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Rapid determination of effective folding agents by sequential cell-free protein synthesis

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ABSTRACT

Cell-free protein synthesis enables the direct translation of genetic information into the corresponding proteins, and thus provides a powerful platform for functional assays of protein-coding genes. In this study, taking advantage of the configurational flexibility of cell-free protein synthesis, we developed a method to study the effect of molecular chaperones on the functional synthesis of recombinant proteins. After the synthesis of a series of molecular chaperones, the reaction mixture for cell-free protein synthesis was reprogrammed for synthesis of *Candida antarctica* lipase B (CalB). The effect of a pre-synthesized molecular chaperone on protein folding was determined by measuring the enzymatic activity of CalB synthesized in the second reaction. Using this sequential expression experiment, we could rapidly determine the molecular chaperones that effectively assisted the functional synthesis of CalB. We believe that the presented strategy will provide a versatile platform for the optimal production of functional proteins, and can also be extended to studies of other interacting proteins.

1. Introduction

The preparation of properly folded recombinant proteins is a fundamental issue across the diverse fields of modern biotechnology. Although the final structure of a nascent polypeptide is primarily determined by its amino acid sequence, various effectors including molecular chaperones and foldases often affect protein folding [1,2]. However, there are no generic rules for determining the appropriate molecular chaperones, and the choice of molecular chaperones for a given target protein often involves laborious co-expression experiments using a panel of known molecular chaperones in a trial-and-error manner.

Cell-free protein synthesis has become widespread in recent years due to its unique advantages of speed and configurational flexibility [3–5]. While conventional batch-type cell-free synthesis systems can only provide analytical amounts of proteins, introduction of dialysis reactors markedly improved the productivity of cell-free protein synthesis. In this reaction scheme, which was originally termed semi-continuous cell-free protein synthesis [6] and now is more commonly referred to as continuous exchange cell-free (CECF) protein synthesis [7], protein synthesis is conducted inside a membrane chamber that is immersed in a feeding solution containing the small molecular substrates required for protein synthesis. As a result of the continuous replenishment of substrates together with the simultaneous removal of byproducts through the dialysis membrane, a CECF reaction routinely continues for several hours with a linear increase in protein accumulation [8–10].

Protein synthesis in a CECF reactor demonstrates that the translational machinery remains functional for a long duration if the initial biochemical conditions are maintained. In this study, as a different approach, we demonstrate that the translational machinery in a once-terminated batch reaction can be re-activated for another round of protein synthesis, by restoring the biochemical environment. When the small molecules in a terminated cell-free synthesis reaction were replaced with fresh substrates by diafiltration, the protein synthesis machinery started to generate proteins again, without a significant loss of translational activity. Using this approach, we sequentially expressed two separate genes to investigate how the protein produced in the first reaction affected the folding of the protein produced in the second reaction. As an exemplary model, *Candida antarctica* lipase B (CalB) was expressed in tandem with various molecular chaperones. While it may appear to be a simpler approach to co-express a molecular chaperone and the target protein, co-expression of multiple genes in a cell-free synthesis system often results in heavily biased protein synthesis [11], and the molecular chaperone and target protein may not both be

Abbreviations: CalB, Candida antarctica lipase B; CECF, continuous exchange cell-free; DsRed, Discosoma sp. red fluorescent protein; pNPP, p-nitrophenyl palmitate; sfGFP, superfolder green fluorescent protein.

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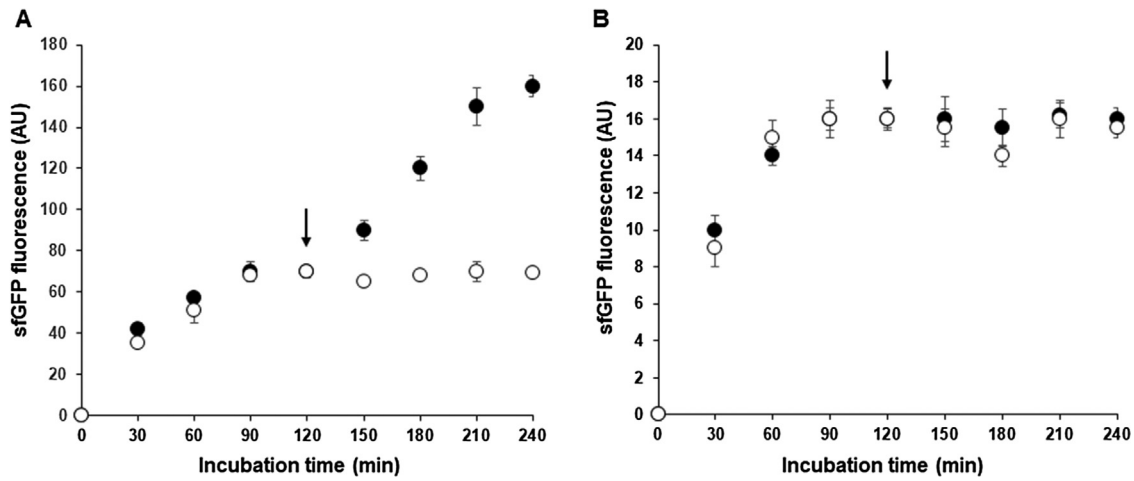

