An Increased Rate of Cell-free Protein Synthesis by Condensing Wheat-germ Extract with Ultrafiltration Membranes

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Wheat-germ extract for cell-free protein synthesis was condensed with ultrafiltration membranes of which the molecular cut-off values were 10 kDa, 100 kDa, and 300 kDa. Reaction conditions of the cell-free system were optimized for the condensed extracts, which needed a higher concentration of creatine phosphate than the uncondensed one, probably due to the increased activity of degradation of ATP and GTP. By using the condensed extract and optimized reaction conditions, the rate of protein synthesis was increased 2- to 3-fold compared with using an uncondensed extract, and about 10-fold compared with conventional conditions. Condensation of the extract with the 300-kDa membrane showed the highest productivity, which was about 30 μg dihydrofolate reductase protein ml⁻¹ h⁻¹. The final amount of synthesized protein was one third of that of a continuous-flow cell-free (CFCF) system reported by Endo et al. [J. Biotechnol., 25, 221-230 (1992)] but the productivity was 5-fold higher than that obtained by the CFCF system.

Cell-free protein synthesis from a cloned DNA fragment presents an alternative way to obtain the translated product without using living cells. Cell-free systems can avoid so many problems such as aggregation of products and cell lysis caused by expression of a toxic gene product. In addition, incorporation of modified and unnatural amino acids into protein has been made possible.²¹ The cell-free system itself was developed in the 1960s, however, a small amount of products has been the primary disadvantage for its application. To overcome this problem, Spirin and co-workers have developed a continuous-flow cell-free translation system, called CFCF in 1988.³¹ In this system, the protein synthesis could be maintained at a constant rate for more than 20 hours. Their results have been recently repeated by other laboratories.¹,⁴,⁵

All of this research was concentrated on extending the period of protein synthesis, but not on increasing the rate of protein synthesis. However, it is very important to shorten the period to get products, particularly in cell-free protein synthesis, because unstable substrates such as nucleotide triphosphates and mRNA are involved. In addition, degradation of products should also be avoided. In this study, we have tried to increase the rate of cell-free protein synthesis by condensing translational extracts prepared from wheat-germ by using an ultrafiltration membrane. We report here that condensing the extract of cell-free system increases the rate of protein synthesis two- to three-fold after readjustment of the reaction conditions.

Materials and Methods

Preparation of wheat-germ extract and its condensation with ultrafiltration membranes. Wheat germ was kindly supplied by Dr. S. Sekiguchi of Nippon Flour Mills Co., Ltd. The extract of wheat-germ for cell-free protein synthesis was prepared as described by Anderson and co-workers⁶⁴ with minor modifications. Wheat germ (4 g) was ground in a chilled mortar with an equal weight of sea sand and 8 ml of a solution containing 20 mM HEPES (pH 7.6, adjusted with KOH), 100 mM potassium acetate, 5 mM magnesium acetate, 2 mM CaCl₂, and 1 mM diethiothreitol (DTT). The homogenate was centrifuged at 30,000 × g for 5 min at 4°C, and its supernatant was passed through a Sephadex G-25 column (2 × 30 cm) equilibrated with 20 mM HEPES (pH 7.6), 120 mM potassium acetate, 5 mM magnesium acetate, and 1 mM DTT at a flow rate of 2-3 ml/min. A fraction of which the absorbance at 260 nm was more than 90 was pooled and recentrifuged at 30,000 × g for 5 min at 4°C. The supernatant was used as a standard extract. The extract of 5 ml was condensed with an ultrafiltration membrane (PM10, YM100, XM300, Amicon, U.S.A.) in a chamber (Model 8050, Amicon) under a nitrogen atmosphere at 4°C for 1 h. The protein concentration of extract was increased about 2.5-fold in each run. All extracts were dispensed into 1.5-ml tubes and immediately stored in liquid nitrogen.

Preparation of mRNA. Plasmid pGEM-DHF-1, which had the dihydrofolate reductase (dhfr) gene of Escherichia coli K-12 under a Sp6 promoter, was used as a template DNA (unpublished result). An mRNA with cap analog was transcribed by using a kit of MEGAscript (Ambion, U.S.A.) as described by Krieg and Melton.²² The transcribed mRNAs were purified with a Nick Spin column (Pharmacia, Sweden), followed by extraction with phenol/chloroform and precipitation with ethanol. The purified RNAs were measured chromatographically using an ion-exchange column (TSK-gel DEAE-NPR, Tosoh, Japan).

