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Cell-free synthesis system suitable for disulfide-containing proteins

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ABSTRACT

Many important therapeutic targets are secreted proteins with multiple disulfide bonds, such as antibodies, cytokines, hormones, and proteases. The preparation of these proteins for structural and functional analyses using cell-based expression systems still suffers from several issues, such as inefficiency, low yield, and difficulty in stable-isotope labeling. The cell-free (or *in vitro*) protein synthesis system has become a useful protein production method. The openness of the cell-free system allows direct control of the reaction environment to promote protein folding, making it well suited for the synthesis of disulfide-containing proteins. In this study, we developed the *Escherichia coli* (*E. coli*) cell lysate-based cell-free synthesis system for disulfide-containing proteins, which can produce sufficient amounts of functional proteins for NMR analyses. Disulfide bond formation was facilitated by the use of glutathione buffer. In addition, disulfide isomerase, DsbC, catalyzed the efficient shuffling of incorrectly formed disulfide bonds during the protein synthesis reaction. We successfully synthesized milligram quantities of functional ^{15}N-labeled higher eukaryotic proteins, bovine pancreatic trypsin inhibitor (BPTI) and human lysozyme C (LYZ). The NMR spectra and functional analyses indicated that the synthesized proteins are both catalytically functional and properly folded. Thus, the cell-free system is useful for the synthesis of disulfide-containing proteins for structural and functional analyses.

1. Introduction

Many pharmaceutical targets are eukaryotic extracellular proteins, such as antibodies, cytokines, hormones, and proteases, and their structural and functional analyses are thus attracting great interest. Most extracellular proteins are stabilized by multiple disulfide bonds formed by the oxidation of pairs of cysteine residues, and protein folding and disulfide bond formation are closely coupled [1]. To date, cell-based expression systems have been widely used to produce proteins for structural and functional analyses. Disulfide bond-containing proteins often form insoluble intracellular aggregates in the cell-based systems, and laborious and inefficient refolding processes are generally required to obtain correctly folded and functional disulfide-containing proteins [2]. Alternatively, secretory production of disulfide-containing proteins is useful, since the formation of disulfide bonds is facilitated in the oxidative extracellular space [3]. However, there are still several problems, such as the inefficient secretion of large eukaryotic proteins, the lack of precise control of the folding environment, and the challenges of selecting the proper combination of the host-vector system and signal peptide [4].

The cell-free protein synthesis system has become one of the standard protein production methods for structural analysis [5]. The cell-free system based on an *Escherichia coli* (*E. coli*) cell extract is suitable for producing stable-isotope labeled proteins for nuclear magnetic resonance (NMR) analyses. The incorporation of the stable-isotope labeled amino acid is more efficient than that in the conventional cell-based method, with less scrambling of the label due to metabolic enzymes [6]. The novel buffer composition with potassium D-glutamate is useful for highly productive uniform stable-isotope labeling [7], and complete and precise amino acid-selective labeling can be achieved by including chemical inhibitors of metabolic enzymes [8]. The dialysis-mode cell-free system can produce milligram quantities of labeled proteins, which are sufficient amounts for structural analyses [9,10]. Many protein structures have been solved with uniformly stable-isotope labeled samples produced by the cell-free system (for example, [11,12]).

Abbreviations: E. coli, *Escherichia coli*; NMR, nuclear magnetic resonance; PEP, phospho*enol*pyruvate; scFv, single chain Fv; BPTI, bovine pancreatic trypsin inhibitor; LYZ, lysozyme C; MWCO, molecular weight cut-off; GSH, reduced glutathione; GSSG, oxidized glutathione; Bz-Arg-pNA, N-benzoyl-L-arginine p-nitroanilide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; mBBr, monobromobimane; HSQC, heteronuclear single quantum coherence.

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In addition to the suitability for structure analysis, the openness of the cell-free system allows direct and flexible optimization of the reaction environment, so that proteins can be synthesized under suitable conditions. For example, the solubility and/or stability of zinc-binding proteins were significantly increased by the addition of proper amounts of its ligand, zinc [13]. Using the advantages of the cell-free system, several laboratories have developed cell-free systems for producing disulfide-containing proteins, such as single chain Fv (scFv) [14–16], lipase B [17], and GM-CSF [18]. Although functional proteins were successfully synthesized in these studies, the productivities achieved by these systems were insufficient for structural analyses.

Recently, the cell-free synthesis of stable isotope-labeled disulfide bond-containing proteins, based on the batch-mode reaction, was reported [19]. We considered the more productive dialysis-mode system to be favorable for the efficient production of disulfide bond-containing proteins. In the present study, we developed the dialysis-mode cell-free system in order to produce milligram quantities of disulfide-containing proteins, especially for NMR analyses. We introduced glutathione buffer to facilitate disulfide bond formation and disulfide isomerase to shuffle incorrectly formed disulfide bonds. Two eukaryotic proteins, bovine pancreatic trypsin inhibitor (BPTI) and human lysozyme C (LYZ), containing 3 and 4 disulfide bonds, respectively, were successfully synthesized by the newly developed system. The synthesized BPTI and LYZ proteins exhibited activities comparable to those of the authentic proteins, and the NMR spectra confirmed their proper folding.

