Development of a Rapid and Productive Cell-free Protein Synthesis System

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Abstract Due to recent advances in genome sequencing, there has been a dramatic increase in the quantity of genetic information, which has lead to an even greater demand for a faster, more parallel expression system. Therefore, interest in cell-free protein synthesis, as an alternative method for high-throughput gene expression, has been revived. In contrast to *in vivo* gene expression methods, cell-free protein synthesis provides a completely open system for direct access to the reaction conditions. We have developed an efficient cell-free protein synthesis system by optimizing the energy source and S30 extract. Under the optimized conditions, approximately 650 μg/mL of protein was produced after 2 h of incubation, with the developed system further modified for the efficient expression of PCR-amplified DNA. When the concentrations of DNA, magnesium, and amino acids were optimized for the production of PCR-based cell-free protein synthesis, the protein yield was comparable to that from the plasmid template.

Keywords: cell-free protein synthesis, PCR, ATP regeneration

INTRODUCTION

With various genome sequencing projects having been completed in recent years, the focus of genomics has now shifted toward the large-scale study of gene expression and function for the integrated understanding of the cellular phenomena at the molecular level. In many cases, the exact role of a genetic sequence needs to be explored through the analysis of its translation product. Accordingly, efficient methods for translating genetic information into protein molecules are required for post-genomic studies to progress.

Due to the presence of well-developed expression systems and purification strategies, *in vivo* protein expression in bacteria has been widely used for the production of recombinant proteins. However, many of the *in vivo* expression steps, including gene cloning, transformation and cell culture, require substantial time and labor. In addition, even if all these steps can be automated and minimized, the intrinsic requirement for cell cultivation imposes a limit on the speed of translating genetic sequences into protein molecules. For this reason, the current *in vivo* expression technology is being outpaced by the exponential growth in sequence information, and such a gap will further widen unless more rapid, parallel protein expression methods are implemented.

In contrast to *in vivo* expression through the direct use of the prepared translational machinery, cell-free protein synthesis by-passes the cell culture steps; thus, greatly reduces the time required to express DNA templates. Since the pioneering work of Nirenberg and Matthaei [1], cell-free protein synthesis has been used as a versatile tool for the analysis of translational process and for the small scale preparation of protein molecules. It also carries a potential to be used for studying the effectors of protein synthesis in a miniaturized format [2-4]. Even though the universal application of cell-free protein synthesis has been limited by the extremely low productivity, the pressing demand in recent years for a high-throughput protein synthesis has motivated many researchers to improve its productivity and reproducibility [5-11]. In this study, the reaction conditions of an *E. coli*-based cell-free protein synthesis system was extensively optimized with respect to the source of S30 extract, energy source for the regeneration of ATP and salt conditions. As a result, a highly productivity cell-free protein synthesis system has been constructed, producing approximately 650 μg/mL of protein product.

In addition, in order to further improve the production of cell-free protein, attempts were made to directly express PCR-amplified genes in the cell-free synthesis system. With the concentrations of template DNA, amino

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acids and magnesium ion optimized, chloramphenicol acetyltransferase (CAT) was expressed at with a productivity almost comparable to that of the plasmid-based reaction. By eliminating the time-consuming steps required for preparation of the template, without compromising the productivity, the proposed method provides a useful platform for the rapid preparation of protein species.

MATERIALS AND METHODS

Materials

The ATP, GTP, UTP, CTP, creatine phosphate, creatine kinase, and *E. coli* total tRNA mixture were purchased from Roche Applied Science (Indianapolis, IN, USA). The L-[U-^{14}C]leucine (11.9 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). *E. coli* strains BL21(DE3) and Rosetta were from Novagen (Madison, WI, USA). All the other reagents were purchased from Sigma. The S30 extract was prepared as previously described [10], with minor modifications. In the reactions using the S30 extract from BL21(DE3) and Rosetta, instead of its exogenous addition, T7 RNA polymerase was expressed during the cultivation of *E. coli* with IPTG induction (1.0 mM) at 0.5 OD_{600}. Cells were harvested 2 h after induction, and used for preparation of the S30 extract.

DNA Templates for Cell-free Protein Synthesis

The plasmid pIVEX2.3CAT, carrying the nucleotide sequence of bacterial chloramphenicol acetyltransferase (CAT) under the control of the T7 promoter, was used as the template for cell-free protein synthesis. For the experiments involving PCR-based cell-free protein synthesis, the pIVEX2.3CAT was amplified with the primer sequences 5′-TCGATCCCGCGAAATTAATACGACTCACTATAGG-3′ (forward) and 5′-CAGCTTCCTTTCGGGCTTTGTTA-3′ (backward), respectively. The PCR products were purified using a PCR-clean up kit (Promega, Madison, WI, USA) prior to their use for protein expression. The amounts and sizes of the PCR products were determined using spectrophotometry and agarose gel electrophoresis.

