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Continuous Cell-Free Protein Synthesis Using Glycolytic Intermediates as Energy Sources

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In this work, we demonstrate that glycolytic intermediates can serve as efficient energy sources to regenerate ATP during continuous-exchange cell-free (CECF) protein synthesis reactions. Through the use of an optimal energy source, approximately 10 mg/ml of protein was generated from a CECF protein synthesis reaction at greatly reduced reagent costs. Compared with the conventional reactions utilizing phosphoenol pyruvate as an energy source, the described method yields 10-fold higher productivity per unit reagent cost, making the techniques of CECF protein synthesis a more realistic alternative for rapid protein production.

Keywords: Glycolysis, ATP regeneration, S12 extract, cell-free protein synthesis, fructose-1,6-bisphosphate

Compared with *in vivo* gene expression methods, cell-free protein synthesis offers excellent speed and flexibility for protein production. Although conventional cell-free protein synthesis systems of batch configuration have suffered from unacceptably low productivity [6, 11], reaction schemes of cell-free protein synthesis have evolved to improve the protein productivity [2, 7, 13, 15]. Among those, the development of the continuous-exchange cell-free (CECF) protein synthesis system [5] has enabled the rapid generation of milligram amounts of proteins *in vitro*. Working on diffusion exchange of reaction components between the feeding reservoir and reaction chamber, the CECF protein synthesis system extends the reaction periods of cell-free protein synthesis, and thus substantially enhances protein productivity [4].

However, the operation of a CECF protein synthesis reactor requires an excessive amount of feeding solution that contains small molecular weight substrates [17, 18], and thus its economical feasibility is limited by the high cost for reagents. In particular, in the composition of the reaction mixture employing conventional energy sources such as phosphoenol pyruvate (PEP) and creatine phosphate (CP), more than half of the total reagent cost is accounted for by energy sources [10].

Recently, Calhoun and Swartz [1] reported the development of a batch cell-free protein synthesis system utilizing glucose as the energy source. It was demonstrated that the glycolytic enzymes present in the cell extract were able to catalyze the glycolysis of glucose to produce ATP, which in turn supported protein synthesis.

We found that the efficiencies of protein synthesis in the glucose-utilizing reactions could be further enhanced when the cell extract was prepared from *E. coli* cells that had been grown in a medium containing a high concentration of glucose and phosphate (2× YTPG medium; see reference 8). Compared with the extract prepared from the cells grown in the absence of additional glucose, most likely through the improved efficiency of ATP regeneration by the highly induced glycolytic enzymes, the extract from the glucose-fortified culture gave rise to an approximately 2-fold increase of protein productivity (Fig. 1). The reaction mixture used for the batch cell-free protein synthesis consisted of the following components: 57 mM Hepes-KOH (pH 8.2), 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 1.7 mM dithiothreitol, 90 mM potassium glutamate, 80 mM ammonium acetate, 12 mM magnesium acetate (except for glucose-utilizing system, 8 mM), 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), 2.1 mM each of the unlabeled amino acids, 10 μM L-[U-^{14}C] leucine (11.3 GBq/mmol), 2% (w/v) PEG (8000), 33 mM energy sources (glucose, glucose-6-phosphate, fructose-1, 6-bisphosphate, 3-phosphoglycerate, phosphoenol pyruvate), 6.7 μg/ml DNA, and 27% (v/v) S12 extract. In the reactions utilizing glucose as an energy source, the Hepes buffer was

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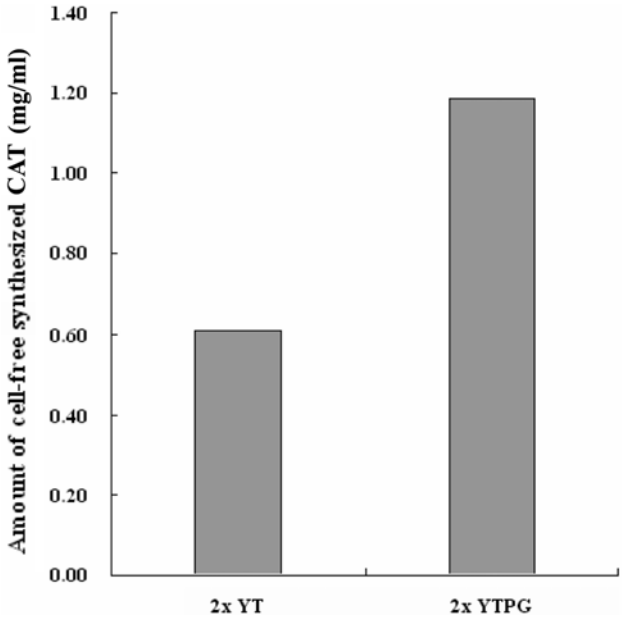

