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Improvement of *Escherichia coli* Cell-Free System by Utilization of Cell Extract Having Additional Property

Problems and Countermeasures

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ABSTRACT

In the *Escherichia coli* cell-free system, the modification of cell extract can be achieved by preparation of the strains carrying additional property or those being induced with a certain gene expression prior to harvesting. In this study, we analyzed the cell-free system with S30 extract containing T7 RNA polymerases (S30 extract-T7pol) prepared from *E. coli* BL21(DE3) strain, which includes T7 RNA polymerase from extrinsic genes by IPTG induction, as a model for the improvement of the cell-free system. The fact that a significant degree of mRNA degradation was observed in the cell-free system with S30 extract-T7pol indicates the increase of ribonuclease activity was an unfavorable influence derived from the cell-extract modification process. We also showed that this influence was settled by the addition of an effective ribonuclease inhibitor, such as copper (II) ion, to the reaction mixture.

Index Entries: Cell-free system; copper ion; ribonuclease; *Escherichia coli*; protein synthesis.

INTRODUCTION

The cell-free protein synthesis system is an effective tool for the preparation of gene products. Both the prokaryotic system using *Escherichia coli* lysate (*1*) and the eukaryotic system using wheat germ lysate (*2*)

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or rabbit reticulocyte lysate (3) have been developed. Moreover, the continuous-flow, cell-free (CFCF) system developed by Spirin and his colleagues (4,5) has achieved the continuous protein synthesis and opens up new possibilities for research and biotechnology (6,7). Since the *E. coli* cell-free system can be conducted by coupled transcription and translation and does not need synthesized mRNAs prior to protein synthesis reaction, it is more useful than the eukaryotic cell-free system. The *E. coli* system also has the possibility that the cell extract is improved by using different strains that have superior property or the cells that expressed the extrinsic genes and accumulated their products. We are, therefore, developing a procedure for improvement of the cell-free system by introducing a cell extract that has an extra function.

In the Zubay method, the cells are harvested in the midlog phase to confer the active ribosomes on cell extract, and the ribonuclease-deficient strains, such as MRE600 (8) and Q13 (9), are used for the source of cell extract to minimize the degradation of RNAs by ribonucleases. By using such cell extract in the thus far cell-free system, the cell-free system has only the properties from such ribonuclease-deficient strains in spite of having high protein synthesis activity. The extra function, however, can be added to cell extract by preparing from *E. coli* strains having extra functions or the expression of interesting genes in the cells.

Since in this improvement we may use *E. coli* strains that are not ribonuclease-deficient strains, or in some cases the expression of interesting genes may be induced after midlog phase, it is predictable that a harmful influence, such as the inactivation of factors relating with protein synthesis or the accumulation of ribonucleases, will occur during the cell-extract modification process. In this study, we used *E. coli* BL21(DE3), which has all ribonuclease activity and carries T7 RNA polymerase genes in chromosomes (10), as the source of cell extract. The expression of T7 RNA polymerase was induced by IPTG after the cell growth reached to midlog phase. Using the cell extract prepared from the BL21(DE3) cells that expressed T7 RNA polymerase, we defined the influence of the usage of ribonuclease-nondeficient strain and the extra gene expression on the cell-free system. We also discussed the effective countermeasures for a disadvantageous influence introduced by the cell-extract modification.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The S30 extract containing T7 RNA polymerase (S30 extract-T7pol) was prepared from *E. coli* BL21(DE3) cells, which expressed T7 RNA polymerase by isopropyl-1-thio-β-D-galactoside (IPTG) induction (11), by the Zubay method (12) with modifications given by Pratt (13). The

plasmid pT7CT contained a chloramphenicol acetyltransferase (CAT) gene under control of T7 promoter, and a Tϕ transcriptional terminator was inserted downstream of the CAT gene. The plasmid pT7CT also contained a *bla* gene.

