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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

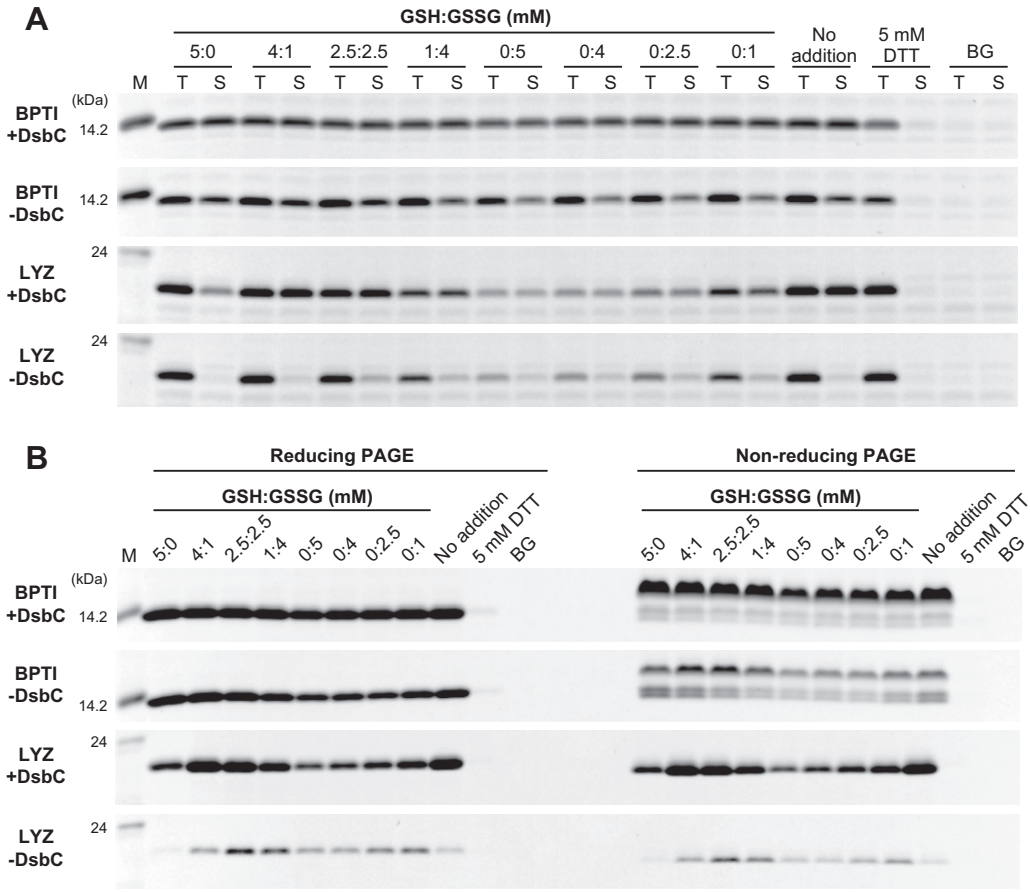

Fig. 1. SDS–PAGE analyses of cell-free synthesized disulfide-containing proteins. BPTI and LYZ were synthesized under various redox conditions with or without DsbC. (A) Total (T) and supernatant (S) fractions (0.2 μl) of the internal solution were analyzed by reducing SDS–PAGE. (B) Partially purified proteins were analyzed by both reducing and non-reducing SDS–PAGE. The glutathione buffers used for the cell-free synthesis are indicated. No addition: the cell-free reaction without glutathione buffer. 5 mM DTT: the cell-free reaction with 5 mM DTT, instead of glutathione buffer. BG: the cell-free reaction without template DNA and glutathione buffer. The gels were stained with Quick CBB Plus.

comparable to that produced under the best conditions, even without glutathione buffer (Fig. 1A, lanes labeled "No addition"). The use of glutathione buffer is still favorable in order to achieve robust control of the redox conditions for reproducible results. Consequently, the addition of DsbC and the use of redox buffer (5 mM GSH or the combination of 4 mM GSH and 1 mM GSSG) are preferable conditions to synthesize functional BPTI by the cell-free system. Under these conditions, approximately 1.8 mg of soluble BPTI were synthesized from 1 ml internal solution.