Cell-free Protein Synthesis and Analysis

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 8.2, 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 1.7 mM dithiothreitol, 80 mM ammonium acetate, 0.17 mg/mL *E. coli* total tRNA mixture (from strain MRE 600), 34 μg/mL L-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 1.5 mM each of the unlabeled amino acids, 0.3U/mL creatine kinase, 67 mM creatine phosphate and 4 μL S30 extract. Depending on the experiment, 0.1 μg plasmid or 0.5 μg PCR-products were used as the templates to direct the protein synthesis. The synthesis reactions were conducted for 2 h in a water bath at 37°C. The synthesized protein was quantified by measuring the TCA-precipitable radioactivities, as described previously [10]. The molecular weight of the expressed protein was confirmed using a 10% Tricine-SDS-polyacrylamide gel [12].

RESULTS AND DISCUSSION

Creatine Phosphate Provides an Enhanced Productivity of Cell-free Protein Synthesis

Phosphoenolpyruvate (PEP) has been the most widely used energy source for the regeneration of ATP in cell-free systems derived from *E. coli* [8]. Conversely, with a few exceptions, creatine phosphate (CP) has been used for ATP regeneration in most systems based on extracts from eukaryotic cells [13,14]. In this study, initially PEP and CP were compared for their efficiency to support ATP regeneration and protein synthesis in the reaction mixture utilizing *E. coli* (A19) S30 extract.

In the presence of 12 mM magnesium acetate, PEP and CP showed different optimal concentrations (33 and 67 mM, respectively). Interestingly, the protein synthesis reached a plateau at 67 mM CP and leveled off, whereas PEP showed a narrow concentration range for maximal protein synthesis, with a sharp decrease observed at higher concentrations (Fig. 1A). Although the final productivity at the optimal CP concentration was similar to that obtained with PEP, the optimal productivity over a wide range of magnesium concentrations offers an advantage for its use as a universal system for the expression of different proteins (it should be noted that the optimal concentration of protein expression varies significantly depending on the species of DNA coding for the target protein).

In addition, a time course analysis of the ATP level indicated that a more stable supply of ATP was achieved with CP (Fig. 1B). This implies that the productivity of protein synthesis can be further improved by optimizing the other reaction parameters. Although the use of higher PEP concentrations also improved the ATP supply (data not shown), as described above, the inhibition of protein synthesis at increased PEP concentrations limited prolonged protein synthesis. It has been postulated that the high chelating constant of PEP in relation to the magnesium ion concentration caused the sequestration of free magnesium ions from the reaction mixture. Based on these results, 67 mM CP was used for the regeneration of ATP during the protein synthesis in the following experiments.

Effect of the Source of the S30 Extract on the Efficiency of Protein Synthesis

The efficiency of protein synthesis in a cell-free synthesis system showed remarkable dependency upon the strain of *E. coli* from which the S30 extract was prepared. For example, compared to the control reaction, where protein synthesis was catalyzed by the S30 extract from

