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A Highly Efficient and Economical Cell-free Protein Synthesis System Using the S12 Extract of *Escherichia coli*

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Abstract We have developed an economical and simple cell-free protein synthesis system that produces milligram quantities of proteins in a milliliter batch reaction. In this system, the S12 extract, which was prepared from glucose-adapted cells, was employed and glucose alone was successfully used for the efficient and stable regeneration of ATP. The ATP level in the reaction mixture remained stable over a remarkably extended reaction period, which enabled prolonged protein synthesis, and the issues associated with proton accumulation and amino acid depletion were simultaneously addressed. Under the reaction conditions established in this study, protein synthesis continued for 6 h and the amount of the accumulated protein reached 1.8 mg/mL. © KSBB

Keywords: cell-free protein synthesis, glucose, chloramphenicol acetyltransferase, NAD, CoA, pH, cysteine

INTRODUCTION

Owing to its excellent speed and flexibility, cell-free protein synthesis is currently accepted as a promising alternative to the conventional *in vivo* gene expression techniques. The various protocols that have been developed over the past decade have greatly extended the applicability of cell-free protein synthesis [1-3]. Among the many efforts that have been made to improve the productivity and cost of cell-free protein synthesis, one of the most notable achievements is the successful use of glucose as the primary energy source [4,5]. The use of glucose not only reduces the cost of the energy source, but also enables a steady supply of ATP over extended reaction periods. Moreover, unlike the energy sources that have been previously used such as phosphoenolpyruvate (PEP) and creatine phosphate (CP), the use of glucose does not result in the accumulation of inorganic phosphate, which can potentially inhibit cell-free protein synthesis by sequestrating magnesium ions [6]. However, the current protocols that involve glucose also have significant drawbacks. First, these methods require the use of expensive co-factors (NAD and CoA), which offsets the economical benefits of using glucose as a cheap energy source. In addition, Calhoun and Swartz [4] have shown that although the use of glucose remarkably improves ATP supply, the productivity of the protein synthesis did not increase as much as expected. In fact, the yield in their glucose-utilizing reaction (0.6 mg/mL) was not higher than cell-free reactions that rely on conventional energy sources such as the PANOx system (0.7 mg/mL) [5].

The aim of this study was to overcome the current limitations in the glucose-based cell-free reactions and to establish a more economical and efficient method for cell-free protein synthesis. This was achieved by first replacing the S30 extract used in the previous study with the recently developed S12 extract [7]. Unlike the conventional S30 extract, the preparation of the S12 extract does not involve high-speed centrifugation or dialysis steps; thus, the endogenous co-factors (NAD and CoA) from the cytosol of the cells remain in the final extract. Therefore, glucose can regenerate ATP in the S12 extract without the addition of expensive co-factors.

In addition, we found that the pH rapidly declined during protein synthesis in the glucose-energized cell-free system. To address this, the buffering capacity of the reaction mixture was fortified, which remarkably improved the productivity of protein synthesis. Finally, protein productivity was further improved by preventing the depletion of key amino acids. As a result, the newly established protocol produced approximately 1.8 mg/mL of protein from a simple batch

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reaction at a remarkably reduced reagent cost when compared to the previous glucose-based methods, as well as the methods that use conventional energy sources.

MATERIALS AND METHODS

Cell-free Protein Synthesis Reactions

The cell-extract (S12 extract) used for catalyzing the cell-free protein synthesis was prepared from the *E. coli* strain BL21 Star™ (Invitrogen, Carlsbad, CA, USA) as described previously [8]. The cells were grown in 2xYT medium (Laboratories Conda, Madrid, Spain) or 2xYTPG medium (2xYT supplemented with 22 mM NaH_2PO_4, 40 mM Na_2HPO_4, and 100 mM glucose).