Cell-free translation system. An initial reaction mixture consisted of 20% volume of extract, 20 mM HEPES (pH 7.6), 100 mM spermidine, 20 μM spermine, 2 mM DTT, 8 mM creatine phosphate, 40 μg/ml creatine phosphokinase (Boehringer-Mannheim), 1 mM ATP, 0.1 mM GTP, 40 μM each amino acid, 1000 U/ml RNase inhibitor (Toyobo, Japan), 75 mM potassium acetate, 0.5 mM magnesium acetate, and variable concentration of in vitro-synthesized mRNA.²³ S-labeled methionine was added for measurement of amino acid incorporation. The concentrations of HEPES, amino acids, creatine phosphate, and extract were varied as indicated.

Analytical methods. The product synthesized in cell-free translation was analyzed by the following two methods. The amount of amino acid incorporated into protein was measured by hot trichloroacetic precipitation.²⁹ Radioactivity was measured by liquid scintillation counting. Alternatively, the enzymatic activity of translated product, dihydrofolate reductase (DHFR), was measured as previously described.¹⁹ One unit of DHFR activity is defined as the amount of enzyme required to reduce 1 μmol of dihydrofolate per min based on a molar extinction coefficient of 12.3 × 10³.³⁴ To measure the turnover number of DHFR synthesized in cell-free system, enzymatic activities with various concentrations of methotrexate were measured by the method of Poe et al.²¹

The translated product was also confirmed by SDS polyacrylamide gel followed by immunoblotting using anti-DHFR serum as described previously.¹⁶

Protein concentration was measured using a Protein Assay Kit (Bio-Rad...
Lab., U.S.A.) with bovine serum albumin as the standard. Acid phosphatase activity of a cell extract was measured using p-nitrophenol phosphate as a substrate as described by Uehara et al.\textsuperscript{14} One unit of the enzyme activity was defined as the amount of enzyme that releases 1 μmol of p-nitrophenol per min at 35°C.

**Results**

**Optimization of reaction conditions for condensed extract by ultrafiltration**

First of all, to know the optimum ratio of a wheat-germ extract to a reaction mixture in cell-free protein synthesis, translated DHFR protein was measured by incorporation of radio-labeled methionine at various concentrations of extract. Figure 1 indicates that the optimum ratio of extract was 30% of the total reaction mixture, and that further increases of this ratio slightly decreased the methionine incorporation. Hence the ratio of extract to reaction mixture was mostly 30% in the subsequent experiments.

Wheat-germ extract passed through a Sephadex column was condensed with an ultrafiltration membrane, PM10, of which the molecular cut-off size was 10 kDa. Protein concentrations of uncondensed and condensed extracts were 20 and 71 mg/ml, respectively. Then protein synthesis using the condensed extract was measured by synthesized DHFR activity. However, there was no increase in the amount of protein synthesized by using the condensed extract compared with the uncondensed one under the reaction conditions listed in Materials and Methods, which were based on the description of Anderson and co-workers (data not shown). Thereby it seemed likely that increasing the mass of extract in the reaction mixture caused lack of some factor(s) or substrate necessary for translation. Then to improve the translational capability of the condensed extract, several reaction conditions were examined.

First, buffer conditions were examined. During two hours of incubation, the pH of translation mixture decreased gradually from 7.6 to 7.4, when the concentration of HEPES was 20 mM. The HEPES concentration of 60 mM was large enough to maintain the pH of the translation mixture for at least three hours.

Next, concentrations of nucleotide triphosphates and amino acids were examined. The translation rate was sensitive to ATP concentration. Either higher or lower concentrations than 1 mM caused a significant decrease in protein synthesis as previously reported by many researchers. The extract concentration did not vary the optimum value of ATP. By contrast, effects of GTP concentration on protein synthesis were dependent on whether the extract was condensed or not. When the uncondensed extract was used, varying GTP concentration from 50 μM to 1 mM had no effect on protein synthesis. When the condensed extract was used, however, the rate of protein synthesis was maximum at 100 μM GTP, and either lower or higher concentrations of GTP reduced protein synthesis substantially (data not shown). Thereby the GTP concentration was fixed at 100 μM in this study, though the reason of the difference of the dependency on GTP concentration between the two extracts remains unclear. Varying the concentrations of amino acids had no striking effects on protein synthesis, but a higher concentration seemed preferable (data not shown).