Transcription and Translation in the Batch System

A typical reaction mixture for a batch system contained, in a total volume of 50 μL, 55 m*M* Tris-acetate (pH 8.2), 70 m*M* KOAc, 32 m*M* NH_4OAc, 10 m*M* $Mg(OAc_2)$, 2 m*M* dithiothreitol (DTT), 10 m*M* phosphoenolpyruvate, 20 μg/mL folinic acid, 2 m*M* ATP, 0.5 m*M* each of GTP, CTP, and UTP, 0.2 m*M* each of 20 amino acids, 1.6% polyethylene glycol-6000, 10 μg/mL rifampicin, 0.2 mg/mL *E. coli* total tRNA (Boehringer Mannheim), 3.8 U/ml pyruvate kinase (Sigma), 15 μg/mL plasmid pT7CT, and an indicated amount of S30 extract-T7pol. Copper (II) acetate was added to the reaction mixture for some experiments as indicated. When mRNAs and proteins were analyzed by the incorporation of radioactive precursors, 308 n*M* [^{3}H]UTP (Amersham, 48 Ci/mmol) and 92.5 μ*M* [^{14}C]leucine (Amersham, 312 Ci/mol), respectively, were added to the reaction mixture. The reaction mixture was incubated at 37°C.

The Protein Synthesis in the CFCF System

The CFCF-coupled transcription and translation system was carried out in a 1-mL reaction chamber that was fitted with a YM100 filter (Amicon) as described before (*11*). The feeding solution, which contained the same components of the reaction mixture except *E. coli* total tRNA, pyruvate kinase, plasmid, and S30 extract, was fed into the chamber from the bottom to the top using an HPLC pump at a 2 mL/h feeding rate. The solution, which filtered through the YM100, was collected every hour.

Analytical Methods

The amount of mRNA was quantified by measuring the [^{3}H]UTP incorporation as described (*11*). The protein synthesis was measured by the [^{14}C]leucine incorporation into acid-insoluble fraction, and CAT activity was measured as described earlier (*11,14*). The synthesized bands have been identified as CAT protein on SDS gel.

RESULTS AND DISCUSSION

The Transcription and Translation in the S30 Extract-T7pol System

The cell-free coupled transcription and translation system using S30 extract-T7pol and pT7CT was conducted. The concentration of S30 extract-

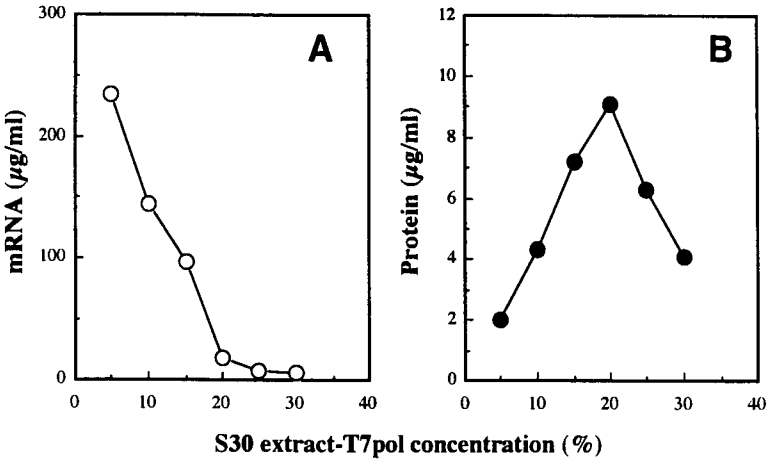

Fig. 1. Effect of S30 extract-T7pol concentration on mRNA (**A**) and protein (**B**) synthesis in the batch system using S30 extract-T7pol and pT7CT. After 40 min of incubation at 37°C, mRNA and protein synthesis were determined using [^{3}H]UTP and [^{14}C]leucine incorporation, respectively.