Fig. 1. Reactivation of translational machinery by diafiltration. After incubation of the reaction mixture primed with the plasmid pK7sfGFP (A) or the PCR-amplified sfGFP gene (B) for 2 h, diafiltration was performed to replenish the small molecular components as described in Section 2.3. The time-course of sfGFP fluorescence was compared between reactions with (filled circles) and without (open circles) the diafiltration step. Arrows indicate the time points for the diafiltrational exchange of reaction components. Measurements were performed in triplicate, and the error bars in the graph represent 95% confidence interval.

efficiently synthesized in the same reaction mixture. Indeed, analyses of CalB expressed in a series of sequential reactions identified the molecular chaperones that markedly improved the functional production of this enzyme. The presented method will provide a versatile platform for screening diverse trans-acting effectors that enhance the functional production of various proteins of interest.

2. Materials and methods

2.1. Materials

ATP, GTP, UTP, CTP, creatine phosphate, creatine kinase and the *E.coli* total tRNA mixture were purchased from Roche Applied Science (Indianapolis, IN, USA). Oligonucleotides for PCR were synthesized by Macrogen (Seoul, Korea). The S12 extract was prepared as described previously [12]. All other reagents were purchased from Sigma (St Louis, MO, USA).

2.2. Preparation of expression templates

Protein-coding sequences of target genes were cloned in the pK7 plasmid between the NdeI/SalI sites [13]. In the experiments for sequential cell-free protein synthesis reactions, the templates for the first reaction were prepared by PCR of the plasmids using the primers targeting the T7 promoter and the twenty nucleotides next to the stop codon (TAA) of the protein-coding sequences. On the other hand, plasmid-cloned genes were directly used as the templates for the second synthesis reactions.

2.3. Cell-free protein synthesis reactions

The standard reaction mixture for cell-free protein synthesis consisted of the following components in the final volume of 150 μL; 57 mM HEPES–KOH (pH 8.2), 1.2 mM ATP, 0.85 mM each of CTP, GTP, and UTP, 2 mM DTT, 0.17 mg/mL *E. coli* total tRNA mixture (from the *E. coli* strain MRE600), 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, 1.5 mM each of 20 amino acids, 2% PEG (8000), 67 mM creatine phosphate, 3.2 μg/mL creatine kinase, and 40 μL of the S12 extract. In addition, 2 μg of plasmid-cloned genes or 10 μg of PCR-amplified genes were used as the template. Cell-free synthesis reactions were conducted in micro tubes in an incubator set at 30 °C for 2 h. For sequential cell-free protein synthesis, the reaction mixture was diafiltered in a centrifugal device (Ultra-0.5 centrifugal filter, Merck Millipore; Billerica, MA, USA) after the first synthesis reaction. After dilution with two volumes of the S12 buffer (10 mM Tris-acetate, pH 8.2, 14 mM magnesium acetate, and 60 mM potassium acetate), the diluted reaction mixture was concentrated to 40 μL (the original volume of the S12 extract in the reaction mixture). After repeating this step three times, the washed translational machinery was supplemented with the second template along with the fresh reaction components in a final volume of 150 μL. To express CalB during the second reaction, a mixture of 4 mM oxidized (GSSG) and 1 mM reduced (GSH) glutathione was added to the reaction mixture in order to provide an oxidative redox environment for the formation of disulfide bonds.

2.4. Analyses of cell-free synthesized proteins

To quantify cell-free synthesized proteins, 0.01 mM L-[U-^{14}C] leucine (11.1 GBq/mmol) was included in the reaction mixture, and trichloroacetic acid-precipitated radioactivity was measured using a liquid scintillation counter (Wallac 1410; PerkinElmer, Waltham, MA, USA), as described previously [13]. The fluorescence of cell-free synthesized superfolder green fluorescent protein (sfGFP) or *Discosoma* sp. red fluorescent protein (DsRed) was measured using a fluorescence micro-plate reader (Victor X2, PerkinElmer). The enzymatic activity of cell-free synthesized CalB was determined using *p*-nitrophenyl palmitate (*p*NPP) following previously described procedures [14].

3. Results and discussion

3.1. Reactivation of translational machinery by diafiltration

In general, cell-free protein synthesis is only sustained for a few hours in a batch reaction. When sfGFP was synthesized from a plasmid-cloned gene in a cell-free protein synthesis system, the rate of protein synthesis began to decrease after 10 min, and plateaued within 2 h. The termination of protein synthesis in a batch reaction can be attributed to various reasons, including substrate depletion, accumulation of inhibitory by-products and/or inactivation of the translational machinery in the cell extract. However, after a first reaction period lasting 2 h, sfGFP synthesis resumed when the small molecules in the reaction mixture were