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REVIEW

Methods for energizing cell-free protein synthesis

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The productivity of cell-free protein synthesis depends critically upon the efficiency of ATP regeneration. In addition, the expensive energy sources used in the conventional cell-free protein synthesis systems account for the major part of the overall cost for the expression of proteins. Therefore, it is essential to implement a cheaper and more efficient ATP regeneration method in order to make cell-free protein synthesis a viable option for industrial protein production. In this article, we review the recent progress in the methodologies for supplying ATP during cell-free protein synthesis in *E. coli* extracts.

[**Key words:** Cell-free protein synthesis; Energy source; ATP; Cell extract; Glycolysis]

Cell-free protein synthesis has become a valuable tool for understanding and analyzing the translation of genetic information into protein molecules. Moreover, due to its outstanding flexibility, simplicity and throughput, this technique is drawing attention to itself as an alternative tool for the genome wide translation of genetic information (1–4).

In addition, the development of different reaction schemes in recent years has enabled the production of milligram levels of proteins in cell-free protein synthesis systems. Examples include the continuous-flow (5), continuous exchange (6, 7) and hollow fiber (8) systems. In parallel, as an option for multiplexed and rapid expression of proteins, the productivity of batch-type cell-free protein synthesis has also been substantially improved (9, 10).

In contrast to the conventional notion that the ribosome and other translation factors would be unstable when taken out of the cells, exceptional longevity of the continuous cell-free protein synthesis reactions indicates that the protein synthesis machinery retains its biological function for long time *in vitro* when proper reaction environments are provided. However, these reaction schemes rely on the continuous supply of substrates and concomitant removal of the by-products of low molecular weight. Therefore, their operation requires excessive amounts of expensive reagents and specific devices (11, 12) and, thus, it is hard to expand their use to the high-throughput production of proteins. In contrast, the conventional systems of batch configuration represent a more viable option for the parallel and rapid expression of proteins. Since the typical batch cell-free protein synthesis system has very low productivity mainly due to short duration of protein synthesis reaction, numerous attempts have been made to identify the factors that limit the duration of protein synthesis in a batch cell-free synthesis reaction. It has turned out that inefficient supply of ATP (adenosine triphosphate) is one of the most critical reasons behind the short duration and low productivity of batch cell-free protein synthesis systems (13, 14). Since the translation of genetic information into protein molecules involves many steps that consume ATP or its equivalents, an adequate and steady supply of ATP is essential for the prolonged synthesis of proteins in a cell-free synthesis system. Traditionally, regeneration of ATP during cell-free synthesis has been dependent upon the substrate-level phosphorylation of ADP using a compound carrying high-energy phosphate bonds (15–18). Due to the presence of numerous phosphatase activities in the cell extract, however, these energy sources undergo rapid degradation even before they are used for ATP regeneration, thereby lowering the yield of proteins synthesis.

In this paper, we review recent efforts to improve the efficiency and economical feasibility of the methods for ATP supply in an *E. coli* extract, which is an essential prerequisite for industrial application of cell-free protein synthesis.

FED-BATCH WISE ADDITIONS OF ENERGY SOURCES AND MAGNESIUM ION

The simplest method to address the problem of substrate depletion would probably be the replenishment of the depleting substrates. Indeed, in the work of Kim and Swartz, depletion of ATP was able to be delayed substantially by repeated additions of energy source. When the energy source (phophoenol pyruvate, PEP) was added to the reaction mixture periodically, duration of protein synthesis was extended from 20 min to 80 min and protein productivity increased accordingly (14).

Since a significant portion of added PEP is degraded by the cell extract, repeated addition of PEP continuously increases the level of inorganic phosphate in the reaction mixture (Fig. 1). It was found that the sequestration of magnesium ions due to the accumulation of inorganic phosphate causes early stoppage of protein synthesis, even when sufficient amount of ATP is present in the reaction mixture. The

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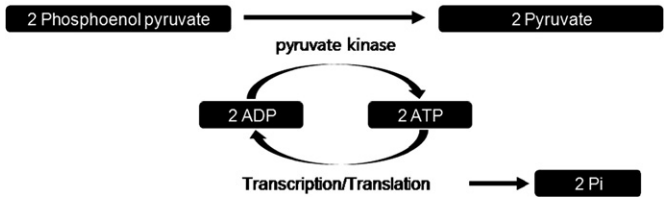

FIG. 1. ATP regeneration using phosphoenol pyruvate (PEP) as an energy source.

phosphate-mediated inhibition could be reversed to some extent by the addition of additional magnesium ion, and the combination of magnesium supplementation and periodic additions of PEP was able to extend protein synthesis up to 2 h. Kim et al. proposed a similar strategy using creatine phosphate as an energy source. Unlike PEP which can be consumed through a number of metabolic enzymes in the cell extract, creatine phosphate is not a natural substrate of the *E. coli* metabolism. Therefore, compared to the metabolizable energy sources, creatine phosphate (CP) can drive the regeneration of ATP with higher efficiency. Nevertheless, as is the case with other phosphate-containing compounds, the utilization of creatine phosphate during cell-free synthesis inevitably causes the accumulation of inorganic phosphate. Based on these, they started protein synthesis reaction with high concentration of CP (80 mM) and periodically provided the reaction mixture with fresh magnesium ions. As a result, protein synthesis reaction continued over 2 h and 1.3 mg/ml of protein was accumulated in the reaction mixture.