T7pol was varied from 5 to 30%. The amount of mRNA and protein synthesized was determined by [^{3}H]UTP and [^{14}C]leucine incorporation, respectively. The results in Fig. 1A show that the amount of mRNA, which was synthesized and remaining in the reaction mixture after 40 min of incubation, decreased remarkably with increase of S30 extract-T7pol concentration. The total amount of protein synthesized after 40 min of incubation increased along with an increasing amount of S30 extract-T7pol up to 20% and then decreased (Fig. 1B). On the contrary, in the case where the cell-free system was conducted with S30 extract from *E. coli* Q13 (HfrH RNaseI$^-$ Met$^-$ Tyr$^-$ [*1*]) (*9*), which was prepared by the modified Zubay method, both amounts of mRNAs and proteins synthesized increased with increase of the S30 extract concentration until it reached 30% (data not shown). These results suggest that S30 extract-T7pol contains higher ribonuclease activity than S30 extract. The amount of synthesized protein in the S30 extract-T7pol system, however, was almost the same level with that in the S30 extract system until the cell-extract concentration reached 20%. Since the amount of mRNA thought to exceed the translational ability and the protein synthesis may be dependent on the translational ability under these cell-extract concentrations, it is implied that S30 extract-T7pol has an equal level of translational ability to S30 extract.

The CFCF Coupled Transcription and Translation in the S30 Extract-T7pol System

In the previous study, we have shown the CFCF system using S30 extract-T7pol and pT7CT continuously produced CAT protein for 15 h (*11*). In the experiment, the CFCF system was conducted under the con-

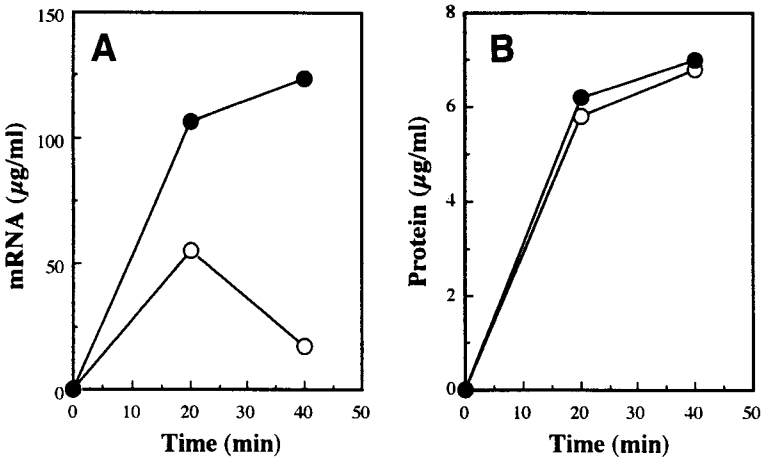

Fig. 2. The mRNA synthesis and remaining (**A**) and protein synthesized (**B**) in the batch system using S30 extract-T7pol and pT7CT, in the absence (open circles) and presence (filled circles) of 0.5 m*M* copper (II) ion. The amount of mRNA synthesized and remaining was determined by [^{3}H]UTP incorporation. Synthesized protein was measured by [^{14}C]leucine incorporation.

dition where the concentration of S30 extract-T7pol was 10% in the reaction mixture. Though the maximum CAT protein synthesis was observed at the S30 extract-T7pol concentration of 20% in the batch system, in the CFCF system the synthesized CAT contained in the fed-out solution was detected during only 10 h of reaction, and the amount of protein synthesized decreased with the reaction time (Fig. 3, white bar). These results indicate that the mRNAs in the reaction chamber might be gradually degraded by the higher ribonuclease activity from S30 extract-T7pol, and the protein synthesis diminished as with the reaction time and terminated at 10 h in the CFCF system with 20% of S30 extract-T7pol.

