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A bilayer cell-free protein synthesis system for high-throughput screening of gene products

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Abstract A high-throughput cell-free protein synthesis method has been described. The methodology is based on a bilayer diffusion system that enables the continuous supply of substrates, together with the continuous removal of small byproducts, through a phase between the translation mixture and substrate mixture. With the use of a multititer plate the system was functional for a prolonged time, and as a consequence yielded more than 10 times that of the similar batch-mode reaction. Combining this method with a wheat germ cell-free translation system developed by us, the system could produce a large amount of protein sufficient for carrying out functional analyses. This novel bilayer-based cell-free protein synthesis system with its simplicity, minimum time and low cost may be useful practical methodology in the post-genome era.

Key words: Cell-free protein synthesis; Bilayer reaction method; *Escherichia coli* coupled transcription–translation; Wheat germ system; High-throughput screening

1. Introduction

A cell-free translation system has been known to have a capacity to synthesize proteins with high speed and accuracy approaching in vivo rates, and it can express proteins that would interfere with cell physiology [1,2]. However, they were generally inefficient because of their instability [3]. We have reported that one of the main causes for the instability of the wheat germ cell-free system is the contamination of wheat germ extract by several inhibitors during the extraction procedure [4,5]. After elimination of these inhibitors, the translation system became very efficient and stable. As we reported, when the cell-free translation was performed by using this extract in Spirin's continuous-flow cell-free protein synthesis system (CFCF), with continuous supply of substrates and removal of small byproducts [6], the translation reaction proceeded for more than 60 h yielding 0.8–4 mg of functionally active proteins per ml of reaction. Though Spirin's CFCF is efficient for materialization of genetic information using efficient cell-free systems such as those from *Escherichia coli* and wheat embryos, they may not be suitable for the massive screening of gene products from the cDNA catalogues. Since the apparatus is equipped with a semi-permeable membrane at the heart of the reaction chamber, the system is complicated mechanically, but for functional analysis of proteins a quantity of only tens of micrograms is sufficient. In contrast, the regular batch-mode reaction works for only a few hours and the amount of synthesized protein is not sufficient for a biochemical analysis. Therefore there is a need to invent a reaction system for the analysis of gene products from cDNA catalogues, which can be simpler and more cost-effective than CFCF and can synthesize sufficient protein quantities better than the batch-mode reaction.

In this article, we successfully demonstrate the bilayer translation method, which is a simplified version of CFCF, in which the substrate mixture is simply overlaid onto the translation mixture. The system was functional for more than 10 times as long when compared to batch-mode reactions, yielding a sub-milligram amount of proteins. The performance of this system and the synthesized protein quantities are high enough for functional analysis of gene products from various cDNA catalogues.

2. Materials and methods

2.1. General

The following procedures were either described or cited previously [4,5,7]: isolation of wheat germs and preparation of the extract, synthesis of mRNA (mRNAs consisted of the sequence cap-AAUACACGGAAUUCGAGCUCGCCCGGGAAAUCUC AAUG (the underlined sequence is the initiation codon) at its 5′-end, a coding sequence, and a 3′-non-coding region of 549 nt and with 100 nt of poly(A)), determination of RNA and protein, estimation of amount of protein synthesized by means of densitometric scanning of the Coomassie brilliant blue (CBB)-stained bands on the gel, and autoradiography. SP6 RNA polymerase, human placental ribonuclease inhibitor (133 U/ml), L-[U-^{14}C]leucine, creatine kinase, spermidine and the 20 amino acids, non-ionic detergent NP-40, and a gene encoding green fluorescent protein (GFP) (pCaMV35S-sGFP(S65T)-NOS3′).