PYRUVATE AS AN ENERGY SOURCE THAT DOES NOT ACCUMULATE INORGANIC PHOSPHATE

Although the aforementioned fed-batch strategy could extend the duration of cell-free protein synthesis significantly, repeated additions of substrates are cumbersome, particularly when parallel expression of multiple proteins is demanded. More importantly, inhibitory effect of the accumulating phosphate cannot be completely reversed by the supplementation of magnesium ions. As was pointed out in the Kim et al.'s report, it appears that different forms of magnesium phosphate are also inhibitory for protein synthesis. The problem of phosphate accumulation will persist as long as ATP regeneration is conducted based on direct substrate-level phosphorylation reactions. Kim and Swartz proposed a novel solution to this intrinsic problem (13): ATP regeneration without accumulation of inorganic phosphate. In this scheme, pyruvate oxidase was introduced into the reaction mixture along with pyruvate (Fig. 2). Acetyl phosphate was generated *in situ* through the condensation of pyruvate and inorganic phosphate, and endogenous acetyl kinase in the *E. coli* extract, then catalyzed the regeneration of ATP. Oxygen is required for the generation of acetyl phosphate and the H_2O_2 produced as a byproduct was sufficiently degraded by endogenous catalase activity. Through the continuous supply of chemical energy, and also through the prevention of inorganic phosphate accumulation, this method was able to extend the duration of protein synthesis up to 2 h. In addition, pyruvate is substantially cheaper than the conventional energy source (PEP). However, because the *E. coli* pyruvate oxidase (E.C. 1.2.2) cannot catalyze the formation of acetylphosphate (it converts pyruvate to acetate instead of acetylphosphate), the conversion of pyruvate into acetylphosphate requires an exogenous enzyme, pyruvate oxidase (E.C.1.2.3.3), from *Lactobacillus* or *Pediococcus* sp. The use of commercial pyruvate oxidase significantly offsets the economic benefits of using pyruvate as an energy source. More importantly, because this enzyme requires molecular oxygen for the oxidation of pyruvate into acetylphosphate, the synthesis reaction cannot be easily scaled-up in a simple batch configuration due to the limitation of oxygen transfer.

PANOX SYSTEM

The requirements for exogenous enzyme and oxygen were able to be eliminated by activating an *E. coli* pathway involving pyruvate dehydrogenase (PDH) and phophotransacetylase (PTA). In the presence of NAD as a cofactor, PDH catalyzes the condensation of CoA and pyruvate to make acetyl-CoA. Subsequently, acetyl-CoA is converted to acetylphosphate by PTA. Since these two enzymes are present in the cell extract, simple additions of the cofactors, NAD and CoA, were sufficient to stimulate the regeneration of ATP from pyruvate in support of cell-free protein synthesis. Moreover, since the utilization of PEP leads to the accumulation of pyruvate, simple addition of the cofactors to the conventional PEP-utilizing cell-free synthesis system substantially improved the efficiency of ATP supply through the secondary ATP regeneration from pyruvate (19). It was also shown to be beneficial to include sodium oxalate, an inhibitor of PEP synthase, in the reaction mixture as it helped retard the non-productive degradation of PEP. Using this ATP regeneration scheme, which was referred to as the PANox system, approximately 300 mg/ml of protein was generated in a single batch reaction.

GLYCOLYTIC INTERMEDIATES AS AN ENERGY SOURCE FOR ATP REGENERATION

Studies on the utilization of pyruvate as an energy source revealed that much of the endogenous enzymes related to energy metabolism remain active in the cell extract. Since pyruvate and PEP are the downstream products of the glycolytic pathway, the successful results of PANox system also inspired the utilization of glycolytic intermediates as an energy source. Accordingly, glucose 6-phosphate (G6P), the first intermediate of the glycolytic pathway has been examined for the possibility of using the glycolytic intermediates as energy sources to fuel protein synthesis machinery. Indeed, when G6P was used under the same reaction conditions as in the PANox system, it supported protein synthesis with a remarkably extended ATP supply. The ATP regeneration with G6P indicates that all of the glycolytic enzymes required to convert G6P into pyruvate are active under the reaction conditions for cell-free protein synthesis. This