The yield of LYZ in the soluble fraction was strongly dependent on the presence of DsbC (Fig. 1A). This result indicated that the disulfide bond exchange reaction catalyzed by DsbC is a critical step in the folding pathway of LYZ during the cell-free synthesis reaction. In contrast to BPTI synthesis, the productivity of LYZ gradually decreased as the GSSG concentration increased. Higher concentrations of GSSG might facilitate cysteine oxidation to cystine in the reaction, before it is used as a substrate for protein synthesis. The partially purified LYZ displayed slightly slower mobility in non-reducing PAGE than in reducing PAGE, indicating the existence of intermolecular disulfide bonds (Fig. 1B). Under the favorable conditions of 4 mM GSH, 1 mM GSSG, and 400 μg/ml DsbC, approximately 3 mg of LYZ were synthesized from 1 ml internal solution (Supplementary Fig. 2). The specific activity of the synthesized LYZ was comparable to that of the authentic enzyme, as described in the subsequent NMR analysis section. Thus, we successfully produced milligram quantities of eukaryotic disulfide-containing proteins, using the dialysis-mode cell-free system.

3.2. Free thiol concentration in the internal solution

We measured the free thiol concentration in the internal solution of the dialysis-mode cell-free protein system. We found that, regardless of the presence of the S30 extract, 10–20% of the free thiols were oxidized after the 6 h incubation, probably by atmospheric O_2 (Fig. 2A). Interestingly, the rapid reduction of GSSG reportedly occurred in the batch-mode cell-free system using phospho*enol*pyruvate (PEP) or glucose as an energy source [21], and the laborious pretreatment of the S30 extract by iodoacetamide or GSSG was indispensable for suppressing the intrinsic reducing activity [21,23]. This significant difference in the redox state arises from that in the energy source, as also suggested in the previous report [19]. In *E. coli*, the cellular redox state is maintained by reducing enzymes, such as glutathione reductase, thioredoxin reductase, and other unidentified reducing enzymes. The use of either PEP or glucose as an energy source in combination with NAD^+ for the cell-free system causes the production of NADH through glycolysis and the TCA cycle, by enzymes in the S30 extract. NADH is most likely to provide electrons to reduce GSSG, leading to a rapid change in the redox state in the cell-free reaction. Our cell-free system uses creatine phosphate and creatine kinase to regenerate ATP, thereby avoiding the problematic reduction of GSSG. Both PEP and glucose activate metabolic pathways causing the scrambling and dilution of stable-isotope labels [19]. Taken together, these findings strongly indicated that creatine phosphate is a much more suitable energy source for the cell-free system, especially for disulfide-containing protein synthesis.

