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Synthesis of milligram quantities of proteins using a reconstituted *in vitro* protein synthesis system

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In this study, the amount of protein synthesized using an *in vitro* protein synthesis system composed of only highly purified components (the PURE system) was optimized. By varying the concentrations of each system component, we determined the component concentrations that result in the synthesis of 0.38 mg/mL green fluorescent protein (GFP) in batch mode and 3.8 mg/mL GFP in dialysis mode. In dialysis mode, protein concentrations of 4.3 and 4.4 mg/mL were synthesized for dihydrofolate reductase and β-galactosidase, respectively. Using the optimized system, the synthesized protein represented 30% (w/w) of the total protein, which is comparable to the level of overexpressed protein in *Escherichia coli* cells. This optimized reconstituted *in vitro* protein synthesis system may potentially be useful for various applications, including *in vitro* directed evolution of proteins, artificial cell assembly, and protein structural studies.

[**Key words:** *In vitro* protein synthesis; PURE system; Green fluorescent protein; Dialysis; Synthetic biology]

Cell-free protein synthesis enables *in vitro* protein synthesis without the requirement of living cells (1–3). Because the cell-free system is independent of cell growth, the synthesis of proteins that may affect cell growth is possible. Furthermore, rapid protein production is possible using a cell-free system, as protein synthesis typically requires only a few hours. Because of these favorable properties, cell-free systems have been used for various applications and basic research.

Among cell-free systems, the PURE system is the only fully reconstituted system in which the minimal components for protein translation are individually purified and reconstituted *in vitro* (4,5). Using this reconstituted system, components can be added or omitted, and the concentration of each component can be altered. This system has enabled the efficient incorporation of unnatural amino acids into peptides (6,7). Because of the inherently low nuclease and protease activity, this system has been used for the *in vitro* evolution of proteins and enzymes (8–10). An additional advantage of the PURE system is the lack of amino acid scrambling, which enables nuclear magnetic resonance structural studies (11,12). However, despite its wide application, there have been few reports on the enhancement of the yield of proteins synthesized using this reconstituted *in vitro* protein synthesis system (13,14).

In this study, we report the optimization of the PURE system, which resulted in the synthesis of greater than 4 mg/mL protein. This optimization was achieved by modifying the component concentrations and utilizing the system in dialysis mode. We have improved the protein yield of this system by including additional components and altering the component concentrations (15,16). Attempts to improve the yield of *in vitro* protein synthesis have been described (13,14). However, the development of a reconstituted *in vitro* protein synthesis system capable of synthesizing a protein at concentrations of greater than 1 mg/mL has not yet been achieved. We evaluated the performance of the optimized system using three different sets of component concentrations determined from the optimization process and demonstrate that milligram quantities of protein can be synthesized.

MATERIALS AND METHODS

DNA and RNA preparation The plasmid DNA used for the *in vitro* transcription-translation system was purified using the Qiagen Plasmid Mini Kit (Qiagen) according to the manufacturer's instructions. Under the control of a T7 promoter, pETG5tag encoding green fluorescent protein (GFP), pET-dhfr (Gene Frontier) encoding dihydrofolate reductase (DHFR), and pET-bgal encoding β-galactosidase were used as expression plasmids. For *in vitro* transcription, pETG5tag was amplified using the primers T7F (5′-TAATACGACTCACTATAGGG-3′) and T7R (5′-GCTAGTTATTGCTCAGCGG-3′) and KOD FX DNA polymerase (Toyobo, Osaka, Japan). The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. RNA was prepared from this PCR product using the MEGAscript T7 Kit (Life Technologies) and purified using an RNeasy kit (Qiagen).

Component preparation Versions 1 and 2 of the system and their components were prepared as previously described (5,15). The protein components constituting version 7 of the system were prepared with additional treatment. Components, with the exception of the ribosome, were stored in stock buffer (50 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 30% glycerol, and 7 mM 2-mercaptoethanol), and the ribosome was stored in 70S buffer (20 mM HEPES-KOH, pH 7.6, 6 mM $Mg(OAc)_2$, 30 mM KCl, and 7 mM 2-mercaptoethanol). All of these components were dialyzed against buffer A (50 mM HEPES, pH 7.6, 10 mM

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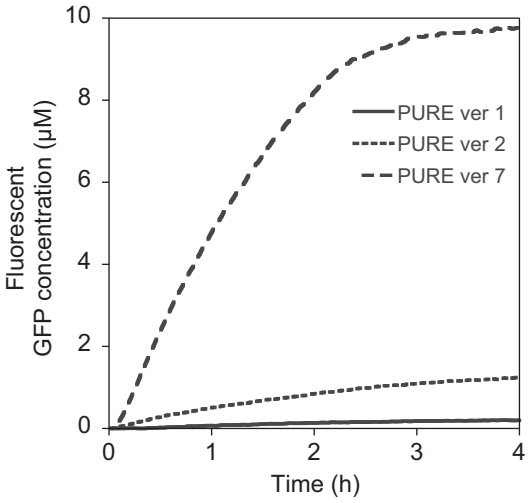

FIG. 1. GFP synthesis using various versions of the reconstituted *in vitro* protein synthesis system. Time course of GFP synthesis in batch mode. RNA was used as a template at a concentration of 200 nM. GFP concentrations were determined from fluorescence intensities using the relationship between the fluorescence and concentration of GFP purified from *E. coli*. Purified GFP was assumed to be 100% active.