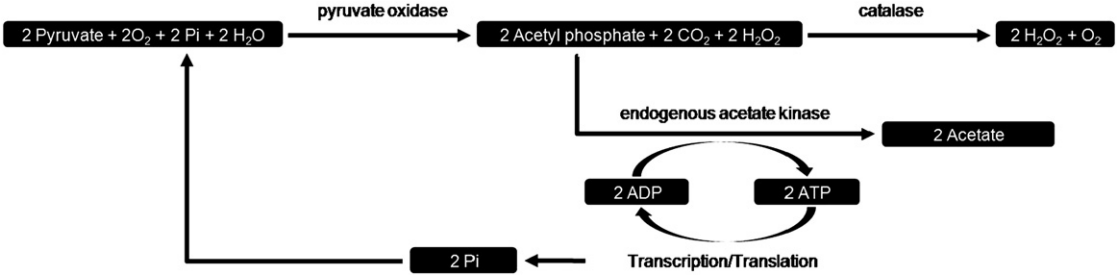

FIG. 2. Use of pyruvate and pyruvate oxidase (Pox) for ATP regeneration during cell-free protein synthesis.

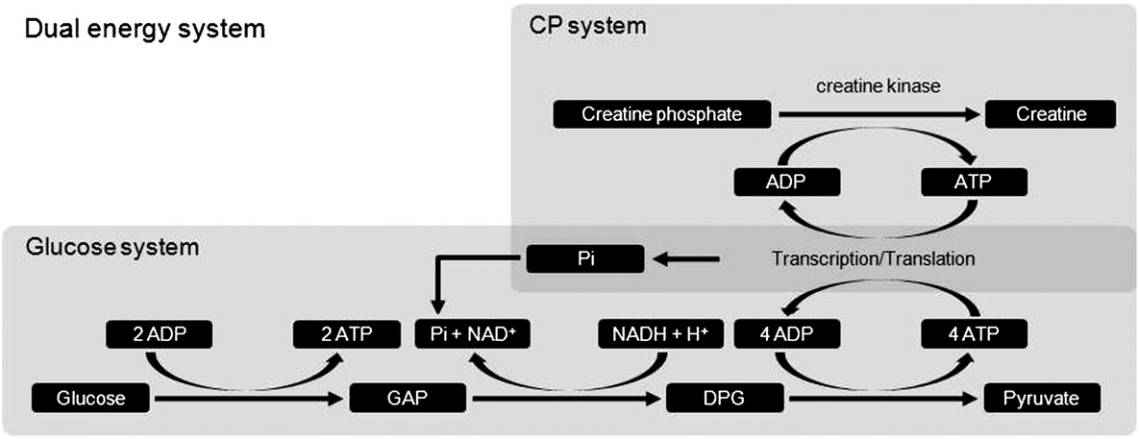

FIG. 3. Combined use of energy sources in the dual-energy system. In the proposed scheme for ATP regeneration, inorganic phosphate from creatine phosphate is recycled for the phosphorylation of a glycolytic intermediate. Therefore, the accumulation of phosphate into the reaction mixture is retarded, and more ATP molecules are provided. Cr, creatine; CP, creatine phosphate; CK, creatine kinase; GAP, glyceraldehyde-3-phosphate; DPG, 1,3-diphosphoglycerate; Pyr, pyruvate; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate.

provides great flexibility when choosing a secondary energy source for protein synthesis. Indeed, it has been shown that the glycolytic intermediates such as FBP, 3PGA could serve as efficient energy sources (19, 20).

USE OF GLUCOSE TO ENERGIZE CELL-FREE PROTEIN SYNTHESIS

In continuation of the works to search for more economical and stable energy sources to energize cell-free synthesis, recently, it was reported that even glucose supports ATP regeneration during cell-free protein synthesis. Although initial attempts with glucose were not successful in producing proteins as much as with other glycolytic intermediates, Calhoun and Swartz found this was due to a rapid decrease of pH in the reaction mixture (21). The productivity of glucose-utilizing cell-free protein synthesis was substantially improved by employing an appropriate buffer system. More recently, Kim and Kim also demonstrated that mg amounts of proteins could be generated by fortifying the buffering capacity of the reaction mixture that used glucose as an energy source (22).