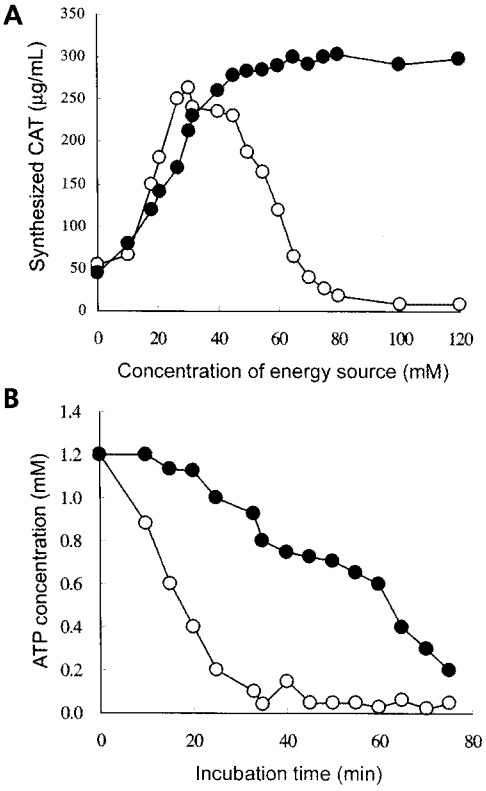

Fig. 1. The optimization of the energy source concentration, as well as the time-course analysis of the ATP concentration for different energy sources. (A) different concentrations of PEP (open circles) or CP (closed circles) were used for the ATP regeneration within a reaction mixture for cell-free protein synthesis. In all of the reactions, the magnesium concentration was fixed at 12 mM. After 2 h of incubation, the quantity of synthesized protein was estimated by measuring the TCA-insoluble radioactivites of the reaction samples. (B) 5 μL samples were taken at the indicated times, and the amounts of residual ATP estimated, as described in Materials and Methods. Reactions were conducted in the presence of 33 mM PEP (open circles) or 67 mM CP (closed circles) as the energy source.

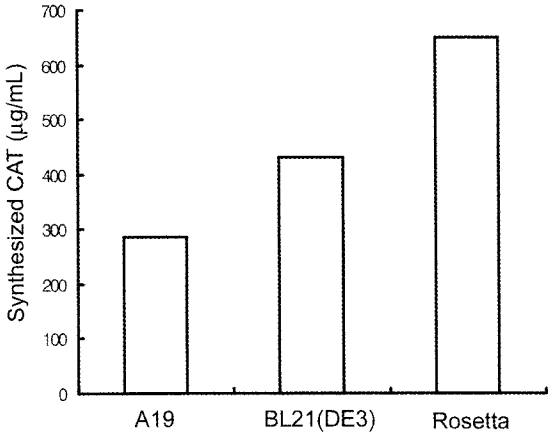

Fig. 2. Dependency of protein synthesis upon the source of S30 extract. S30 extracts were prepared from the indicated *E. coli* strains, and the efficiency of protein synthesis examined in the standard reaction mixture containing 67 mM CP.

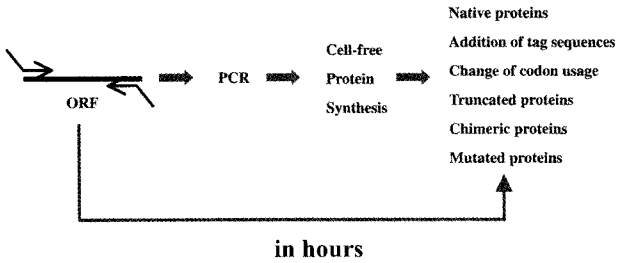

Fig. 3. Possible applications of the PCR-based cell-free protein synthesis.

the strain A19, under the same reaction conditions, the S30 extract from the strain BL21(DE3) gave a significantly increased production of CAT (Fig. 2). In addition, the protein synthesis was further improved when the extract from the Rosetta strain, which carries the same genetic background as BL21(DE3), but harbors the plasmids coding for rare tRNAs [pRARE (argU, argW, ileX, glyT, leuW, and proL)], was used, indicating that a sufficient presence of rare tRNAs is also important for improving the productivity of the cell-free synthesis (Fig. 2). As a result, with the use of the S30 extract from Rosetta, with the optimal concentration of CP, approximately 650 μg/mL of CAT was produced in 2 h.

Optimization of Reaction Conditions for the Protein Synthesis Directed by PCR Products

As described above, the optimization of the energy source and S30 extract led to remarkable enhancement of the protein synthesis in a cell-free system, which can rapidly generate hundreds of micrograms (per milliliter) of protein products. However, even though cell-free protein synthesis greatly shortens the time for the translation of genetic information into proteins, the growing of cells for the cloning and amplification of the DNAs is still required as long as the synthesis reaction is directed by plasmid templates. Time- and labor-intensive steps of plasmid preparation cause a discrepancy between the template preparation and translation of the prepared templates. The direct use of PCR-amplified genes as templates for protein synthesis will solve this problem. The rapid preparation of the expression templates, based on PCR methods, and their subsequent expression in a cell-free synthesis system will eliminate the requirement of cell growth for template amplification as well as protein expression. Furthermore, the rapid amplification of modified target genes and their direct expression will accelerate the generation of various mutant proteins, which will greatly enhance the throughput of protein engineering and screening (Fig. 3). However, even though several reports have described the successful cell-free expression of PCR products [15-17], the efficiency of cell-free synthesis is generally very low when linear templates are used as the expression templates.