Finally, protein synthesis reactions were done with various creatine phosphate concentrations under thus improved conditions as shown in Fig. 2a). The condensed extract had a higher optimum concentration of creatine phosphate than an uncondensed extract. Figure 2b) shows a course of protein synthesis in 8 mM or 20 mM creatine

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Effects of Condensation of Extract on Translation.

Reactions were done in 20 μl containing 7.5 μg ml \textit{dfr} mRNA, [\textsuperscript{15}S] methionine at 30°C for 60 min. The protein concentration of the extract used in this experiment was 23.2 mg/ml.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Influence of Creatine Phosphate on Translation.

\( a \) Assay mixtures containing 30% volume of condensed (●) or uncondensed (○) extract with various concentrations of creatine phosphate were incubated for 1 h, and their DHFR activities were assayed.

\( b \) Course of protein synthesis at an optimum concentration of creatine phosphate. Assay mixtures containing 30% volume of condensed (●, ▲), or uncondensed (○) extract were incubated with 20 mM (○), or 8 mM (▲, ▼) creatine phosphate. The concentration of \textit{dfr} mRNA was 17 μg/ml.
Table I. Summary of Reaction Conditions for Cell-free Protein Synthesis with Wheat-germ Extracts

<table>
<thead>
<tr>
<th></th>
<th>Standard (Anderson)</th>
<th>For uncondensed extract</th>
<th>For condensed extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES KOH, pH 7.6</td>
<td>20 mM</td>
<td>60 mM</td>
<td>60 mM</td>
</tr>
<tr>
<td>ATP</td>
<td>1 mM</td>
<td>1 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>GTP</td>
<td>0.02 mM</td>
<td>0.1 mM</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>8 mM</td>
<td>8 mM</td>
<td>20 mM</td>
</tr>
<tr>
<td>Amino acids</td>
<td>0.025 mM</td>
<td>0.16 mM</td>
<td>0.16 mM</td>
</tr>
<tr>
<td>Extract</td>
<td>30%</td>
<td>30%</td>
<td>30%</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of Concentration of mRNA on Translational Reaction Using Condensed Extract.

Reaction mixtures with condensed with PM10 membrane (■) and uncondensed (○) extracts with various dhfr mRNA were incubated for 60 min and samples were taken for DHFR assay. The relative activity is expressed as a percentage of the maximum activity of the mRNA concentration used. Reaction conditions except for the concentration of mRNA are shown in Table I.

Fig. 4. Course of Protein Synthesis Using Condensed Extracts under Optimized Reaction Conditions.

Profiles of in vitro synthesized DHFR activity using uncondensed (○) and condensed extracts with XM300 (■), YM100 (▲), and PM10 (■) are shown. Reaction conditions were as listed in Table I. The concentration of dhfr mRNA was 28 μg/ml.

Table II. Protein Concentration and Acid Phosphatase Activity of Wheat-germ Extract

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Protein concentration (mg/ml)</th>
<th>Phosphatase activity (mU/ml)</th>
<th>Specific activity (mU/mg-protein)</th>
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</thead>
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<tr>
<td>Uncondensed</td>
<td>24.3</td>
<td>353</td>
<td>14.5</td>
</tr>
<tr>
<td>PM10</td>
<td>65.3</td>
<td>1100</td>
<td>16.9</td>
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<tr>
<td>YM100</td>
<td>66.8</td>
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<td>15.4</td>
</tr>
<tr>
<td>XM300</td>
<td>66.8</td>
<td>858</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Fig. 4 shows a course of protein synthesis using the condensed extract with PM10 membrane and the improved conditions with optimized mRNA concentration (28 μg/ml). By these modifications, condensing wheat-germ extract increased both the protein synthesis rate and the final amount of the product.

Condensation of wheat-germ extract with other membranes

Wheat-germ extract was condensed with YM100 membrane (molecular cut-off value is 100 kDa), or XM300 (molecular cut-off value is 300 kDa) under the same conditions for the PM10 membrane. There was no significant difference in the protein concentration among these condensed extracts and that with PM10 (Table II), as well as in optimum reaction conditions. Figure 4 shows a course of protein synthesis using thus condensed extracts. The extract condensed with YM100 showed a similar profile as that with PM10. By contrast, the extract condensed with XM300 was more active than others.

As seen in Fig. 2, protein synthesis was greatly dependent on the creatine phosphate concentration, so that the phosphatase activity of extracts seemed to be one of the key factors that determine the productivity of cell extracts. Although there are many phosphatases that degrade ATP and GTP in wheat-germ extract under the conditions of cell-free protein synthesis, the rate of ATP consumption was increased at lower pH (data not shown) suggesting involvement of acidic phosphatase activity in the ATP consumption. Therefore we measured the activity of each extract as shown in Table II. XM300 extract had lower acid phosphatase activity than any other condensed extracts, and moreover its specific activity was lower than an uncondensed extract.