2.2. Materials

An *E. coli* coupled transcription–translation kit (The Rapid Translation System, TRS_{500}) was purchased from Roche Molecular Biochemicals, Germany. Plasmids carrying genes encoding sarcosine oxidase from *Arthrobacter* sp. TE1826 (soxA) [8], *Bam*HI from *Bacillus amyloliquefaciens* H [9], KOD from archaeon *Pyrococcus* sp. strain KOD1 [10], and estrogen receptor protein (ER) from the human can-

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Abbreviations: CBB, Coomassie brilliant blue; FAD, flavin adenine dinucleotide; GFP, green fluorescent protein; KOD, archaeon *Pyrococcus* sp. strain KOD1 DNA polymerase; ER, estrogen receptor protein

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cer cell line MCF-7 [11] were kindly provided by Dr. T. Imanaka (Osaka University). Authentic *Bam*HI (specific activity 600 U/μg) and sarcosine oxidase (specific activity 0.0125 U/μg) were purchased from Toyobo Biochemicals, Japan.

2.3. Cell-free translation

Batch-mode GFP synthesis in the *E. coli* S-30 cell-free kit was carried out using vector GFP according to the instruction manual. Batch-mode wheat germ cell-free protein synthesis was performed as previously reported with slight modifications [5]. In brief, 25 μl of reaction mixture contained 12 μl of extract (thus 48% (v/v)); final concentrations of the various ingredients are: 24 mM HEPES/KOH pH 7.8, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, 0.45 mg/ml creatine kinase, 2 mM dithiothreitol, 0.4 mM spermidine, 0.3 mM of each of the 20 amino acids including [^{14}C]leucine (2 μCi/ml) where indicated, 2.7 mM magnesium acetate, 100 mM potassium acetate, 50 μg/ml of deacylated tRNA prepared from wheat embryos, 0.05% NP-40, 0.005% NaN_3 and 20 pmol of mRNAs. The extract was not treated with micrococcal nuclease since we did not observe any positive effect on the treatment. Incubation was done at 26°C. For the bilayer systems, as shown in Fig. 1A, the substrate mixture (50 μl), containing ingredients described above except for mRNA and creatine kinase, was carefully overlaid onto the translation mixture (10 μl) containing each 20 pmol mRNA in 96 wells of the titer plate and incubated at 26°C for the indicated period of time.

2.4. Determination of enzymatic activities of the products

Sarcosine oxidase (EC 1.5.3.1) was purified and activity was determined using the 4-amino-antipyrine peroxidase system [8]. Activity of the restriction enzyme *Bam*HI was measured using λDNA as a substrate, products were separated on 1% agarose gel and visualized by ethidium bromide [9].

3. Results and discussion

The drawbacks associated with cell-free translation systems, such as the low yield of translation product due to the presence of several inhibitors in the extract and the short duration of the translation reaction, have recently been overcome by eliminating the endogenous translation inhibitors [5] and using continuous flow reaction, CFCF [6]. As we reported earlier [5], our newly developed wheat germ cell-free system synthesizes the functionally active proteins efficiently and could be an efficient alternative system for the synthesis of gene products from a given cDNA catalogue. The principle of CFCF involves the continuous supply of amino acids and energy components and removal of small byproducts through a semi-permeable membrane that mimics the normal cell physiology. From a practical point of view, however, points such as the massive screening of gene products and the instrumentation of the process, there is a need to invent mechanically simpler methods rather than using such complicated membrane systems.