In this study, attempts were made to improve the pro-

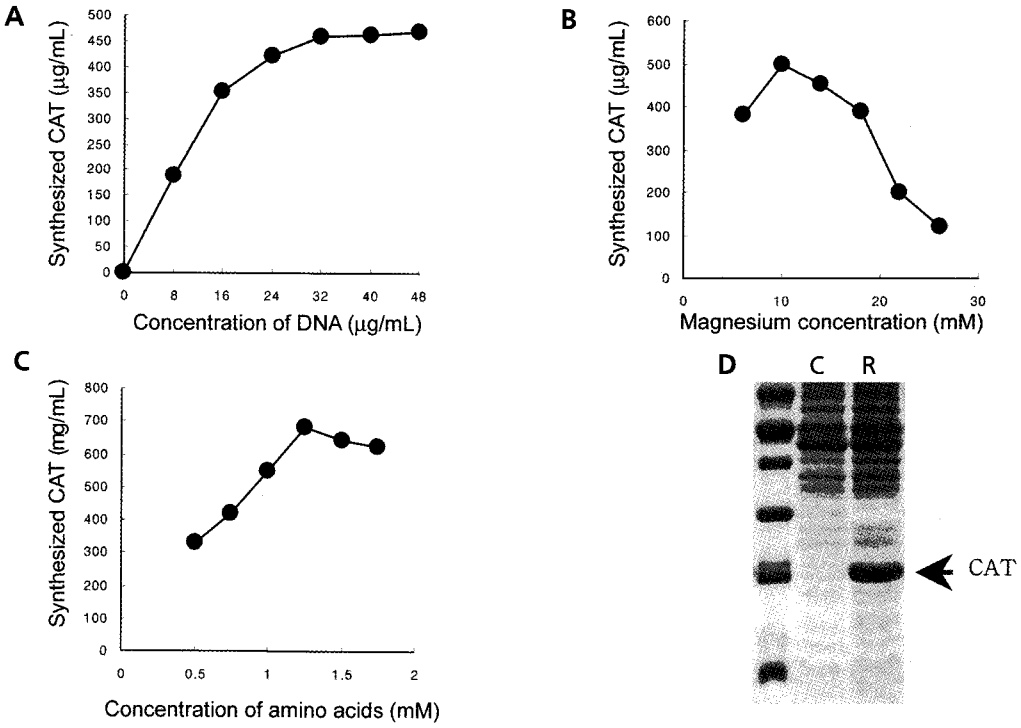

Fig. 4. Optimization of the PCR-based cell-free protein synthesis in relation to the concentrations of DNA, magnesium, and amino acids. The template for cell-free protein synthesis was prepared by PCR from plasmid pIVEX2.3CAT, as described in Materials and Methods. (A) in the presence of 10 mM magnesium acetate and 1 mM amino acids, different concentrations of the PCR-product were added to the reaction mixture, and the quantity of cell-free synthesized protein analyzed. (B) in the presence of 32 μg/mL DNA and 1 mM amino acids, different concentrations of magnesium acetate were added to the reaction mixture, and the final quantity of synthesized protein analyzed. (C) in the presence of 32 μg/mL DNA and 12 mM magnesium acetate, different concentrations of amino acids were added to the reaction mixture, and the quantity of cell-free synthesized protein analyzed. (D) the quantity of cell-free synthesized protein in the presence of 32 mg/mL DNA, 12 mM magnesium acetate and 1.25 mM amino acids was analyzed on a SDS-PAGE gel (lane R), as described in Materials and Methods. Lane C: control reaction without the DNA template. Synthesized protein (CAT) is indicated by an arrow.

ductivity of PCR-based cell-free protein synthesis through extensive optimization of the reaction conditions. Among the reaction parameters examined; shown in Fig. 4, the concentrations of DNA, magnesium and amino acids had significant effects on protein synthesis in the cell-free system; their optimum values were quite different from those in the plasmid-based reaction. For example, the quantity of protein synthesized continued to increase in proportion to the amount of PCR-amplified DNA added; upto 32 μg/mL, while the protein synthesis was leveled off on the addition of more than 6 μg/mL of plasmid DNA. The optimal concentration of magnesium (10 mM) was substantially lower than with the plasmid templates (16 mM). In the presence of optimal concentrations of these components, the productivity of the PCR-based cell-free synthesis reactions became comparable to that of the conventional reaction directed by plasmid templates, which was a rather unexpected. We note that substantially larger amounts of the PCR products were required to obtain the maximal yield, and assumed the presence of an excessive amount of PCR products compensated for their degradation in the cell-free extract.

We expect that the system develop in this study will find versatile application in various field of post-genomics research where the rapid, parallel expression of protein molecules is required.

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