2. Materials and methods

2.1. Cell-free synthesis

The dialysis-mode of the cell-free protein synthesis system was used in this study [9,10]. A small-scale dialysis unit (30 μl internal solution and 300 μl external solution) was used to optimize the reaction conditions [20]. The compositions of the internal and external solutions in this study were based on the D-glutamate system [7], with several modifications as follows. DTT was excluded from the S30 extract, the amino acid solution, and the S30 buffer. NH_4OAc and cAMP were omitted, and the concentration of polyethylene glycol 8000 was reduced from 4% to 2% (w/v). To remove the DTT, the S30 extract was dialyzed 4 times in a dialysis tube (Spectra/Por Biotech Regenerated Cellulose Dialysis Membrane molecular weight cut-off (MWCO): 15 kDa) (Spectrum, USA) against 50 volumes of DTT-free S30 buffer at 4 °C for 60 min. Various concentrations of reduced (GSH) and/or oxidized glutathione (GSSG) (Nacalai Tesque, Japan) were added to both the internal and external solutions, to optimize the redox conditions. In addition, to facilitate the disulfide bond isomerization, 400 μg/ml of the *E. coli* disulfide isomerase DsbC was added to the internal solution. DsbC was prepared basically according to the previous report [21]. The cell-free synthesis was performed at 30 °C for 6 h.

2.2. Synthesis of uniformly ^{15}N-labeled proteins

For the synthesis of the uniformly ^{15}N-labeled BPTI and LYZ, the unlabeled amino acids in both the internal and external solutions were replaced by 3 mg/ml ^{15}N-labeled algal amino acid solution, supplemented with 1 mM L-[^{15}N]cysteine, 1 mM L-[^{15}N]glutamine, 1 mM L-[^{15}N]tryptophan, and 2 mM L-[^{15}N]asparagine. To facilitate proper disulfide bond formation, 4 mM GSH, 1 mM GSSG, and 400 μg/ml DsbC were added to the internal solution, while 4 mM GSH and 1 mM GSSG were added to the external solution. The internal solution (9 ml) in a dialysis tube (Spectra/Por 7 MWCO: 15 kDa, Spectrum) was dialyzed against the external solution (90 ml), at 30 °C for 6 h with gentle shaking [20]. All of the stable-isotope labeled compounds were purchased from Taiyo Nippon Sanso (Japan). Plasmid construction, protein purification, free thiol concentration analyses, disulfide bond confirmations, and activity measurements of ^{15}N-labeled BPTI and LYZ are described in the supplementary material.

2.3. NMR analysis

The ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) spectra were acquired from 0.3 mM uniformly ^{15}N-labeled BPTI, in 20 mM MES (pH 6.0), 100 mM NaCl, and $^{2}H_2O$ (10% v/v), and from 0.3 mM uniformly ^{15}N-labeled LYZ in 90% H_2O/10% $^{2}H_2O$, with the pH adjusted to 5.0 with HCl. NMR measurements were performed with an AVANCE 600 MHz spectrometer equipped with a CryoProbe (Bruker BioSpin, Germany). The HSQC spectra were measured with 16 scans per increment, 128 complex points in t_1 (^{15}N), and 512 complex points in t_2 (^{1}H) at 298 K for ^{15}N-labeled BPTI, and with 16 scans per increment, 64 complex points in t_1 (^{15}N), and 512 complex points in t_2 (^{1}H) at 310 K for ^{15}N-labeled LYZ. The HSQC spectra were processed with the TopSpin software (Bruker BioSpin).

3. Results and discussion

3.1. Cell-free synthesis of disulfide-containing proteins

Proper disulfide bond formation plays an important role in the folding of many secretory proteins. In order to facilitate disulfide bond formation, we controlled the redox conditions of the dialysis-mode of the cell-free system by introducing glutathione buffer. Moreover, the *E. coli* disulfide isomerase DsbC was added to the internal solution, to shuffle improperly formed disulfide bonds. Two eukaryotic proteins, BPTI and LYZ, were synthesized under different redox conditions in the absence or presence of DsbC. The internal solutions were harvested after the 6 h reaction, separated into total and soluble fractions by centrifugation, and analyzed by SDS–PAGE (Fig. 1A).

Most of the BPTI was soluble in the presence of 400 μg/ml DsbC under all redox conditions, except for 5 mM DTT. Without DsbC, however, the amounts of BPTI in the supernatant fraction decreased to 60–80% of those of the total fractions under all of the redox conditions, based on the gel band intensity analysis. This result suggested that BPTI containing incorrectly paired disulfide bonds was prone to precipitate during cell-free synthesis. BPTI was partially purified from the internal solution using TALON resin, and was further analyzed by reducing and non-reducing SDS–PAGE. The difference in the mobility indicates the presence of disulfide bonds (Fig. 1B). On the non-reducing gel, small amounts of faster-migrating bands, particularly for the BPTI synthesized without DsbC, were observed. These bands might represent misfolded BPTI with incompletely or improperly formed disulfide bonds, rather than the truncated protein, because only one major band was detectable on the reducing gel. Comparable amounts of these smaller bands were also observed for commercial authentic BPTI, as compared to the partially purified BPTI synthesized with DsbC, suggesting that the addition of DsbC is sufficient for the proper folding of BPTI (data not shown). BPTI synthesized without DsbC exhibited lower activity than that expected from its yield, as compared to BPTI synthesized with DsbC (Supplementary Fig. 1). Considering that misfolded BPTI with incorrect disulfide bonds is inactive [3,22], this result suggested that the disulfide bonds are apt to be incorrectly paired when synthesized without DsbC. The amount of soluble BPTI synthesized in the presence of DsbC was