The S30 Extract-T7pol System Conducted with Copper (II) Ion

We have recently shown that cooper (II) ion acts as an inhibitor of ribonucleases in the cell-free system (*15*). In the previous study, we used *E. coli* strain Q13, which lacks the activity of typical nonspecific ribonuclease (*E. coli* ribonuclease I) (*9*) as the source of S30 extract. In contrast, the *E. coli* strain BL21(DE3) used in this study has normal ribonuclease activity. To confirm that copper (II) ion also acts as an effective ribonuclease inhibitor in the S30 extract-T7pol cell-free system, we conducted the batch system in the presence of 0.5 m*M* of copper (II) ion.

The amount of mRNAs that was synthesized and remaining in the reaction mixture was remarkably increased by the addition of 0.5 m*M* copper ion (Fig. 2A). Since the protein synthesis was almost equal in the presence and the absence of copper (II) ion (Fig. 2B), the factors relating

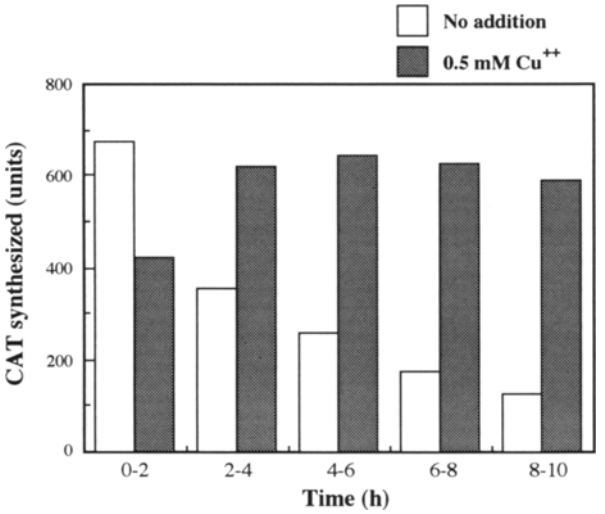

Fig. 3. The protein synthesis in the CFCF-coupled transcription and translation system using S30 extract-T7pol and pT7CT, in the absence (white bars) and presence of 0.5 m*M* copper (II) ion (black bars). S30 extract-T7pol concentration was 20% (40 A_{260} U/mL). Solution filtered out was collected every 1 h, and the CAT activity in each fraction was measured. The values show the total amount of protein synthesized during each 2 h of reaction period.

to protein synthesis might not be influenced by copper (II) ion at this concentration. It is supposed that the amount of synthesized protein did not increase in the presence of copper ion because the amount of mRNAs might exceed the translational ability even in the cell-free system, which was conducted in the absence of copper ion. These results indicate that copper (II) ion can effectively reduce the ribonuclease activity, including *E. coli* ribonuclease I, and it prevents the mRNA degradation in the S30 extract-T7pol system.

We also examined the CFCF system using S30 extract-T7pol in the presence of 0.5 m*M* of copper (II) ion. The S30 extract-T7pol was added to a final concentration of 20%. The results in Fig. 3 show that the protein synthesis continued well for at least 10 h in the CFCF system with copper ion (black bars), whereas the protein synthesis did not continue in the CFCF system conducted at this concentration without copper (II) ion (white bars). These results clearly indicate that copper (II) ion can prevent the mRNAs degradation by higher ribonuclease activity from the cell extract and stabilize the protein synthesis in the CFCF system in which the cell extract having high ribonuclease activity is used.

CONCLUSION

In this article, we have demonstrated that the high ribonuclease activity is the most unfavorable influence derived from the improvement of

the cell-free system by introducing the cell extract having an extra function. The reduction of the ribonuclease activity, therefore, is the key to the success in the cell-free extract modification. The addition of 0.5 m*M* of copper (II) ion is effective for the prevention of mRNA degradation. Any reactions related to protein synthesis were not influenced by copper (II) ion at this concentration (*15*). We then showed that the cell-free system using modified cell-free extract, which contained higher ribonuclease activity, was successfully conducted by reducing the ribonuclease activity. Thus, the cell-free system using the modified cell-free extract can perform its own effectiveness satisfactorily by combining with the method of the reduction of ribonuclease activity.

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