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Automated system for high-throughput protein production using the dialysis cell-free method

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ABSTRACT

High-throughput protein production systems have become an important issue, because protein production is one of the bottleneck steps in large-scale structural and functional analyses of proteins. We have developed a dialysis reactor and a fully automated system for protein production using the dialysis cell-free synthesis method, which we previously established to produce protein samples on a milligram scale in a high-throughput manner. The dialysis reactor was designed to be suitable for an automated system and has six dialysis cups attached to a flat dialysis membrane. The automated system is based on a Tecan Freedom EVO 200 workstation in a three-arm configuration, and is equipped with shaking incubators, a vacuum module, a robotic centrifuge, a plate heat sealer, and a custom-made tilting carrier for collection of reaction solutions from the flat-bottom cups with dialysis membranes. The consecutive process, from the dialysis cell-free protein synthesis to the partial purification by immobilized metal affinity chromatography on a 96-well filtration plate, was performed within ca. 14 h, including 8 h of cell-free protein synthesis. The proteins were eluted stepwise in a high concentration using EDTA by centrifugation, while the resin in the filtration plate was washed on the vacuum manifold. The system was validated to be able to simultaneously and automatically produce up to 96 proteins in yields of several milligrams with high well-to-well reliability, sufficient for structural and functional analyses of proteins. The protein samples produced by the automated system have been utilized for NMR screening to judge the protein foldedness and for structure determinations using heteronuclear multi-dimensional NMR spectroscopy. The automated high-throughput protein production system represents an important breakthrough in the structural and functional studies of proteins and has already contributed a massive amount of results in the structural genomics project at the RIKEN Structural Genomics/Proteomics Initiative (RSGI).

Introduction

The completion of genome sequencing projects has provided comprehensive collections of gene sequence data from many prokaryotes and eukaryotes [1–3]. Utilizing the genome data and cDNA resources from many organisms, structural genomics and proteomics projects are now in progress to understand the structures and functions of proteins on the proteomic scale [4,5].

Structural and functional studies of proteins usually require the preparation of protein samples, which is one of the bottlenecks for these studies. To overcome the bottlenecks, high-throughput methodologies must be developed to produce numerous proteins in sufficient quantities for the studies. Although protein production using recombinant technology based on cells has been well developed and is widely used, many intrinsic limitations still remain with high-throughput protein production. The conventional cell-based methods for producing proteins consist of many processes—cloning a cDNA of interest into an expression plasmid vector, transforming bacteria (e.g., *Escherichia coli*) with the vector, growing the transformants in culture medium, and subsequently purifying the protein from the bacterial cells—which are time-consuming and labor-intensive procedures. Thus, the methods of protein production using living cells are not fully compatible with the vast majority of high-throughput systems.

As an alternative to cell-based protein synthesis, cell-free protein synthesis technologies have been developed for protein production since the late 1980s to the early 1990s by researchers,

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including us [6–8]. Through the principal breakthroughs of improvements in protein yields in the cell-free protein synthesis by our group and other researchers [9–11], cell-free protein synthesis technologies have also been established as one of the standard methods for protein production [12–15]. The cell-free methods allow us to use PCR-amplified linear DNA fragments directly as templates for protein synthesis, thus bypassing most of these labor-intensive steps [16–22]. Therefore, the cell-free methods permit high-throughput protein production using fully automated systems.

Cell-free protein synthesis has been performed using several types of modes. In the batch mode, the reaction is carried out in a tube or the well of a microplate, which contains all of the necessary components for the protein synthesis as well as the synthesized products and by-products. Although the batch mode is amenable to an automated system, the protein production with the batch mode has a relatively low yield, which was far from that required on a productive scale for structural and functional studies [23,24]. To increase the productivity, we and other researchers improved a cell-free method by optimizing the components in the cell-free reaction mixture and by continuous-flow or continuous-exchange (i.e., dialysis) of the reaction mixture against a feeding solution containing low-molecular-weight substrates [6–10,20,22,24,25]. The dialysis cell-free protein synthesis has been performed using various reactors, including a dialysis tube clamped at both ends [20] and commercially available devices, such as the DispoDialyzer® from Spectrum Laboratories, Inc. [10], the Slide-A-Lyzer® MINI Dialysis Unit from PIERCE [26], and the RTS Continuous Exchange Cell Free (CECF) Kit from Roche [27]. These reactors were not adapted for automated instruments to achieve high-throughput protein production. Therefore, the dialysis mode of cell-free protein synthesis has not become automated, in spite of its advantages for protein production.

