chaperone-enriched S30 extract mixture was used as an extract. For both panels A and B, C and L indicate the circular plasmid and the linear template, respectively. T, total EPO translated; S, soluble EPO. Lane M contains molecular weight markers (14.3 and 30 kDa), and the uppercase letter P represents the band corresponding to EPO.

Table 2. Gene-specific PCR Primer Sets[a]

Protein	Sense Primer	Antisense Primer
CAT	5′-GTTTAACTTTAAGAAGGAGATATACAT**ATG GAGAAAAAAATCACTGGATATACCA**-3′	5′-CTTTGTTAGCAGCCGGTCGACTTA**CGC CCCGCCCTGCCACTCATCGCAG**-3′
EPO	5′-GTTTAACTTTAAGAAGGAGATATACAT **ATGGGGGTGCACGAATGTCCTG**-3′	5′-CTTTGTTAGCAGCCGGTCGACTTA**TCT GTCCCCTGTCCTGCAG**-3′

CAT, chloramphenicol acetyl transferase; EPO, erythropoietin.
[a]Gene-specific priming sequences are bolded.

expression vector, which cannot support the expression in the prokaryotic expression system, was used in the first gene-specific PCR. The *CAT* gene in pK7-*CAT* (1) was subcloned to pUC 19 using *Nde*I/*Sal*I sites. The plasmid generated was named pUC19-CAT and was used as a template plasmid for the first *CAT* gene-specific PCR. p64T-EPO (5) was used as a template plasmid for the first *EPO* gene-specific PCR.

The first gene-specific PCR was performed in a 50 µL reaction containing 1× PCR buffer (iNtRON Biotechnology, Seongnam-si, Korea), 250 nM each corresponding sense and antisense primer, 50 ng corresponding template plasmid, and 3 U *pfu* DNA polymerase. The first gene-specific PCR was performed using the following profile: 94°C for 4 min, then 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and then a final extension of 72°C for 7 min. The first-round PCR product was directly used in the subsequent second overlap extension PCR without purification.

For the second overlap extension PCR, N- and C-terminal DNA fragments were prepared by oligonucleotide annealing. The sense oligonucleotide for the N-terminal DNA fragment was 5′-CTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAA TAATTTT**GTTTAACTTTAAGAAGGAGATATACAT**-3′, and the antisense oligonucleotide for the N-terminal DNA fragment was 5′-**ATGTATATCTCCTTCTTAAAGTTAAAC**AAAATTATTTC TAGAGGGAAACCGTTGTGGTCTCCCTATAGTGAG-3′. The sense oligonucleotide for the C-terminal DNA fragment was 5′-AGA GGCCCCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCA GCAGCCAACTCAGCTTCCTTTCGGG**CTTTGTTAGCAGCC GGTCGAC**-3′, and the antisense oligonucleotide for the C-terminal DNA fragment was 5′-**GTCGACCGGCTGCTAACAAAG**CCC GAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAA TAACTAGCATAACCCCTTGGGGCCTCT-3′. The overlapping regions with the first gene-specific PCR product are represented in bold, and the overlapping regions with the second overlap extension PCR primer are underlined. After oligonucleotide annealing, without purification, the annealed DNA fragments were diluted to the desired concentration with water.

As a template for the second overlap extension PCR, the first gene-specific PCR product was used. The second overlap extension PCR was performed in a 200 µL reaction containing 1× PCR buffer, 400–650 ng the first gene-specific PCR product, 480 nM each sense outer primer (5′-GCGAAATTAATACGACTCACTATAGGGAG ACCACAA-3′) and antisense outer primer (5′-CAAAAAACCC CTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGC-3′), 12 nM each N- and C-terminal DNA fragment, and 12 U *pfu* DNA polymerase. The overlapping regions with the DNA fragment are underlined. The second overlap extension PCR was performed using the following profile: 94°C for 4 min, then 30 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min, and a final extension at 72°C for 7 min.