The cell-free protein synthesis reactions were carried out in a 1.5 mL microtube that was placed in a water bath set at 37°C. The plasmid pK7CAT, which encodes the chloramphenicol acetyltransferase (CAT) between the T7 promoter and the T7 terminator, was used as the template. The standard reaction mixture consisted of the following components: 57 mM of HEPES-KOH (pH 8.2), 1.2 mM of ATP, 0.85 mM each of CTP, GTP, and UTP, 2 mM of DTT, 0.17 mg/mL of *E. coli* total tRNA mixture (from strain MRE600), 90 mM of potassium glutamate, 80 mM of ammonium acetate, 8 mM of magnesium acetate, 20 mM potassium phosphate dibasic (pH 7.2), 34 μg/mL of L-5-formyl-5, 6, 7, 8-tetrahydrofolic acid (folinic acid), 2.1 mM each of 20 amino acids, 2% (w/v) of PEG (8000), 33 mM of glucose, 10 μM of L-[U-^{14}C] leucine (11.3 GBq/mmol), 6.7 μg/mL of DNA, and 27% (v/v) of cell-extract (S12 extract). Depending on the experiments, the concentrations of glucose and HEPES-KOH (pH 8.2) were varied. The amount of synthesized protein was estimated by measuring the radioactivity of insoluble TCA using a WALLAC 1410 liquid scintillation counter (Pharmacia LKB, Sweden), as previously described [9].

Determination of Glucose Concentration

The quantitative analysis of glucose was carried out using a commercial glucose assay kit (GAGO-20, Sigma) with minor modifications. Samples of the reaction mixture were centrifuged at 12,000 rpm for 1 min. Five μL of the supernatant was diluted 40-fold in water. The subsequent steps were conducted according to the manufacturer's protocol.

Phosphatase Activity Assay

The phosphatase activity in the cell-extract was determined using a commercial phosphatase activity assay kit (BioAssay Systems, Hayward, CA, USA). The assay was conducted according to the manufacturers' instructions after diluting the cell-extract 5-fold in solution A (10 mM Tris-acetate buffer (pH 8.2), 14 mM magnesium acetate, 60 mM potassium glutamate).

Measurements of Residual ATP Concentration and pH

A firefly luciferase assay was used to determine the ATP concentration, as previously described [10]. The pH of the reaction mixture during cell-free protein synthesis was monitored using a micro-combination pH-electrode (InLab423, Mettler-Toledo GmbH, Switzerland).

RESULTS

Enhanced Glucose Utilization, ATP Supply, and Protein Synthesis in the Cell-extracts from Glucose-adapted Cells

In order to facilitate the use of glucose and also to decrease the phosphatase activity, cell-extracts were prepared from *E. coli* cells BL21-Star™ (DE3) that had been grown in the 2xYT medium supplemented with glucose and inorganic phosphate (2xYTPG). While the cell growth rate was similar to cells grown in the 2xYT medium, the cell-extracts prepared from cells grown in the 2xYTPG medium ($S12_{2xYTPG}$) exhibited substantially lower phosphatase activity (Fig. 1A), which is in agreement with a previous report [11]. In addition, glucose molecules were used more efficiently for the regeneration of ATP in the $S12_{2xYTPG}$ extract, most likely due to the enrichment of glucose-metabolizing enzymes. When the synthesis was started in 33 mM of glucose, the complete depletion of glucose took approximately 40 and 80 min in $S12_{2xYTPG}$ and $S12_{2xYT}$, respectively (Fig. 1B). The time-course analysis of ATP and synthesized protein concentrations demonstrated that the accelerated glucose consumption in the $S12_{2xYTPG}$ extract led to the enhanced regeneration of ATP and, in turn, a higher rate of protein synthesis (Figs. 1C and 1D).

Prolonging Cell-free Protein Synthesis in the $S12_{2xYTPG}$ Extract at a Stable and Constant pH

The successful development of continuous cell-free protein synthesis systems [12,13] clearly demonstrated that ribosome and other translation factors are quite stable *in vitro* and remain functional over tens of hours. Therefore, in theory, the longevity of a batch cell-free protein synthesis reaction should be governed by the continuous supply of energy (ATP) and substrates (amino acids). However, the use of conventional energy sources inevitably causes the accumulation of inorganic phosphate in the reaction mixture; thus, the longevity of the protein synthesis reaction has been historically limited by the sequestration of magnesium ions. In contrast, glucose does not result in the accumulation of inorganic phosphate; therefore, we initially expected that prolonged cell-free protein synthesis could be achieved simply by adding a large amount of glucose to the reaction mixture.