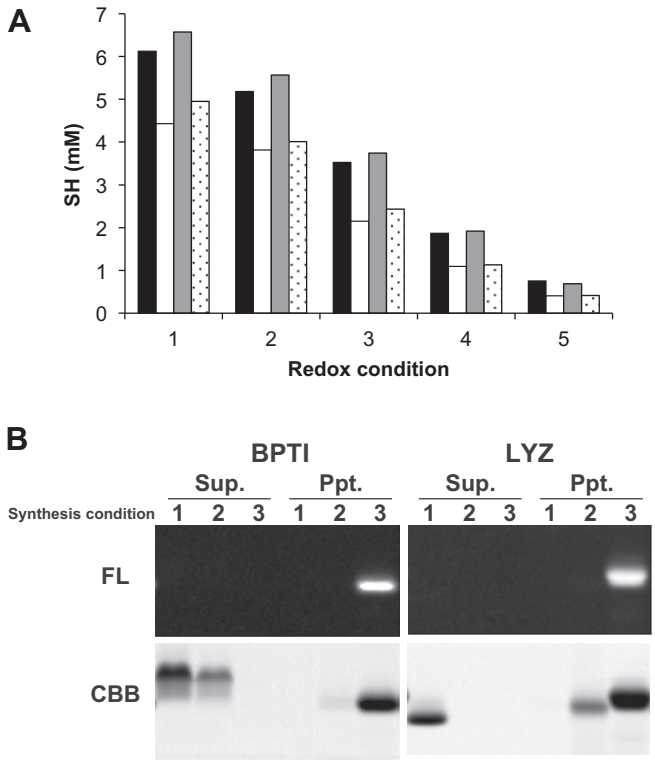

Fig. 2. (A) Free thiol concentration of the internal solution. Small-scale dialysis reactions were performed in the presence or absence of S30 extract under the following conditions: (1) 5 mM GSH, (2) 4 mM GSH and 1 mM GSSG, (3) 2.5 mM GSH and 2.5 mM GSSG, (4) 1 mM GSH and 4 mM GSSG, (5) 5 mM GSSG. The free thiol concentrations of the internal solutions before and after the 6 h incubation were measured and are indicated as follows: in the presence of S30 extract before (black) and after incubation (white), and in the absence of S30 extract before (gray) and after incubation (dotted). (B) Confirmation of disulfide bonds. BPTI and LYZ were synthesized under three conditions: (1) 4 mM GSH, 1 mM GSSG, and 400 μg/ml DsbC, (2) 4 mM GSH, 1 mM GSSG, (3) 5 mM DTT. The precipitate in the internal solution (Ppt.) and the partially purified supernatant fraction (Sup.) were denatured by 8 M urea and modified by the thiol-specific fluorescent probe, mBBr, to detect free thiol groups. The samples were analyzed by 15% non-reducing SDS–PAGE. The mBBr-modified proteins were detected by fluorescence (FL). Unmodified and mBBr-modified proteins were detected by staining the gel with Quick CBB Plus (CBB).

3.3. Confirmation of disulfide bonds

To confirm the existence of disulfide bonds in the BPTI and LYZ proteins synthesized by the cell-free system, the proteins were modified by a thiol-specific fluorescent probe, mBBr, and then were analyzed by non-reducing SDS–PAGE (Fig. 2B). No fluorescence was observed on the proteins synthesized under the preferable conditions (4 mM GSH, 1 mM GSSG, and 400 μg/ml DsbC), indicating that the cysteine residues of these proteins formed disulfide bonds. In the case of BPTI synthesized with 4 mM GSH and 1 mM GSSG, a large portion of the protein was soluble and no fluorescence was observed, demonstrating that, to a certain extent, properly paired disulfide bonds were spontaneously formed even in the absence of DsbC. No fluorescence was observed for the precipitated BPTI, which exhibited different mobility in the non-reducing SDS–PAGE from that of the soluble BPTI. This result suggested that incorrectly paired disulfide bonds were formed in the precipitated BPTI, due to the lack of DsbC. In the case of LYZ synthesized with 4 mM GSH and 1 mM GSSG, the protein was totally insoluble and no fluorescence was observed. Its mobility in the gel was different from those of the LYZ proteins synthesized under the other two conditions: 4 mM GSH, 1 mM GSSG, and 400 μg/ml DsbC, and 5 mM DTT. These results suggested that LYZ formed incorrectly paired disulfide bonds due to the lack of DsbC, resulting in precipitation. Fluorescent bands were observed for both BPTI and LYZ synthesized with 5 mM DTT, which were totally precipitated. These proteins seemed to be misfolded due to the failure of disulfide bond formation under the reducing conditions. Consequently, these results demonstrated that the cysteine residues of BPTI and LYZ synthesized by the cell-free system under the optimal conditions formed proper disulfide bonds, and DsbC was important to facilitate proper protein folding, by catalyzing the disulfide bond shuffling reaction.