To convert DHFR activity into the total weight of the protein, the turnover number of in vitro-synthesized DHFR was measured by a titration assay with methotrexate as described in Materials and Methods. Since DHFR has only one site for binding methotrexate, the specific activity of DHFR was calculated to be 35 U/mg similar to that previously reported by Poe et al. Accordingly more than 30 μg protein was synthesized in a one-ml reaction mixture by using the condensed wheat-germ extract and optimized conditions.

To get direct evidence for the protein obtained in our system, 15 μl of the reaction mixture was electrophoresed on a polyacrylamide gel, followed by immunoblotting with anti-DHFR antibody, as described in Materials and Methods. Figure 5 shows that the reaction mixture containing approximately 0.5 μg DHFR protein was detected with the antisera (lane 3), but no band was detected without incubation (lane 2).
Fig. 5. Immunoblotting of in vitro Synthesized Protein against Anti-DHFR Antibody.
Fifteen µl of reaction mixture of cell-free protein synthesis at 0 hour (lane 2) and 80 min of incubation (lane 3) was electrophoresed and immunoblotted with anti-DHFR antibody. Lane 1 was 1.5 µg DHFR protein purified from E. coli cells.

Discussion

The results presented here demonstrate that the condensation of wheat-germ extract is able to increase the productivity of cell-free protein synthesis, if reaction conditions are optimized for the condensed extract. First we examined raising up the content of wheat-germ extract in a cell-free reaction mixture. Figure 1 shows that the amount of extract added to a reaction mixture has a limit. Any in vitro translation system has an optimum magnesium ion concentration around 1.5 mM. Since wheat germ extract is prepared in the presence of 5 mM magnesium, increasing the ratio of extract more than 30% leads to excess of magnesium. On the other hand decreasing the magnesium concentration in preparing extracts resulted in a significant loss of activity of the extract (data not shown). Therefore the maximum ratio of extract should be 30% of the total reaction mixture, which suggests a further increase in concentration of translational machinery is impossible without condensing extracts.

Under standard conditions described by Anderson and co-workers, the effects of the condensation of extract on translational activities were not seen. But the condensed extract needed a larger amount of creatine phosphate than the uncondensed extract to get maximum productivity. Our preliminary experiments showed that ATP was rapidly consumed by wheat-germ extract, and sequentially transformed to diphosphate-, monophosphate-, and nucleoside forms even in the presence of a translation inhibitor (data not shown). Some phosphatases are therefore likely to work in the reaction mixture. Condensation of extracts is thought to increase the amount of these enzymes in the reaction mixture. The rate of the ATP consumption might increase accordingly, consequently stopping translational reaction early as shown in Fig. 2b). In addition, the condensed extract needs a larger amount of mRNA than the uncondensed extract, as shown in Fig. 3, possibly due to the increase of ribonucleases in the condensed extracts.

Although molecular weights of most translational components involving initiation factors are less than 300 kDa, the highest productivity was obtained in the extract condensed with XM300 that passes proteins smaller than 300 kDa. XM300 membrane was also used in the CFCC system in which the translational reaction continued for more than 20 hours, suggesting that the factors necessary for translation are incorporated into a "super translational complex" during protein synthesis. Since a similar complex is thought to exist in vivo, it might remain during extraction and the condensation procedure.

Moreover, XM300 extract showed the lowest specific activity of acid phosphatase as listed in Table II. Although the low activity of acid phosphatase cannot totally explain the high productivity of this extract, it seems to represent the level of factors unfavorable to protein synthesis, such as nucleases and proteases. By using membranes with large molecular cut-off size, the relative amount of such factors to translational machinery might be reduced. Therefore, it is very important to know the molecular characteristics of phosphatases, nucleases, proteases, and other things that reduce cell-free protein synthesis, and to develop a method to quickly separate translational machinery from these factors, in order to get highly active extracts.

The amount of DHFR protein with the condensed extract using XM300 membrane was about 30 µg per one ml reaction volume in one hour. In the CFCC system, about 100 µg protein was obtained during 20 hours or more. Accordingly the total amount of products in our system was one third of that obtained in CFCC system, however, the productivity was 5-fold higher than the CFCC system.

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References