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Research paper

Synthesis of 2.3 mg/ml of protein with an all *Escherichia coli* cell-free transcription–translation system

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ABSTRACT

Cell-free protein synthesis is becoming a useful technique for synthetic biology. As more applications are developed, the demand for novel and more powerful *in vitro* expression systems is increasing. In this work, an all *Escherichia coli* cell-free system, that uses the endogenous transcription and translation molecular machineries, is optimized to synthesize up to 2.3 mg/ml of a reporter protein in batch mode reactions. A new metabolism based on maltose allows recycling of inorganic phosphate through its incorporation into newly available glucose molecules, which are processed through the glycolytic pathway to produce more ATP. As a result, the ATP regeneration is more efficient and cell-free protein synthesis lasts up to 10 h. Using a commercial *E. coli* strain, we show for the first time that more than 2 mg/ml of protein can be synthesized in run-off cell-free transcription–translation reactions by optimizing the energy regeneration and waste products recycling. This work suggests that endogenous enzymes present in the cytoplasmic extract can be used to implement new metabolic pathways for increasing protein yields. This system is the new basis of a cell-free gene expression platform used to construct and to characterize complex biochemical processes *in vitro* such as gene circuits.

1. Introduction

In vitro protein synthesis is continually expanding its range of applications [1]. Originally developed for fundamental studies, cell-free expression systems are nowadays used for evolutionary biology [2,3], enzymes bioengineering [4,5], NMR-based structural and high-throughput proteomics [6–8], nanobiotechnology [9–13], industrial and medical applications [14,15]. *In vitro* screening of molecular interactions and production of novel potential therapeutics can also be easily performed with these systems [16–19]. In the past decade, DNA-dependent cell-free expression has also become a valuable technique for synthetic biology [20]. Cell-free expression systems are used to rapidly construct, prototype and validate synthetic gene circuits and biological reaction networks [21–26], to emulate complex biological processes [27], and as systems for machine learning optimization [28]. Cell-free protein synthesis is being developed as open platforms for designing and understanding biological systems using reductive bottom-up approaches [29–31].

Recently, cell-free transcription–translation (TX–TL) was used to construct larger self-assembled biological systems. An all *Escherichia coli* platform was used to produce T7 phages [32], including DNA replication and packaging, demonstrating the potential of cell-free TX–TL to construct complex systems from scratch with genome-sized DNA programs. Ribosomes were synthesized using hybrid T7-based *in vitro* TX–TL [33]. Such achievements, carried out with kits capable of producing 1 mg/ml of protein, encourage the development of more powerful *in vitro* TX–TL, to engineer even larger biochemical systems such as evolvable artificial cells [34–36].

One way to increase cell-free protein production of batch mode TX–TL reactions is to improve energy regeneration and byproducts recycling. The recycling of inorganic phosphate (iP) produced during cell-free TX–TL is essential to increase the yield of synthesized protein as its accumulation feedbacks negatively on the reactions [37,38]. Recently, recycling of iP was carried out with maltodextrin, an oligosaccharide hydrolyzed in glucose and glucose-1-phosphate by the enzyme maltodextrin phosphorylase [39]. Using maltodextrin, up to 1.7 mg/ml of protein was synthesized with a T7 hybrid system [40].

In this work, we describe the development of an all *E. coli* cell-free TX–TL system capable of 2.3 mg/ml of protein production in

Abbreviations: TX–TL, transcription–translation; iP, inorganic phosphate; 3-PGA, 3-phosphoglycerate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NAD, beta-nicotinamide adenine dinucleotide; cAMP, adenosine 3′,5′-cyclic monophosphate; DTT, dithiothreitol; reGFP, recombinant enhanced green fluorescent protein.

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batch mode reactions. The crude extract is prepared in 12 h from a commercial strain. We demonstrate that maltose can be substituted to maltodextrin to recycle iP. We show that maltose also improves ATP regeneration by coupling to 3-phosphoglycerate (3-PGA) metabolism. Cell-free expression lasts up to 10 h in batch mode. Measurements of pH change, kinetics of iP and ATP concentrations are presented. The amount of synthesized green fluorescent protein is measured by fluorescence and SDS-PAGE.

2. Materials and methods

2.1. Crude extract preparation

The crude extract was prepared as described previously [41] using the BL21 Rosetta2 strain and with the following slight modifications. S30A buffer composition: 14 mM Mg-glutamate, 60 mM K-glutamate, 50 mM Tris buffered with acetic acid to pH 8.2. S30B buffer composition: 14 mM Mg-glutamate, 150 mM K-glutamate buffered to pH 8.2 with Tris. The lysate was prepared with a cell press at a pressure of 16,000 LB.

