Biochemical Engineering Journal 138 (2018) 156–164

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Review

Cell-free protein synthesis for producing 'difficult-to-express' proteins

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ARTICLE INFO

Article history:
Received 19 March 2018
Received in revised form 6 July 2018
Accepted 12 July 2018

Keywords:
Cell-free protein synthesis
Cancer therapeutics
Antimicrobial peptides/proteins
Antibodies
Membrane proteins
Non-standard amino acids

ABSTRACT

Biomanufacturing requires the stable production of active biological molecules including proteins. However, a significant percentage of genes cannot be expressed efficiently in a cell-based system due to cellular homeostasis. Upon overproduction, proteins often form inclusion bodies by becoming insoluble, lose biological activities due to improper folding, or are easily degraded by proteases. Many molecular tools and protocols have been established to improve the production yield of 'difficult-to-express' proteins in cell-based systems; however, *in vivo* approaches have a substantial limitation in that the host strains should be maintained healthy for stable protein production and folding. Cell-free protein synthesis (CFPS) offers superior advantages in synthesizing 'difficult-to-express' proteins due to reaction environment openness and no cell viability constraints. In CFPS systems, active protein production can be maximized by optimizing the reaction environment with selective supplements of positive effectors and the elimination of negative effectors. This review summarizes current strategies for the high-quality production of difficult-to-express proteins including toxic cancer therapeutics, antimicrobial peptides/proteins, toxins, vaccines, antibodies, membrane proteins, and proteins containing nonstandard amino acids.

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https://doi.org/10.1016/j.bej.2018.07.013
1369-703X/

1. Introduction

Cell-free protein synthesis (CFPS) is a protein-producing methodology that does not involve the use of living cells [1,2]. In 1961, Nirenberg and Mattaei utilized CFPS to decode nucleotide triplets known as codons that are translated to specific amino acids [3]. However, at the early stage of CFPS, its limitations, such as a short reaction duration, low protein production yield, and small reaction volume, impeded CFPS as a viable protein synthesis methodology in industrial applications [4,5]. CFPS technology has recently been revived due to its outstanding characteristics, which enable flexible programming of the protein synthesis system [6] as well as the engineering of proteins due to the open nature of the cell-free environment [7,8]. In the past two decades, CFPS platforms have been significantly improved by increasing protein yield and duration, improving protein solubility, and decreasing production cost [5,8,9]. CFPS technology should be further developed for industrially relevant operations [10]; however, CFPS can also provide a superior or alternative biological platform for developing advanced biomanufacturing processes for recombinant proteins. This review focuses on reviewing the proteins that are difficult to produce in a cell-based system but can be efficiently synthesized *via* cell-free systems.

2. Cell-free protein synthesis

2.1. *Challenges for* in vivo *protein production*

Recombinant protein production *in vivo* is widely used and studied. However, some proteins remain difficult to express due to poor growth of the host cell, inclusion body formation, protein inactivity, and low production yield [11]. Difficult-to-express proteins include cancer therapeutics, vaccines, antimicrobial peptides/proteins, toxins, membrane proteins, antibodies, proteins with non-standard amino acids, and others. Many molecular tools and protocols have been established to improve the production yield of difficult-to-express proteins *in vivo*, including optimizing nutrient conditions, lowering temperature, co-expressing chaperones, and engineering host strains and expression vectors [11]. However, *in vivo* approaches have a substantial limitation in that the host strains should be maintained healthy for stable protein production and folding. Cells may inhibit toxic protein production or may inactivate the toxicity of the protein because they would otherwise be killed [12]. For example, producing membrane proteins often perturb the central metabolism of the cells and repress cell growth [13,14]. In producing antibodies, microorganisms do not provide sufficient post-translational modifications, such as glycosylation, which is crucial for antibody activities, and mammalian cells require long processing times and present elevated costs when producing antibodies [15]. Developing biological platforms to synthesize active forms of difficult-to-express proteins in high yields will advance investigations into new mechanisms as well as their utility in various applications.

2.2. *Preparation of cell-free protein synthesis*

CFPS utilizes transcriptional/translational machineries from crude cell extracts derived from prokaryotic, plant, and mammalian cells [7] or reconstituted purified transcriptional/translational components [16]. Crude extracts are prepared by lysing the cells followed by removing cellular debris and large molecules, such as genomic DNA, *via* multiple rounds of washing and centrifugation. It is critical to mimic the cytoplasmic environment to enable and enhance CFPS [17]. Hence, energy sources, cofactors, buffers, salts, nucleotides, and amino acids are mixed together with the extract. After adding DNA gene template in either circular plasmid or linear PCR fragment forms and incubating at a proper temperature, *in vitro* transcription and translation reactions occur simultaneously, resulting in protein production (Fig. 1). Proteins are synthesized within several hours, yielding a μg/mL scale batch reaction; however, continuous-flow cell-free systems *via* feeding buffers containing nucleotide building blocks [18] and continuous-exchange cell-free (CECF) settings using an integrated dialysis membrane allow a prolonged reaction lifetime (up to several days) and a higher protein yield (up to several mg/mL) [19,20].

2.3. *Advantages of cell-free protein synthesis for producing proteins*

Compared to protein production *in vivo*, CFPS offers compelling benefits in producing proteins that are toxic or difficult to produce in cells [5] with the following advantages. Some cell-based approaches for improving difficult-to-express protein production can be directly applied to enhance protein production *in vitro,* as the same transcriptional and translational machineries are used in CFPS. These efforts include lowering the temperature to increase the active fraction [21], adding/expressing chaperones to facilitate protein folding [22], regenerating co-factors to energize the cell-free reaction [23], optimizing codon usage of the gene of interest to enhance translation [24], and adding/expressing protein disulfide isomerase (PDI) to facilitate disulfide bonds for folding [25] (Fig. 1). Genome engineering that selectively removes negative effectors in the cell extract can also improve the efficiency of CFPS [20,26,27].

The openness of the cell-free systems allows unique environmental control and freedom of design, which further optimize protein production (Table 1) and are not possible in a cell-based system. First, the cell-free reaction enables the addition or synthesis of new components at precise concentrations. For example, protein production could be optimized by changing the transcription and translation components in the CFPS reaction [5], and the addition of high-molecular crowding agents, such as polyethylene glycol, can increase protein yields [28,29]. Second, CFPS systems are not constrained by cell viability requirements and are therefore impervious to the cytotoxic nature of some proteins. Third, CFPS systems can use linear DNA fragments (*e.g.*, polymerase chain reaction products) for target gene expression, which avoids time-consuming gene cloning steps commonly required for *in vivo* protein synthesis and grants access to a large library of mutants without being limited by transformation efficiency [30]. In addition, CFPS can facilitate the high-yield production of proteins with post-translational modification by site-specific nonstandard amino acid incorporation [31] and enables the production of membrane-anchored proteins by making membrane mimics [32]. The advantages of CFPS address challenges in producing difficult-to-express proteins, which we describe below.

3. Producing 'difficult-to-express' proteins

3.1. *Proteins that are toxic to the production host*

Some proteins are toxic to the host cells upon production, leading to a low production yield *in vivo*. The production and purification of such toxic proteins can be achieved by solubilizing and refolding proteins from inclusion bodies; however, the active portion of the protein is significantly decreased [33]. In contrast, the CFPS platform allows toxic protein production with a high yield and optimal capability because it does not require cell viability constraints.