This was achieved by taking advantage of using a simple bilayer reaction method. As illustrated in Fig. 1A, the method is very simple: the substrate mixture was overlaid onto the translation mixture that contained the same ingredients as a conventional batch-mode reaction. We first tested the protein-synthesizing capacity of this bilayer apparatus by using the commercially available *E. coli* coupled transcription–translation system programmed with a gene encoding GFP. It was interesting to see translation in the bilayer apparatus that continued to incorporate [^{14}C]leucine into the protein for up to 17 h. Under similar conditions, when the reaction was performed using regular batch-mode protein synthesis, protein synthesis ceased after only a few hours (Fig. 1B). A similar incubation, but which was started after mixing of the two layers, resulted in a low incorporation. This might be simply due to shortage of mRNA supply followed by six times dilution of transcription elements in the reaction mixture. To examine this possibility, the experiments were designed in a way that allowed incubation first for 2 h (allowing synthesis and accumulation of mRNA in the batch-mode reaction), after which the substrate mixture was added and mixed well (arrow). As can be seen, significant improvement in protein synthesis was observed, indicating that the bilayer incubation method is in fact effective to keep the translation reaction going for longer periods, thus yielding large amounts of protein. Longevity of the bilayer system can be explained as it enables the continuous supply of substrates, and removal of small byproducts through a phase between the translation mixture and substrate mixture by diffusion. We have examined the ratio of the substrate mixture to the translation mixture systematically and have found that a 5:1 volume ratio gave the best result (results not shown). In order to compare the efficiency of the bilayer method with the other type, mRNA-directed protein synthesis, we then applied it to a wheat germ cell-free system. As shown in Fig. 1C, the bilayer system was efficient for a prolonged period of time yielding much larger amounts of product compared to the batch-mode system. When we used another well having a vessel diameter of 7 mm (0.385 cm^2), instead of 3 mm (0.071 cm^2), the productivity was significantly increased (Fig. 1C). The results can be explained by the fact that a larger surface area between the phases could lead to a better exchange of substrates and small byproducts, thus resulting in a better protein synthesis efficiency. Thus both the ratio of volume of the substrate mixture to that of the translation mixture and the area between the two phases are important parameters for the efficiency of the method.

After establishing the performance of the bilayer system using GFP as a model gene, we next addressed a practical application of the method. To do this, we selected several genes whose products are sometimes difficult to express in vivo. Those are genes for *Bam*HI from *B. amyloliquefaciens* H as an example of restriction enzymes, sarcosine oxidase from *Arthrobacter* sp. TE1826 (soxA), KOD from archaeon *Pyrococcus* sp. strain KOD1, and ER from the human cancer cell line MCF-7. Each mRNA, having the cap and poly(A) tail transcribed from the expression plasmid carrying one of the cDNAs, was translated in the bilayer reaction system in the same way as above. The reaction mixture was incubated for 15 h and was then separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). As shown in Fig. 2A, CBB staining already indicates intense bands on the gels (arrowheads), indicating each of the proteins was produced in a large amount. Although CBB staining did not show a clear ER band due to overlap with an endogenous protein band (lane 5), the autoradiogram clearly shows the product (lane 6). The amount of products in 3 μl reaction volume (which corresponded to 0.5 μl in the batch reaction) estimated by densitometric scanning of each band was as follows: 0.2, 0.1, 0.2 and 0.1 μg for sarcosine oxidase, *Bam*HI, KOD and ER respectively. We checked the solubility of the products by brief centrifugation (30 000 × *g* for 15 min) and found that all of the products were recovered in the supernatant fraction (data not shown). These results demonstrated that the present method is capable of materializing the genetic information in an amount that is sufficient for the functional