However, we found that increased glucose concentrations led to a substantial reduction in protein synthesis despite the substantially enhanced ATP supply. By measuring the pH during the glucose-energized reaction, we found that the pH

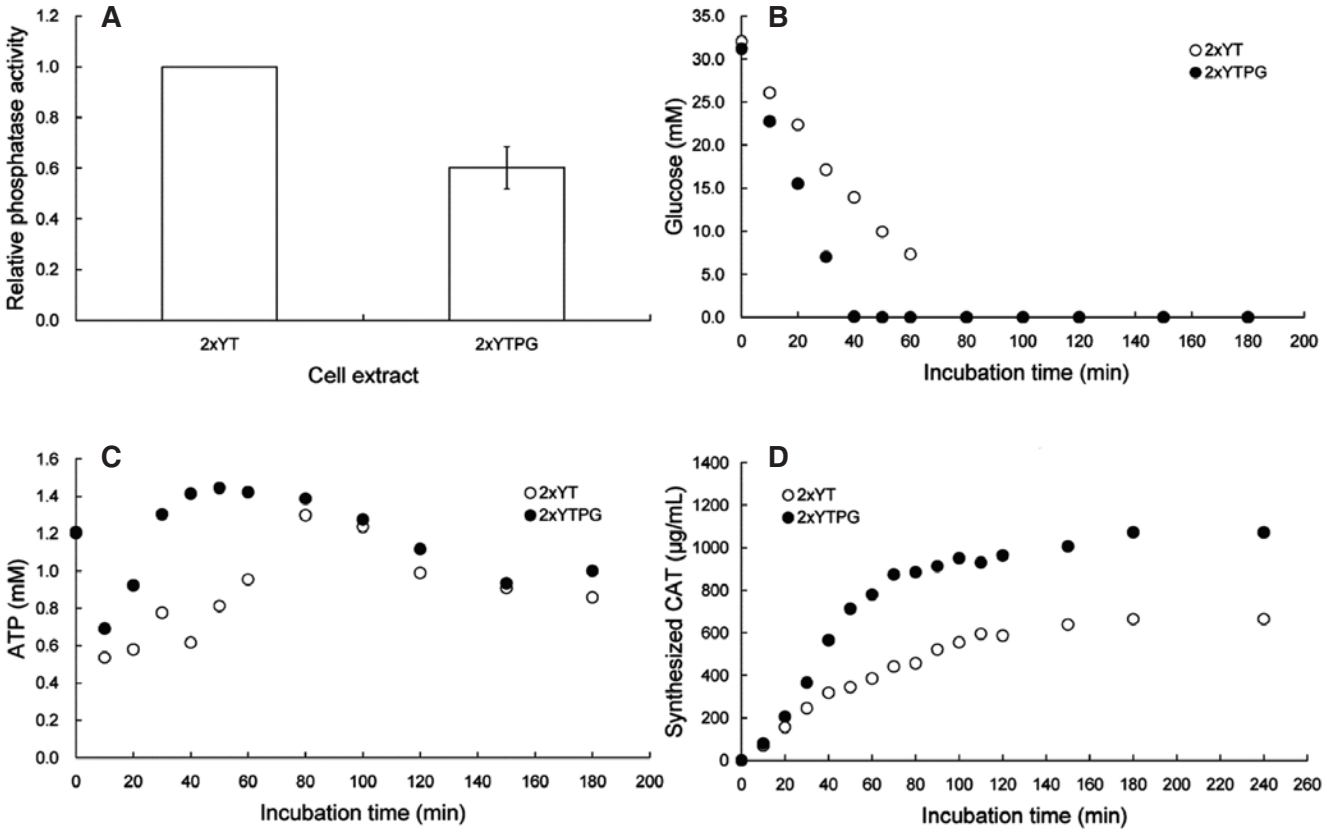

Fig. 1. Comparison of the cell-extracts prepared from 2xYT and 2xYTPG. Relative phosphatase activity (A), the rate of glucose consumption (B), the efficiency of ATP regeneration (C), and translational rate (D).