CECF Protein Synthesis

Three commercially available plastic wares, Centriprep (Millipore, Billerica, MA, USA), Ultra-15 (Amicon, Billerica, MA, USA), and DispoDialyzer (Spectrum, Rancho Dominguez, CA, USA), were used as a CECF format reactor. These plastic wares have an ultrafiltration membrane (molecular weight cut-off of 10,000) that creates two compartments in the reaction vessel: (*i*) a reaction compartment and (*ii*) a feeding compartment. In all cases, the volume of translation mixture was 500 µL. The composition of translation mixture in the reaction compartment was the same as described previously (3). To

label the proteins translated, when necessary, a FluoroTect Green$_{Lys}$ in vitro Translation Labeling System (Promega, Madison, WI, USA) was used, and this was added only to the translation mixture, not the external buffer. The feeding compartment was filled with 5 mL external buffer. The *E. coli* total transfer RNA (tRNA) mixture, creatine kinase, and extract were not included in the external buffer, because these components cannot pass across the membrane. For expression of CAT and EPO from a circular plasmid, pK7-*CAT* and pK7-s*EPO* were used, respectively. pK7-s*EPO* was constructed by PCR subcloning the human *EPO* gene from p64T-*EPO* to pK7-*CAT* using *Nde*I/*Sal*I sites. The final concentration of a circular plasmid used in the cell-free reaction was 6.7 µg/mL. For expression from a linear template, the second overlap extension PCR product without purification was directly added to a cell-free reaction. The concentration of linear template in a cell-free system is approximately 1.3 µg/mL (approximately at equimolar concentration). After adding a DNA template, the reaction mixture was incubated for 8 h at 37°C.

Analysis of Protein Translated and Amount of mRNA Present in Cell-free Reaction Mixture

The protein translated and the content of soluble protein in a total protein translated were analyzed by fluorescent gel scanning as described previously (3). Messenger RNA (mRNA) present in the cell-free reaction mixture was isolated as described previously (6), and the amount of mRNA was analyzed by urea-polyacrylamide gel electrophoresis (PAGE) (7). In vitro-transcribed mRNA samples were prepared using a Riboprobe in vitro transcription system (Promega).

RESULTS

Comparison of the Level of Protein Yield From a Linear Template in the Different Extract-Using CECF Systems

Three CECF systems were constructed using the commercially available plastic wares including Centriprep, Ultra-15, and DispoDialyzer. Because these CECF systems worked well and no significant difference in the translational activity among these CECF systems was observed, the Centriprep was used as the plastic ware for construction of a CECF system in all subsequent experiments. The effect of the volume of external buffer on the translational activity was examined, and it was negligible in the range of 5–15 mL (data not shown). Therefore, 5 mL external buffer were used in all subsequent experiments.

As shown in Figure 1A, CAT was successfully expressed from a linear template in the CECF system using the RNase E-deficient S30 extract (S30Star). Yet the level of CAT yield from a linear template was significantly decreased in the CECF system using the normal S30 extract (S30BL) (Figure 1A).

To examine the amount of mRNA present in the translation mixture, urea-PAGE was carried out. Although the in vitro transcription level from a linear template was higher than that from a circular plasmid (Figure 1B) and the concentrations of each template applied in cell-free reactions were the same as those in the in vitro transcription, the subsistent amount of mRNA transcribed from a circular plasmid was higher than that from a linear template in any translation mixture (Figure 1B). Also, the subsistent amount of mRNA transcribed from a linear template in the normal S30 extract-based translation mixture was much less than that in the RNase E-deficient S30 extract-based translation mixture (Figure 1B).

Enhanced Production of Soluble Protein with a Linear Template

Conventional prokaryotic cell-free protein synthesis of the aggregation-prone proteins originated from eukaryotes frequently results in rapid aggregation, although to a lesser extent compared with in vivo expression. For enhanced soluble production of aggregation-prone protein, the use of chaperone-enriched cell-free system was reported (3). This chaperone-enriched system was applied to the CECF system. EPO was produced from a linear template in the RNase E-deficient and chaperone-enriched CECF system. Even though the expression of EPO from a linear template was negligible in the normal S30 extract-based CECF system (data not shown), EPO was successfully expressed from a linear template in the RNase E-deficient S30 extract-based cell-free system (Figure 2). The highest amount of soluble EPO was obtained with the RNase E-deficient and molecular chaperone-enriched S30 extract mixture. The obtainable soluble EPO from the linear template was estimated to be roughly 70% of that from the circular plasmid in this RNase E-deficient and molecular chaperone-enriched extract mixture-based CECF system (Figure 2B).