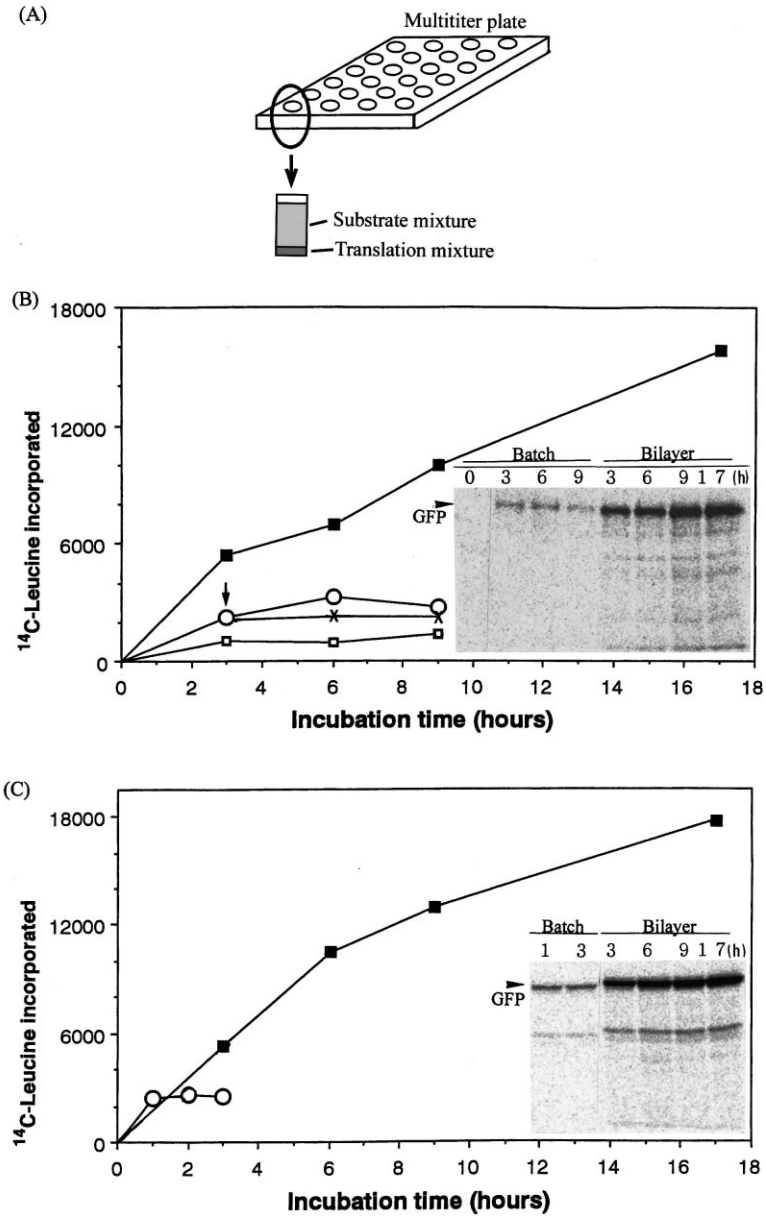

Fig. 1. A novel bilayer cell-free protein synthesis. A: Schematic illustration of the method. GFP syntheses were performed using *E. coli* S-30 coupled transcription–translation system (B), and wheat germ cell-free system as described in Section 2 (C). In both experiments incubations were carried out by regular batch-mode reaction (□), the bilayer method (○), bilayer but mixed (■), and first 2 h by batch-mode then overlaid substrate solution onto the translation mixture (×). For the measurement of [^{14}C]leucine incorporation, samples were vortexed and hot trichloroacetic acid-insoluble radioactivity in 5 μl in the batch reaction or 30 μl in the bilayer reaction, thus adjusted amount of extracts in each system. The diameter of wells used was 3 mm unless noted in the text. The inset shows autoradiograms and arrowheads marks GFP.

analysis, and is a way to check the solubility without using radiolabeled amino acids. One of the most commonly asked questions for cell-free translation systems is whether the system produces functionally active protein. We addressed this point directly by determining enzyme activities of synthesized products. As shown in Fig. 2B,C, when purified sarcosine oxidase was incubated in the presence of flavin adenine dinucleotide (FAD), the protein has practically the same level of specific activity as the authentic enzyme produced in vivo (B). Another interesting observation is that the activity of the enzyme was completely dependent on added FAD, indicating the enzyme synthesized was in the apo-form rather than the holo-enzyme. In view of the fact that one can obtain only those proteins as holo-enzymes when expressed in vivo, the cell-free system provides a valuable tool for the preparation of enzymes in the apo-form. Some of the enzymes are toxic to host cells when expressed in vivo and it has been necessary to have a suitable design of the system. Restriction enzymes are one of those examples. The assay of enzyme activity of the synthesized *Bam*HI without purification showed that the protein retained full activity and produced identical digestion patterns as the commercially available one (Fig. 2C). In fact, incubation with systematically diluted samples of the substrate and subsequent densitometric determination of the DNA digestion confirms that the cell-free translated product has about two-fold higher specific activity than the authentic