$Mg(OAc)_2$, 100 mM potassium glutamate (K-Glu), and 7 mM 2-mercaptoethanol) using a microscale dialyzer (Nippon Genetics) with a molecular weight cut-off of 3000 Da at 4°C overnight. When the components precipitated as a result of dialysis, 3 M K-Glu was added until the pellets were dissolved. All protein components were concentrated to greater than 10 mg/mL using an Amicon concentrator (Millipore). Protein concentrations were calculated from their absorbances at 280 nm. The concentrated proteins were flash frozen in liquid nitrogen and stored at −80°C. These protein components were subsequently used to assemble version 7 of the system.

Protein synthesis using the reconstituted *in vitro* protein synthesis system For proteins synthesized in batch mode, plasmid DNA was added to the reaction mixture supplemented with 4 units of RNasin (Promega). For dialysis reactions, 50 μL of the reaction mixture was placed in a microscale dialyzer (Nippon Genetics) and dialyzed against 200 μL of buffer, which contained all system components except for proteins and tRNAs. All reactions were performed at 37°C. Time course measurements of GFP synthesis were performed using a temperature-controlled fluorometer (Mx3005P; Agilent) and a 492/516-nm excitation/emission filter set.

When necessary, *in vitro* protein synthesis was performed using ^{35}S-methionine (^{35}S-Met), and the quantity of synthesized protein was determined from the intensity of the corresponding band in autoradiograms of SDS-polyacrylamide gels. Briefly, various volumes of radiolabeled ^{35}S-Met were spotted onto a filter paper to obtain a standard curve of the relationship between the volume of ^{35}S-Met and the signal intensity obtained in the autoradiogram. Subsequently, an appropriate volume of ^{35}S-Met (e.g., p μl/μL) was added to the reaction mixture in which a large excess of cold Met (0.3 mM) was present. After the synthesis reaction, a portion of the sample was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The volume of ^{35}S-Met (e.g., q μl/μL) was determined from the band intensity in the autoradiogram using the standard curve. The concentration of incorporated Met was calculated using the equation $0.3 \times (q/p)$ (mM), and we obtained the concentration of the synthesized proteins by dividing this value by the number of Met residues in the sequence of each synthesized protein.

RESULTS AND DISCUSSION

We prepared three versions of the reconstituted *in vitro* protein synthesis system (versions 1, 2, and 7), each of which contain different component concentrations. The performance of each system was evaluated by synthesizing GFP using 200 nM RNA as a template at 37°C (Fig. 1A). Version 7 demonstrated 49-fold higher GFP fluorescence after 3 h compared with that of version 1. A list of component concentrations is provided in Table S1. Below, we describe the details of each version of the system.

Version 1 was prepared according to the original study by Shimizu et al. (4), and version 2 was prepared based on two previous studies (15,16). HrpA (ATP-dependent RNA helicase) and Tig (trigger factor) were decided to be included in version 2 of the system based on a comprehensive analysis using the PURE system that indicated a beneficial role for these components in GFP synthesis (16). Additionally, the concentrations of 69 system components were systematically altered to improve the GFP yield (15), resulting in the set of concentrations used in version 2. Beginning with version 2, we first omitted chloride and glycerol from the system because an absence of these components has been shown to increase GFP yield (unpublished results). We then altered the concentration of the ribosome by threefold and increased the concentration of the other protein components, with the exception of EF-Tu (34 components in total), by approximately fourfold. Additionally, peptidyl-tRNA hydrolase (Pth) (17) was included because it was found to improve the yield by 1.2-fold. As a result of these modifications, an 8-fold increase in fluorescent GFP yield was observed for version 7 compared with that of version 2 (Fig. 1). The translation rate was determined for version 7 (Fig. 2), which synthesized GFP at a rate of 4 amino acid residues/min/RNA. We postulate that the majority of the improvement is derived from the improvement in the elongation step. This is because the increase in the EF-Tu concentration of the version 1 system to that of version 2,

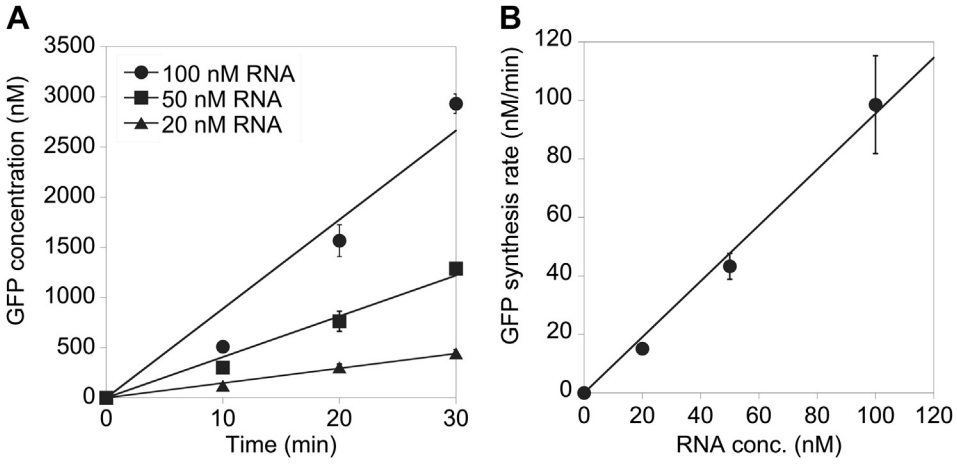

FIG. 2. The rate of protein synthesis using the optimized system. (A) GFP synthesis was performed using different concentrations of RNA, and the initial rate was calculated from the band intensity in the autoradiogram. All experiments were performed in duplicate. (B) The relationship between RNA concentration and the initial rate. The translation rate was calculated from the slope of the linear regression of the data, resulting in an elongation rate of 0.96 ± 0.06 GFP molecules/min/RNA. Because GFP is 240 residues long, the rate is equivalent to 3.84 amino acid residues/s/RNA.