DISCUSSION

In this article, the CECF system was modified for rapid and preparative-scale soluble protein production from PCR-generated DNA. The linear template was prepared through a two-step PCR method based on overlap extension PCR. In this method, DNA fragments are used for eliminating the requirement of a long primer containing both the gene-specific priming sequence and all regulatory elements necessary for transcription and translation. The use of a relatively short primer reduced the formation of primer-dimers compared with the case with a long primer (data not shown). According to this method, regardless of the gene of interest, the same DNA fragments and outer primers required in the second overlap extension PCR can be used, and only the first-round gene-specific PCR primer is dependent upon the gene of interest.

To construct the modified CECF system, the RNase E-deficient and molecular chaperone-enriched extract derived from BL21 Star (DE3) was used as an extract. Because T7 RNA polymerase synthesizes mRNA more rapidly than *E. coli* RNA polymerases, transcription from the T7 promoter is uncoupled from translation in the *E. coli*-based cell-free system. This results in mRNA transcripts unprotected by ribosomes, which are then subject to enzymatic degradation by endogenous RNases (8). The *E. coli* BL21 Star (DE3) strain contains a mutation in the gene encoding RNase E (*rne*131), which is one of the major sources of this mRNA degradation (9), and this strain significantly improves the stability of mRNA transcripts and increases protein expression yield from T7 promoter-based vectors. The use of the RNase E-deficient extract derived from BL21 Star (DE3) significantly increased the obtainable amount of protein from a linear template (Figure 1). This indicates that the depletion of RNase E activity from an extract is effective in the enhancement of protein yield from a linear template.

In the conventional cell-free system using a normal S30 extract, the severely reduced level of protein yield from a linear template is a common drawback (Figure 1A), and this may come from both the instability of a linear template and the degradation of its

transcript by RNase E. As shown in Figure 1B, although the same kind of S30 extract was used, the subsistent amount of mRNA transcribed from a circular plasmid was higher than that from a linear template in any translation mixture, indicating that the reduced subsistent amount of mRNA is basically caused by the instability of a linear template in the translation mixture. Although the same template was used, the amount of mRNA present in the normal S30 extract-based translation mixture compared with RNase E-deficient S30 extract-based translation mixture was smaller (Figure 1B). This smaller quantity of mRNA present in the translation mixture, including a normal S30 extract, may be mainly due to degradation by RNase E.

This enzymatic degradation of mRNA by RNase E seems to give a severe reduction in protein expression yield in the normal S30 extract-based cell-free system, because this normal S30 extract-based translation mixture has a much higher RNase E activity compared with the RNase E-deficient S30 extract-based translation mixture. The more efficient expression with a circular plasmid compared with a linear template in the normal S30 extract-based cell-free system can be explained as follows. Commonly, there is a saturated level of mRNA for efficient in vitro protein expression. At higher mRNA levels than the saturated point, as long as the subsistent mRNA level is higher than the saturated point, the level of protein yield is not critically concerned with a decrease in the level of mRNA by RNase E activity. However, at lower mRNA levels than the saturated point, the level of protein yield is significantly dependent upon a decrease in the level of mRNA by RNase E activity. The level of mRNA transcribed from a linear template is much lower than that from a circular plasmid due to the instability of a linear template in the translation mixture, and the mRNA level with a linear template may be below the saturated mRNA level. Therefore, the effect of RNase E on decreased protein expression yield is more severe in the case with a linear template than with a circular plasmid. This assumption is plausible in the case with the normal S30 extract-based cell-free system using a linear template, and it is in accord with the results presented in Figure 1. We can conclude that the use of RNase E-deficient extract is effective in the enhancement of protein expression from a linear template in the CECF system through providing high levels of mRNA present in the translation mixture.

For this article, to increase the obtainable amount of soluble protein from the linear template, the RNase E-deficient and molecular chaperone-enriched extract, which contains several molecular chaperones expressed endogenously not supplemented exogenously, was used as an extract. This resulted in the enhanced soluble protein production through increasing the soluble protein content (Figure 2). It indicates that the enhancement of the soluble protein portion by the assisting of molecular chaperones is also effective in obtaining a high yield of soluble protein from a linear template in the CECF system.

This modified CECF system can be applicable to rapid and preparative-scale soluble protein preparation from a linear template for characterization studies, functional assays, or structural analysis.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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