3.4. NMR analyses

Uniformly ^{15}N-labeled BPTI and LYZ were synthesized by the dialysis-mode cell-free system in the presence of 4 mM GSH, 1 mM GSSG, and 400 μg/ml DsbC, and were then purified to homogeneity by a combination of several chromatography steps. The final yields of BPTI and LYZ from 9 ml of the internal solution were 7.1 mg and 11.0 mg, respectively, which are sufficient amounts for

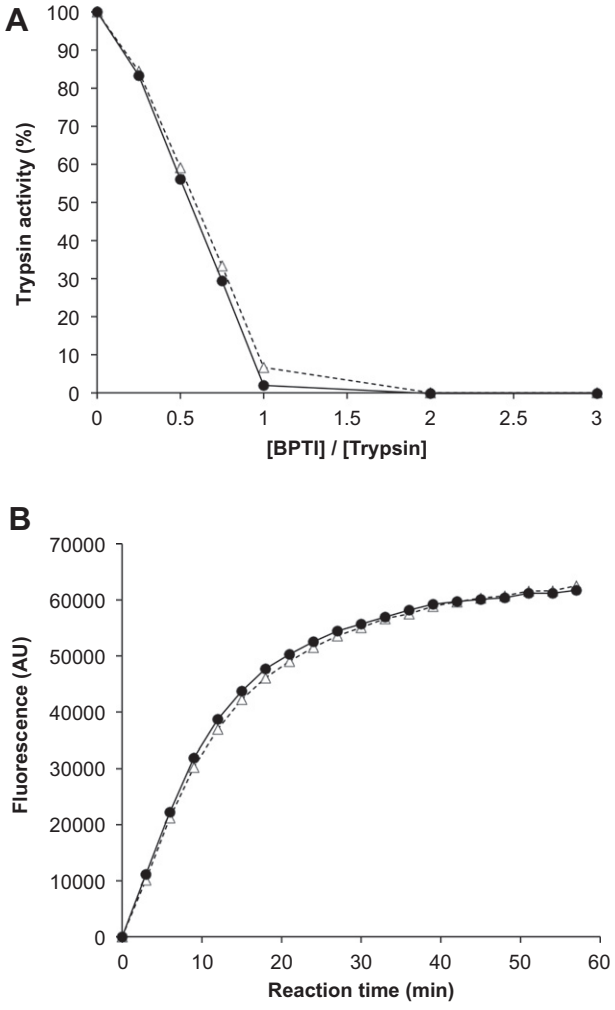

Fig. 3. Activity measurements. The activities of the cell-free synthesized ^{15}N-labeled BPTI and ^{15}N-labeled LYZ were compared with the activities of their authentic proteins. Data points indicate the average of three independent experiments. (A) Trypsin inhibition activities of the cell-free synthesized ^{15}N-labeled BPTI (filled circles) and the BPTI standard (open triangles) are shown. The residual trypsin activity is indicated as the fraction of that of the reference sample containing only trypsin. (B) The time course of the activity of the cell-free synthesized ^{15}N-labeled LYZ (filled circles) and the standard protein (open triangles). The enzyme concentration was 0.2 μM.

NMR structural analyses. We first compared the activities of these cell-free synthesized proteins to those of their authentic counterparts, and confirmed that both proteins exhibited essentially the same specific activities as the authentic ones (Fig. 3). Furthermore, each synthesized protein generated a sharp single peak with a nearly identical retention time to the authentic protein in the reverse phase HPLC analysis, suggesting that these cell-free synthesized proteins folded into the native structures (Supplementary Figs. 3 and 4). We then measured the ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled BPTI and LYZ, and well-dispersed sharp cross peaks were observed for both proteins (Fig. 4). Comparisons with the published NMR spectra of BPTI (BMRB entry 5359) and LYZ [24] demonstrated that the structures of these proteins synthesized by the cell-free system were almost the same as those of the native proteins. The spectral dispersion of LYZ gradually decreased by adding 20 mM DTT, providing further evidence that the cell-free synthesized LYZ contained disulfide bonds (data not shown). These results indicated that fully functional disulfide-containing proteins that are especially suitable for structural analyses can be synthesized.

Many disulfide-containing secretory proteins exert their functions through interactions with their specific targets. The precise analysis of the interaction site, on the basis of the tertiary structure, is a crucial issue. In combination with advanced stable-isotope labeling techniques, such as the SAIL method [25], methyl-selective protonation [26], and site-directed stable-isotope labeling [27], the cell-free system developed in this study is expected to contribute to NMR analyses of large disulfide-containing proteins and their complexes, including antibody-antigen interaction analyses for antibody drug development.