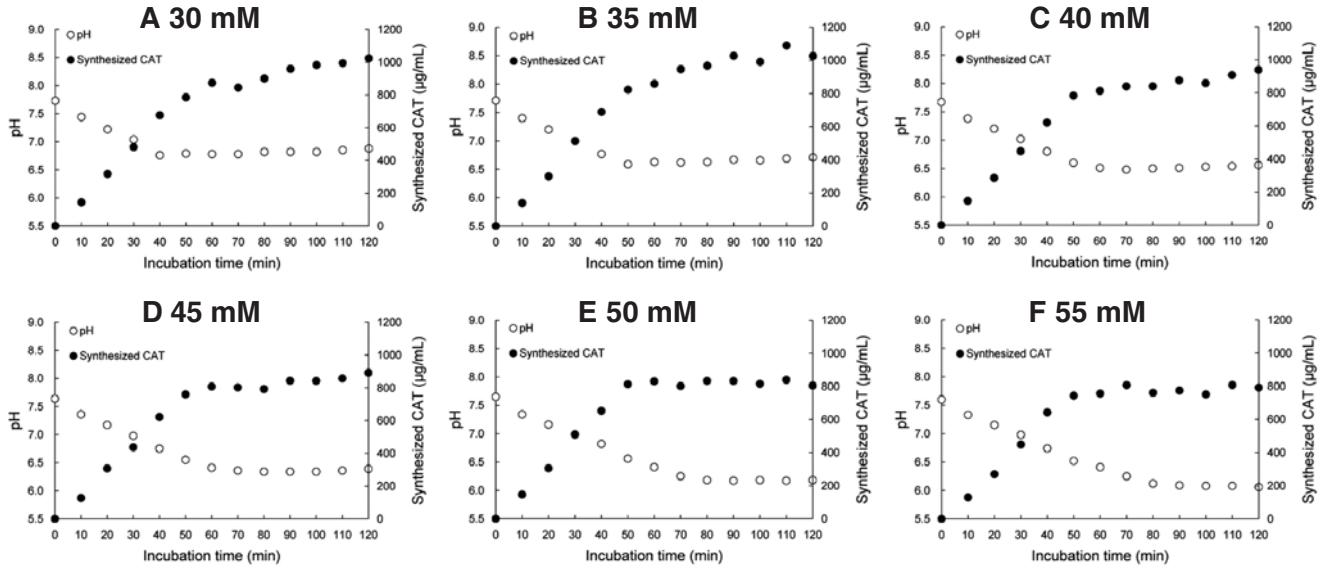

Fig. 2. Time-course of pH and protein synthesis at different initial glucose concentrations. The reactions were carried out under standard conditions with various glucose concentrations (30~55 mM). The amount of expressed protein and the change in pH were measured as described in Materials and Methods.

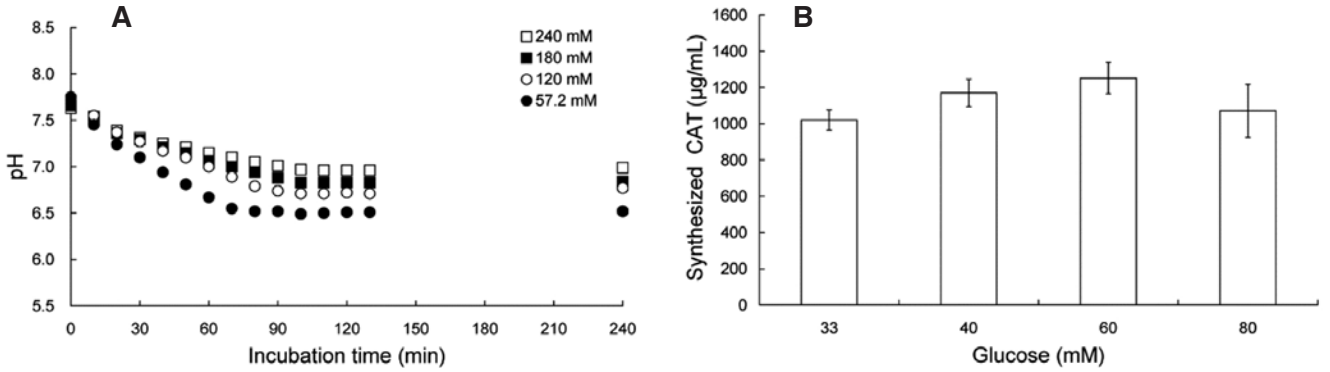

Fig. 3. Stably maintaining pH during cell-free protein synthesis with increased buffer (HEPES-KOH) concentration. (A) The cell-free protein synthesis reaction was conducted in the presence of different concentrations of HEPES-KOH (57.2~240 mM). The initial glucose concentration was set at 33 mM. The pH of the reaction mixtures was measured with a micro-combination pH-electrode as described in Materials and Methods. (B) The plasmid pK7CAT was incubated in a cell-free reaction mixture containing 240 mM HEPES-KOH. Initial glucose concentrations were varied and the amount of cell-free synthesized CAT was determined from the radioactivity of insoluble TCA. The results were averaged from two separate experiments.