DUAL ENERGY SYSTEM

Use of glucose as a cheaper energy source offers a prospect for the application of cell-free protein synthesis in the production of industrial proteins. During its conversion into pyruvate through the glycolytic pathway in the S30 extract, one molecule of glucose can generate 2 molecules of ATP, and there are sufficient evidences showing that at least part of pyruvate molecules are consumed for ATP regeneration through oxidative phosphorylation pathway. However, since the consumption of ATP is required to prime the glycolytic pathway, ATP concentration in the reaction mixture is relatively low during the initial phase of incubation. A compromised approach was designed by Kim et al. to handle the issues of ATP supply and phosphate accumulation simultaneously (Fig. 3; 23). Through the use of a mixture of creatine phosphate and glucose for the regeneration of ATP, inorganic phosphate from creatine phosphate was recycled to drive glycolytic pathway, thereby generating additional ATP molecules with a retarded rate of phosphate accumulation during cell-free protein synthesis. As a result, protein synthesis was prolonged up to 3 h, accompanied by an enhanced yield of protein synthesis. The amount of the synthesized protein (CAT, chloramphenicol acetyltransferase) was 2–3 times higher than that from the reactions using creatine phosphate or glucose as the sole energy source.

CONCLUDING REMARKS

Despite its versatility as a rapid route to protein expression, the requirements for expensive reagents have limited the applications of

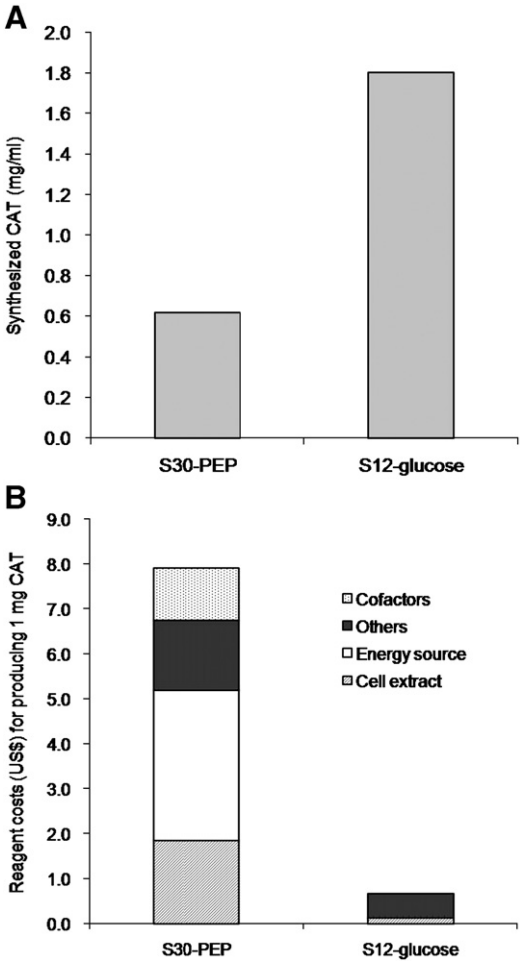

FIG. 4. (A) Productivity of cell-free protein synthesis using S30-PEP or S12-glucose. (B) Comparison of the total reagent costs for producing 1 mg protein (CAT) in S30-PEP and S12-glucose cell-free protein synthesis reactions.

cell-free protein synthesis, especially when large amounts of proteins are required. Unlike the fermentation processes where transformed cells produce recombinant proteins by utilizing substrates and energy generated *in vivo* from inexpensive carbon- and nitrogen sources, cell-free synthesis methods has required the addition of expensive substrates as well as the isolated translation machinery to carry out amino acid polymerization.

In this review, we surveyed the recent achievements in the development of more efficient and economical ATP regeneration methods to energize cell-free protein synthesis. Starting from the direct use of phosphate bond-containing chemicals (acetylphosphate, PEP and creatine phosphate), various attempts have been made to minimize cost and maximize protein productivity. At the present, glucose seems to be the most promising energy source for conducting cell-free protein synthesis in an industrial scale. The cost for glucose is almost negligible compared to conventional energy sources and protein synthesis reaction lasts longer since inorganic phosphate is not accumulated in the reaction mixture. While the requirements for cofactors (NAD and CoA) can offset the economical benefits of using glucose substantially, recently reported S12 extract system allows glucose to be used without the additions of cofactors, thereby dramatically improving the economical feasibility of cell-free protein synthesis (24). Unlike the typical S30 extract, preparation of the S12 extract does not involve a dialysis step, and the residual cofactors in the S12 extract are sufficient to catalyze the oxidation of glucose during protein synthesis. The S12 extract brings additional benefits to the economics and scalability of cell-free protein synthesis by reducing the overall processing time and cost for extract preparation. Mainly by skipping the pre-incubation and dialysis steps, the time and reagent cost for extract preparation was reduced by 60% and 80%, respectively. Compared to the conventional reactions using PEP in the S30 extract, use of glucose in the S12 extract reduces the cost for cell-free protein synthesis as much as 12 folds (Fig. 4).

We expect that the productivity of cell-free protein synthesis can further be improved through genetic or biochemical approaches to direct more ATP molecules towards protein synthesis. This will include the development of customized *E. coli* strains whose extract carries minimal activities of non-specific degradation of energy sources.

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