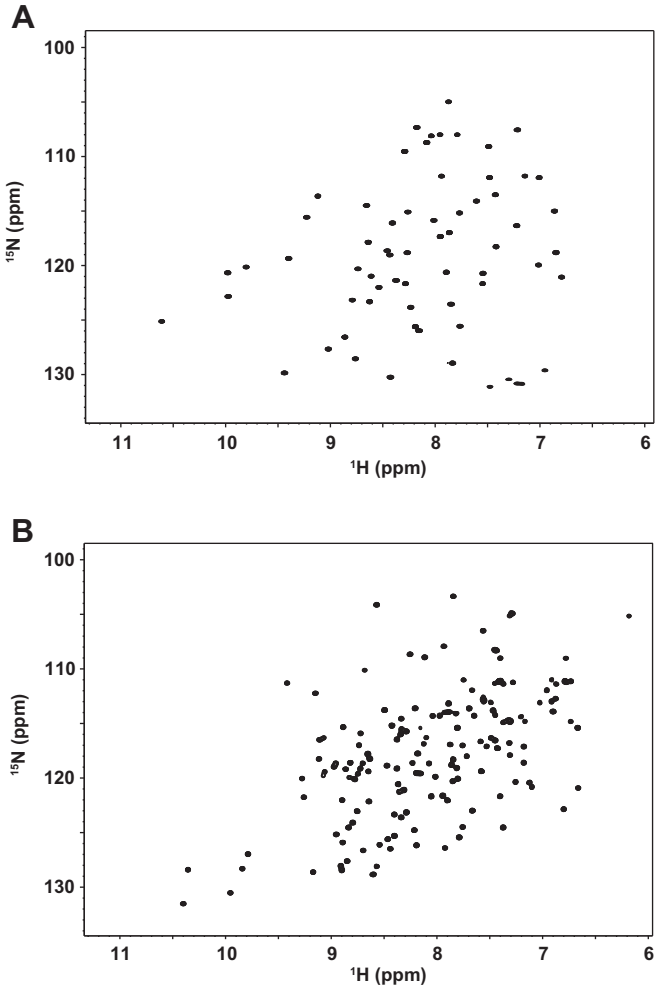

Fig. 4. NMR analyses of ^{15}N-labeled BPTI and LYZ. The ^{1}H-^{15}N HSQC spectra of the uniformly ^{15}N-labeled (A) BPTI and (B) LYZ.

In this study, we described the cell-free synthesis of proteins containing multiple disulfide bonds. Catalytically active BPTI and LYZ were successfully synthesized through the optimization of the redox conditions with glutathione buffer and the promotion of the disulfide bond shuffling reaction by DsbC. During the optimization of the redox conditions, we examined 5 different types by using the following glutathione buffers: (1) 5 mM GSH, (2) 4 mM GSH and 1 mM GSSG, (3) 2.5 mM GSH and 2.5 mM GSSG, (4) 1 mM GSH and 4 mM GSSG, and (5) 5 mM GSSG. One of these conditions renders the synthesis of an active fraction in most cases, in our experience. The addition of DsbC was usually highly effective in improving the yield of the properly folded and catalytically active fraction. Creatine phosphate was shown to be a much more suitable energy source than PEP [21] or glucose [18] for disulfide-containing protein synthesis as well as stable-isotope labeling [19]. We obtained 1.8–3.3 mg of purified proteins from 1 ml reaction solutions by using the dialysis-mode cell-free system (Supplementary Figs. 1 and 2), whereas approximately 5-fold lower amounts (0.3–0.7 mg) of purified disulfide-containing proteins were obtained from 1 ml of the batch-mode cell-free reaction in the previous report [19]. Therefore, this new cell-free protein synthesis system, dedicated to the expression of disulfide-containing proteins, will certainly be useful for structural biology studies as well as other analyses, including protein engineering.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.107.

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