Fig. 1. Use of a cell extract prepared from glucose-enriched culture increases protein productivity in a cell-free protein synthesis reaction utilizing glucose as an energy source.
The 2×YT medium consistsed of 16.0 g/l of tryptone, 10.0 g/l of yeast extract and 5.0 g/l of sodium chloride. One hundred mM glucose, 22 mM NaH_2PO_4, and 40 mM Na_2HPO_4 were added to the 2×YT medium to prepare the 2×YTPG medium. The amounts of the cell-free synthesized proteins were estimated from TCA-insoluble radioactivity using a liquid scintillation counter (WALLAC 1410) as described previously [8].

replaced with 90 mM Bis-Tris buffer (pH 6.5) and 20 mM sodium phosphate (dibasic, pH 7.2) was included. The S12 extract was prepared as described previously [9] except that the 2×YTPG medium was used to grow the cells. As the template for cell-free protein synthesis reactions, the plasmid pIVEX2.3d-CAT that encodes the sequence of bacterial chloramphenicol acetyltransferase (CAT) under the control of the T7 promoter was used. The amounts of total and active protein products were determined as described previously [9].

Since the cost for glucose is almost negligible compared with those of conventional energy sources, the use of glucose improves the economics of cell-free protein synthesis. In particular, the benefits of using a cheap energy source will be maximized in the CECF protein synthesis reactions where greater amounts of energy sources are required.

For this reason, we next attempted to apply the established reaction conditions to the operation of a CECF protein synthesis reaction. Reactions were conducted in a commercial CECF protein synthesis device (ProteoMaster System, Roche Diagnostics GmbH, Germany). The CECF protein synthesis device consists of a reaction chamber (1 ml) and a feeding reservoir (10 ml), which are separated from each other by a dialysis membrane. After filling the compartments with the reaction mixture and the feeding solution (identical to the reaction mixture except that the volumes for cell extract, tRNA, and DNA were replaced with water), respectively, the reactions were initiated by placing the filled device in an incubator that provides reciprocal agitation at 800 rpm.

Disappointingly, unlike the cases of using the conventional energy sources (PEP and CP) where several fold increases of product accumulation have been reported [3, 16], the glucose-utilizing CECF protein synthesis reaction did not produce substantially higher amounts of proteins than a batch reaction. Since the regeneration of ATP during the glycolysis is a multistep process involving many intermediate compounds, it was assumed that glycolytic intermediates generated from glucose were diffused out of the reaction