of the reaction mixture continually decrease throughout the reaction period at a rate that varied depending on the initial glucose concentration. Interestingly, the cessation of protein synthesis coincided with the time at which the pH of the reaction mixture reached a certain point (ca. 6.5; Fig. 2). Based on these results we hypothesized that the short duration of protein synthesis in the glucose-based cell-free synthesis reactions occurred because of the rapid decrease in pH.

Therefore, we attempted to extend the protein synthesis reaction by slowly decreasing the pH of the reaction. When the initial concentration of (HEPES-KOH) was increased from 57 to 240 mM, the pH of the reaction mixture was maintained above 7.0 throughout the entire incubation period (Fig. 3A). Moreover, in the presence of a higher concentration of buffer, the duration of protein synthesis was remarkably extended. More specifically, protein synthesis in reaction mixtures that contained higher concentrations of glucose (60 mM) and HEPES-KOH (240 mM) continued for 3 h and produced 1.2 mg/mL of CAT (Fig. 3B).

Improving Productivity by the Optimization of Cysteine Concentration

From the measurements of the TCA-insoluble radioactivity of the reaction samples, it was estimated that on average approximately 40% of the initially added amino acids were polymerized into protein molecules under the newly optimized reaction conditions (60 mM glucose and 240 mM HEPES). Since a number of amino acids are subject to nonproductive degradation or metabolic conversion in cell-extracts [14,15], it was hypothesized that certain amino acids may have been depleted during the course of the reaction. Therefore, we examined the effect of increasing the initial concentration of amino acids on protein synthesis. Surprisingly, the yield of the cell-free synthesized CAT increased to 1.8 mg/mL when the reaction mixture was supplemented with 4 mM of cysteine. However, the addition of other amino acids did not significantly affect protein yield (Fig. 4).

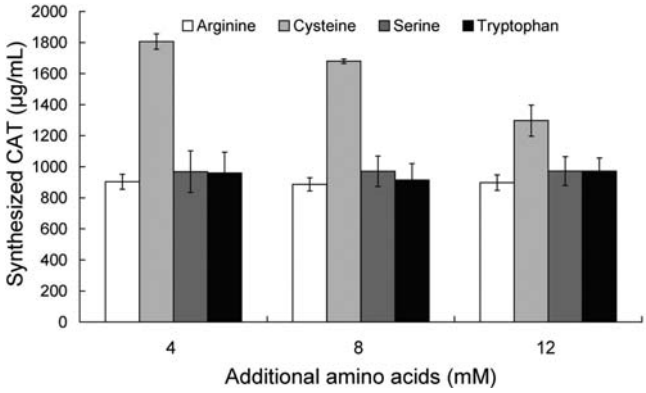

Fig. 4. Effect of amino acids supplementation on the yield of cell-free protein synthesis. The reaction mixture (240 mM HEPES, 60 mM glucose) was supplemented with varying concentrations of arginine, cysteine, serine, or tryptophan. After 6 h of incubation, the amount of cell-free synthesized CAT was determined from the radioactivity of insoluble TCA. The results were averaged from two separate experiments.

DISCUSSION

The use of glucose as an energy source greatly reduces the reagent cost of cell-free protein synthesis, thereby removing one of the major obstacles for the widespread application of cell-free protein synthesis. However, previously established glucose-based methods required the co-addition of NAD and CoA, which offset the economical advantages of using glucose. In addition, the use of glucose as an energy source in cell-free protein synthesis results in the accumulation of