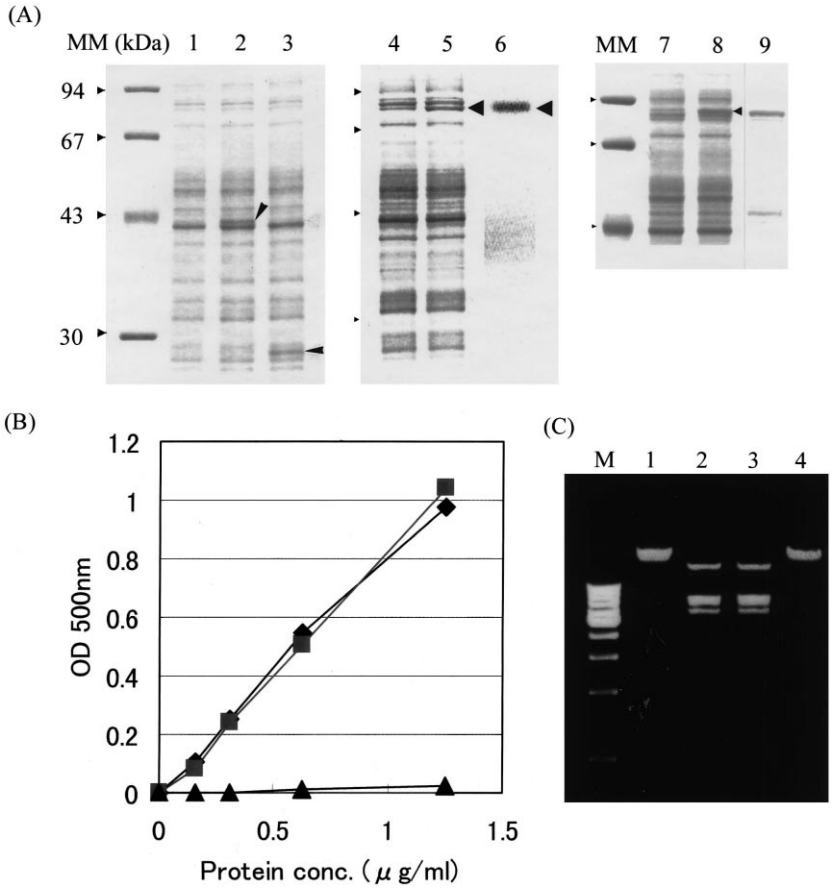

Fig. 2. High-throughput materialization from cDNAs (A) and their enzymatic activities (B,C). A: Incubations were carried out separately in the bilayer systems for 15 h using wells (diameter 7 mm). Reaction mixtures were vortexed and proteins in each 6 μl of samples was separated on SDS–PAGE and stained with CBB. Sarcosine oxidase (lane 2), *Bam*HI (lane 3), ER (lane 5, and lane 6 is its autoradiogram), KOD (lane 8, and lane 9 is 20 000×*g* supernatant fraction from 75°C heat-treated sample), and lanes 1, 4, 7 are from samples incubated without mRNA. Arrowheads marked each gene product. B: Sarcosine oxidase purified from the reaction mixture and enzyme activity was measured in the presence (■) or in the absence of FAD (▲). Authentic enzyme was determined in the absence of FAD (◆). C: *Bam*HI activity assayed with λDNA as a substrate. Molecular weight markers (lane M), λDNA (lane 1), substrate was incubated with the reaction mixtures carried out in the presence (lane 2) or absence of mRNA (lane 4). The amount of the product was estimated by densitometric scanning of the band on the gel and its equivalent amount of authentic *Bam*HI (2 ng) was assayed similarly (lane 3) as a control.

enzyme (data not shown). In addition, it is worth mentioning here that there was very little digestion of the λDNA substrate when the assay was performed using reaction mixture incubated in the absence of mRNA. This result confirms that our wheat extract contained little deoxyribonuclease, if any. This characteristic is extremely valuable towards the invention of PCR-directed efficient cell-free protein synthesis. Since linear DNA is unstable against deoxyribonuclease attack, invention of cell-free systems that contain very low activity has been awaited.

A methodology for high-throughput materialization of genetic information through massive screening is an essential step towards the post-genome era. Our improved wheat germ system, together with the present bilayer cell-free system, may provide a means for inventing an automated instrumentation of cell-free protein synthesis for high-throughput screening of cDNA catalogues.

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