2.2. Cell-free reaction in batch mode

The reaction mixture was prepared as described before [20]. The reaction buffer is composed of: 50 mM Hepes pH 8, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/ml tRNA, 0.26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 1 mM DTT, 2% PEG8000. A typical cell-free reaction is composed of 33% (volume) of *E. coli* crude extract. The other 66% of the reaction volume are composed of the plasmids and the reaction buffer containing the nutrients. The amino acid concentration was adjusted to either 1.5 mM or 3 mM of each of the 20 amino acids. Mg-glutamate and K-glutamate concentrations were adjusted according to the plasmids used (60 mM K-glutamate and 5 mM Mg-glutamate for pBEST-OR2-OR1-Pr-UTR1-deGFP-T500, 20 mM K-glutamate and 5 mM Mg-glutamate for the T7 cascade). The concentration of synthesized deGFP was determined from a calibration curve (Ex 485 nm and Em 528 nm) done with pure recombinant eGFP (reGFP) purchased from Cell Biolabs Inc. End-point fluorescence was measured in a 384 format well plate using a BioTek plate reader. deGFP synthesis kinetics were measured in a 96-well plate sealed with a plastic cap to avoid evaporation. Cell-free reactions (5–15 μl) were incubated at 29 °C.

2.3. Plasmids

The plasmids used in this study have been described before [20]. The plasmid pBEST-OR2-OR1-Pr-UTR1-deGFP-T500 was used for cell-free protein synthesis based on the endogenous core RNA polymerase and sigma factor 70. The plasmids pBEST-p15A-OR2-OR1-Pr-UTR1-T7rnap-T500 and pIVEX2.3d-deGFP were used for the expression of deGFP through the T7 transcriptional activation cascade. pH kinetics measurements were performed using pBEST-OR2-OR1-Pr-UTR1-Luc-T500. Plasmids were quantified with the fluorescent assay QuantiFluor (Promega).

2.4. SNARF-5F pH change kinetic measurements

pH of cell-free reactions was measured with the SNARF-5F dye, 5-(and-6)-carboxylic acid (Invitrogen). The dye was dissolved in DMSO at 10 mM and stored at −20 °C. Before usage, the dye was diluted 100 fold in water and used at 100 μM in the reaction mixture. The dye was excited at 514 nm and the ratio of the emissions at 580/640 nm was used to monitor the pH. The pH change was monitored in real-time (cell-free reaction incubated at 29 °C in a 384-well plate). To avoid interference between the fluorescence signals of the dye and the expressed protein, a plasmid encoding for the non-fluorescent protein Luciferase was used for expression.

2.5. iP and ADP/ATP assays

A spectrophotometry assay for inorganic phosphate was performed according to a procedure described in the literature [42]. Briefly, the protocol is based on absorbance measurements at 850 nm of the phosphomolybdate complex reduced by ascorbic acid at pH 5 with zinc in solution. Measurements were taken with Genesys10UV spectrophotometer (Thermofisher). The ADP/ATP assay was done using the ApoSENSOR ADP/ATP ratio assay kit (BioVision). The assay was performed in a 96 format well plate using a plate reader (BioTek). For both assays, aliquots were collected during cell-free reaction and diluted accordingly before each measurement.

2.6. SDS-Page

A 12% polyacrylamide gel was prepared according to standard procedures. To quantitatively estimate the total amount of deGFP (25.4 kDa) synthesized, known amounts of rEGFP (29 kDa, Cell Biolabs Inc) were added into each sample to be analyzed. A blank cell-free reaction was used for subtracting the background for quantification of deGFP and reGFP. The image analysis was done with the software ImageJ.

2.7. Visual display of data

Histograms are used for endpoint measurements (after 10–12 h of incubation), lines are used for kinetics.

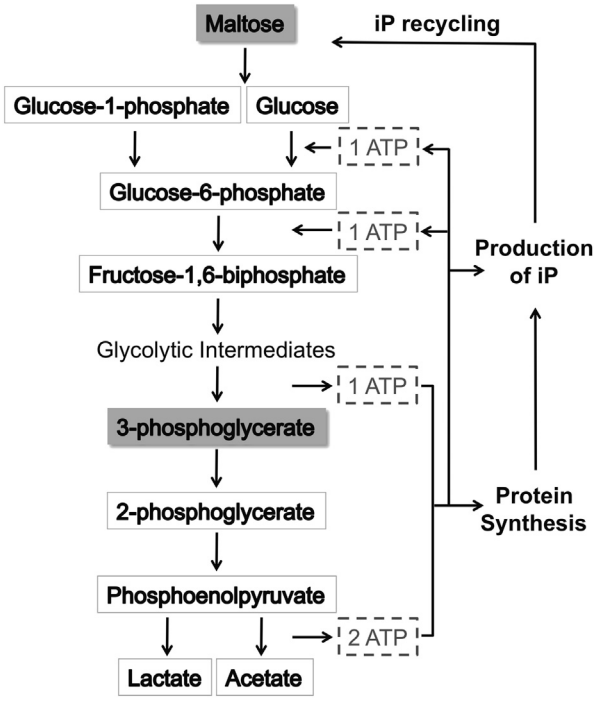

Fig. 1. Schematic illustration of the maltose-based metabolism for recycling inorganic phosphate (iP) and regenerating ATP. Chemical components added to cell-free reactions are shaded in gray.