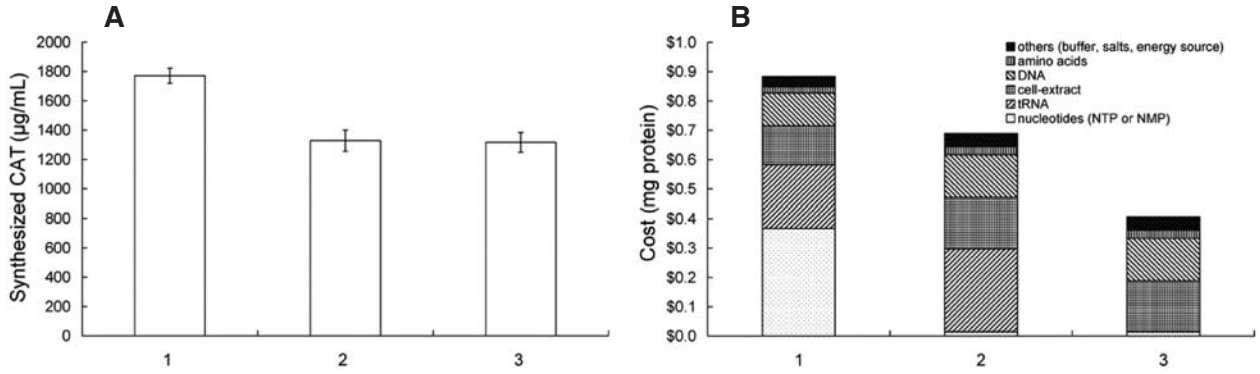

Fig. 5. Comparison of protein productivity (A) and reagent costs for producing 1 mg of protein (B) in different versions of the cell-free protein synthesis system. (A) System 1 (240 mM HEPES, 60 mM glucose, 1.2 mM ATP, 0.85 mM each of GTP, CTP, and UTP, and 0.17 mg/mL of *E. coli* total tRNA mixture); System 2 (240 mM HEPES, 60 mM glucose, 1.2 mM AMP, 0.85 mM each of GMP, CTP, and UMP, and 0.17 mg/mL of *E. coli* total tRNA mixture); System 3 (240 mM HEPES, 60 mM glucose, 1.2 mM AMP, 85 mM each of GMP, CMP, and UMP, and no additional tRNA). (B) The reagents costs for producing 1 mg of CAT. Cost analysis was conducted based on the reagent prices listed in the Sigma and Roche Applied Science catalogues.

organic acids, which substantially decreases the pH of the reaction [4]. Unfortunately, this side effect limits the duration of the protein synthesis reaction and deteriorates the efficiency of protein synthesis.

In the present study, we employed the S12 extract instead of the conventional S30 extract, which eliminated the need for additional co-factors. Since the preparation of the S12 extract does not involve any dialysis steps, the co-factors were retained and used for the oxidation of glucose. In particular, the S12 extract prepared from the glucose-adapted cells showed a remarkably improved efficiency of ATP regeneration with an accelerated glucose-consumption rate. In addition, we found that the translation activity of the cell-extract was severely impaired when the pH of the reaction mixture dropped below 6.5. Therefore, to address this issue the buffering capacity of the reaction mixture was increased, which greatly enhanced the productivity of the glucose-utilizing cell-free protein synthesis.

Finally, when the reaction was supplemented with 4 mM of cysteine a 50% increase in productivity was observed. Recently, Calhoun and Swartz [15] developed a genetic approach to stabilize amino acids in a cell-extract by deleting several genes (tryptophanase, *tnaA*; arginine decarboxylase, *speA*; serine deaminase, *sdaA*, *sdaB*; glutamate-cysteine ligase, *gshA*) from the *E. coli* chromosome. Using this approach they showed that the all amino acids were stabilize for a reaction time of 3 h. We expect that the addition of amino acids used in this study would be unnecessary if their mutant strains were used in the preparation of the S12 extract.

Nucleotide triphosphates (NTPs) would account for the highest proportion of the total cost of reagents in the glucose-based cell-free protein synthesis method developed in this study. Since it was previously shown that nucleotide monophosphates (NMPs) can be charged with phosphate for the *in situ* generation of NTPs in the *E. coli* S30 extract [5], we also replaced the NTPs in our reaction mixture with NMPs. Although the protein productivity was slightly reduced when NMPs were used, this alternative method further decreased the cost of cell-free protein synthesis since NMPs are substantially less expansive than NTPs. To further reduce protein production costs, tRNA was removed from reaction mixtures containing NMPs. Interestingly, the amount of synthesized protein was not affected by the absence of the tRNA mixture (Fig. 5). The results presented in this study suggest that cell-free protein synthesis may be used as a realistic alternative for the large-scale production of proteins.

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