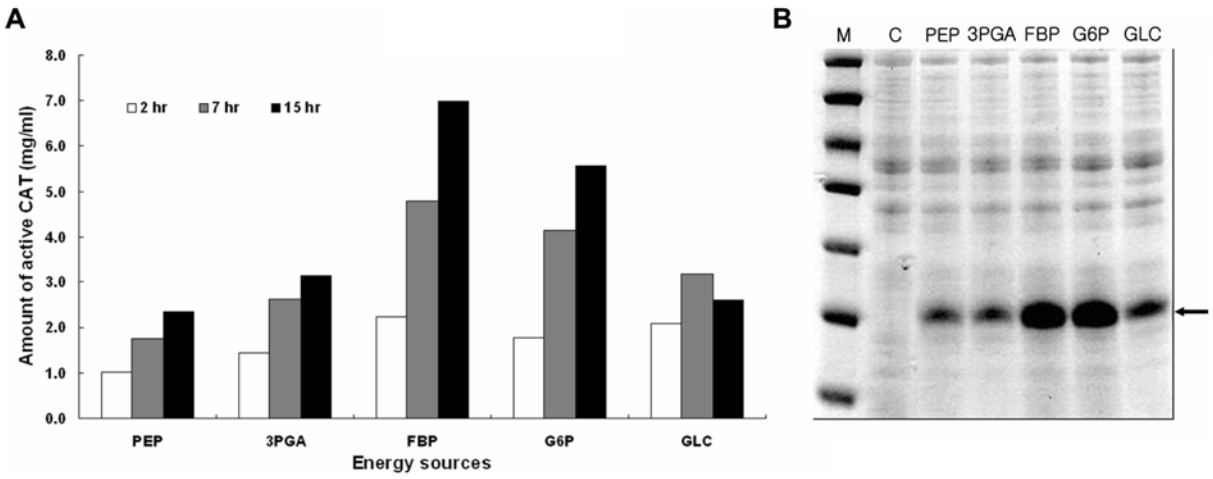

Fig. 2. CECF protein synthesis reactions with different energy sources.
A. Time-course of the accumulation of active CAT in the reaction chamber. **B**. SDS-PAGE analysis of the reaction mixture after 15 h of incubation. M, protein molecular weight markers; C, control reaction without DNA.

chamber, which limited ATP regeneration and protein synthesis.

However, interestingly enough, we found that prolonged protein synthesis by the CECF protein synthesis reaction was achieved when glycolytic intermediates were used as the primary energy sources instead of glucose. As shown in Fig. 2, for example, when glucose was replaced with fructose-1,6-bisphosphate (FBP) either in the reaction mixture or feeding solution, continuous increase of protein accumulation was observed in the reaction chamber. Based on the intensity of the bands on a Coomassie Blue-stained gel, after 15 h of incubation, approximately 10 mg/ml of protein product (CAT) was produced from the CECF protein synthesis reaction using FBP as the energy source. Enzymatic assay of the synthesized protein indicated that approximately 70% of total product was active. Although not as effective as FBP, other glycolytic intermediates were also able to support the continuous protein production in the CECF protein synthesis reaction with varying efficiencies. These results correlate well with a previous report where it was shown that the use of FBP substantially enhanced protein synthesis through improved supply of ATP [10]. It remains intriguing why glucose was not able to be used for supporting the CECF protein synthesis reaction whereas the use of the subsequent glycolytic intermediates was effective. It might be related to the different conversion yields at each of the multiple steps in the glycolytic pathway. For example, if the conversion rate of glucose to glucose-6-phosphate (G6P) is substantially slower than the diffusion rate while the downstream steps are faster, the reaction mixture using glucose will be depleted of any energy sources, which will lead to a cessation of protein synthesis. If this is the case, use of a cell extract prepared from a hexokinase-overexpressing strain can be considered for the efficient supply of ATP using glucose.

Apart from the exact mechanisms of ATP supply in the reaction mixture, as summarized in Fig. 3, our results demonstrate that the protein productivity and economical feasibility of the continuous cell-free protein synthesis reaction can be greatly improved by employing appropriate glycolytic intermediates (FBP, in this experiment) as the energy source to regenerate ATP. We also expect that the efficiency of ATP supply can be further improved through the use of the cell extracts from *Escherichia coli* strains whose glycolytic pathways have been engineered to minimize the generation of inhibitory products [12, 14].

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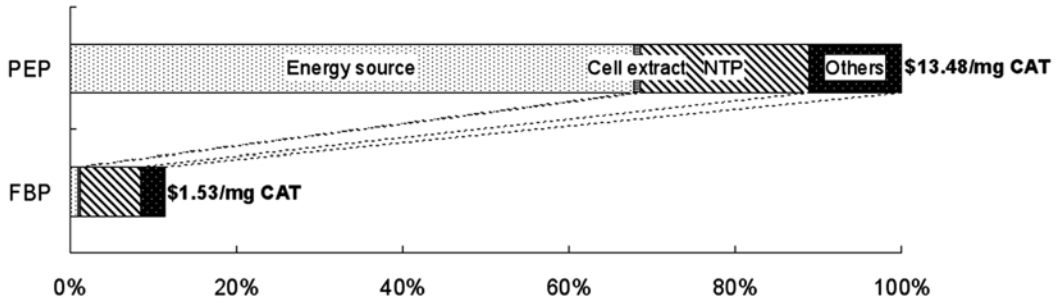

Fig. 3. The total reagent costs for producing 1 mg of CAT in a CECF protein synthesis system using PEP and FBP as the energy sources.

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