We now report the development of a dialysis reactor suitable for an automated system and a fully automated protein production system based on a Tecan Freedom EVO® 200 workstation. The automated system is able to synthesize simultaneously up to 96 histidine-tagged proteins using dialysis cell-free protein synthesis, and the proteins are subsequently purified using immobilized metal affinity chromatography (IMAC[1]), in yields of several milligrams. The automated protein production system is a valuable tool for high-throughput protein production for structural and functional analyses.

Materials and methods

Linear DNA construct for cell-free protein synthesis

The plasmid pK7-Ras, containing the T7 promoter and the gene encoding the human c-Ha-Ras protein, was used [9]. A linear DNA construct of the Ras protein (NHis-Ras) fused with a Histidine Affinity Tag (HAT) (Clontech Laboratories, Inc., USA) was produced by a two-step PCR method from the pK7-Ras plasmid, and was used as a template for the cell-free protein synthesis [20,21].

Cell-free protein synthesis

Cell-free protein synthesis with the automated system was performed in the dialysis mode, using the linear DNA construct. An S30 extract from the *E. coli* BL21-CodonPlus®-RIL strain (Stratagene) grown at 30 °C and the reaction solution for the dialysis cell-free system were prepared as described previously [22,28]. The cell-free pre-mixed solution was composed of the buffers, the substrates, creatine kinase, T7 RNA polymerase, and the S30 extract. The external feeding solution was composed of the buffers and the substrates, except for the enzymes and the S30 extract. The negative control (no DNA) consisted of adding an aliquot of Milli-Q® ultra-pure water (Millipore Corp., USA) to the cell-free reaction mixture instead of the DNA template. The cell-free protein synthesis was performed at 30 °C for 8 h with horizontal shaking.

Dialysis reactor

The dialysis reactor, composed of a lid, a dialysis cup, a 6-well cup holder plate, a splash prevention baffle, and a 1-well reservoir, is schematically represented in Fig. 1(A). The reservoir, the lid, and the baffle were made of acrylic resin, while the cup holder plate was made of polystyrene resin. The components, except for the dialysis cup, were rinsed with nuclease-free ultra-pure Milli-Q water to remove possible inhibitors of the enzymatic reaction, and then were reused. The dialysis cup was disposable and assembled with a cylindrical outer sleeve, a cylindrical inner sleeve, and a dialysis membrane, as shown in Fig. 1(A). The inner and outer sleeves were composed of a polymer alloy combining an acrylonitrile butadiene styrene (ABS) with a polycarbonate resin. The dialysis membrane was a regenerated cellulose Spectra/Por® 2 Dialysis Membrane (MWCO 12–14 kDa; Spectrum Laboratories, Inc., USA). The membrane was held between the two sleeves and was attached as a flat surface to the bottom of the cup (Fig. 1(A)). The bottom of the inner sleeve extends slightly beyond the bottom of the outer sleeve, to avoid the trapping of air pockets between the membrane and the feeding solution (Fig. 1(B)). The effective area of the dialysis membrane was ca. 5.3 cm^2. The dimensions of the footprint of the reservoir were considered to meet the ANSI/SBS standards published by the Society for Biomolecular Sciences (SBS) [29], so the reactor is compatible with commercially available robotic equipment.

The reactor can be easily assembled before use according to the construction scheme shown in Fig. 1(A). The dialysis cups on the cup holder plate were immersed in the feeding solution (Fig. 1(B)). The protein synthesis was carried out within the dialysis cup. The reaction solution in each of the six cups was 1 mL, and the feeding solution in the reservoir was 60 mL, and thus a total of 6 mL of the reaction solution was dialyzed against 10 volumes of the feeding solution.

Immobilized metal affinity purification

IMAC purification was performed using Ni Sepharose™ High Performance resin (GE Healthcare), which has high binding capacity and is pipettable with a disposable tip due to its small particle radius. The resin was pre-equilibrated with a wash buffer (20 mM sodium phosphate, 750 mM NaCl, pH 8.0). The sample binding to the resin was performed in a deep-well plate, and then the resin suspension with the supernatant of the reaction solution was transferred to a TurboFilter™ 96 filtration plate (QIAGEN) residing on a vacuum manifold, using disposable tips. The resin was washed three times with the wash buffer on the vacuum manifold. The bound proteins were eluted by stepwise centrifugation with 10 and 50 mM EDTA buffers, containing 50 mM sodium phosphate, 300 mM NaCl, and 0.02%(w/v) sodium azide, pH 8.0.

Automated instruments

The automated system for the dialysis cell-free protein synthesis and protein the purification procedure was developed based on a Freedom EVO 200 liquid handling workstation (Tecan Group Ltd.,

[1] *Abbreviations used:* CV, coefficient of variation; EDTA, ethylenediamine tetraacetic acid; IMAC, immobilized metal affinity chromatography; MWCO, molecular weight cut off; NMR, nuclear magnetic resonance; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.