J Struct Funct Genomics (2006) 7:93–100
DOI 10.1007/s10969-006-9012-1

ORIGINAL PAPER

Cell-free synthesis of zinc-binding proteins

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Received: 16 June 2006 / Accepted: 2 October 2006 / Published online: 5 December 2006

Abstract Cell-free protein synthesis has become one of the standard methods for protein expression. The cell-free method is suitable for the synthesis of a protein that requires a ligand for its enzymatic activity and/or structure formation and stabilization, since it is an open system, which allows us to add the proper ligand to the reaction mixture. A large number of proteins that require zinc for their function are involved in diverse cellular processes, including transcription, DNA replication, metabolism, and cell signaling. In this study, we analyzed the effects of zinc on the cell-free synthesis of plant-specific zinc-binding transcription factors. The solubility and/or stability of the proteins were significantly increased in the presence of the proper concentration of zinc during the cell-free reaction. NMR analyses confirmed that correctly folded proteins were synthesized by the cell-free method. These results indicate that the cell-free method can be used to synthesize correctly folded and functional zinc-binding proteins.

Keywords *Arabidopsis thaliana* · Cell-free protein synthesis · In vitro translation · Zinc-binding protein · Structural genomics

Abbreviations

SPL	*SQUAMOSA* promoter binding protein-like
DBD	DNA-binding domain
HSQC	Heteronuclear single quantum coherence
CAT	chloramphenicol acetyltransferase

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Introduction

Cell-free (or in vitro) protein synthesis has become one of the standard methods for protein expression, and it has several advantages over the conventional cell-based (or in vivo) methods. PCR-amplified linear DNA fragments can be directly used for a cell-free protein synthesis reaction, freeing us from the

time-consuming cloning and fermentation steps [1]. The cell-free method fits automated protein preparation pipeline, using robotics with multi-well plates. The cell-free method with large-scale dialysis can synthesize milligram quantities of protein samples [2], so it is useful for producing $^{13}C/^{15}N$-labeled protein samples for NMR spectroscopy [3] and seleno-methionine substituted protein samples for X-ray crystallography [4, 5]. For these reasons, the cell-free method is quite suitable for structural genomics and proteomics, which require the preparation of an enormous number of protein samples [6].

Since the cell-free method is an open system, which allows us to add the proper ligand to the reaction mixture, it is suitable for the synthesis of a protein that requires a ligand for its enzymatic activity and/or structural stabilization. The activities of cell-free synthesized thioredoxin reductase and glutathione reductase from *Escherichia coli* were reportedly increased by the addition of the cofactor, flavin adenine dinucleotide [7]. Therefore, the cell-free method can be used as a high-throughput expression system for ligand-binding proteins.

A large number of proteins require zinc for their function. Bioinformatics report stated that 2,800 human proteins, about 10% of the human proteome, are potentially zinc-binding [8]. Among the 654 target proteins of the New York Structural GenomiX Research Consortium, 30 proteins (4.58%) were revealed to be zinc-binding by a quantitative analysis of their X-ray fluorescence signals [9]. Zinc-binding proteins are involved in diverse cellular processes, including transcription, DNA replication, metabolism, and cell signaling [10]. In many cases, the Zn^{2+} ions are the key structural component of zinc-binding proteins, and thus are essential for their function.

For example, the *Arabidopsis thaliana* SPL (*SQUAMOSA*-promoter binding protein-like) family consists of 12 plant-specific transcription factors with a highly conserved DNA binding domain (SPL-DBD) [11], which is suggested to bind zinc [12]. The C-terminal WRKY domain of the *A. thaliana* WRKY4 protein (WRKY4-C) contains the conserved WRKYGQK sequence and a $CX_{4-5}CX_{22-23}HXH$ zinc-binding motif [13]. Yamasaki et al. reported the solution structures and the specific DNA binding activities of SPL4-DBD, SPL7-DBD [14], SPL12-DBD [15], and WRKY4-C [16]. These structures are characterized by their novel types of zinc-binding folds. It should be noted that all of the protein samples were prepared by the cell-free method, but the details of the condition were different from those in the present optimized condition. The effects of zinc on the cell-free synthesis were not analyzed in these previous studies, whereas, in the present study, we analyzed the effects of zinc on the cell-free synthesis of plant-specific zinc-binding transcription factors in detail. Thus, we found that the proper concentration of $ZnSO_4$ in the reaction significantly increased the solubility as well as the yield of zinc-binding proteins.

Materials and methods

Construction of plasmids

The DNAs encoding SPL4-DBD (Leu51–Glu131), SPL7-DBD (Val135–Ala220), SPL9-DBD (Ile71–Ala151), SPL12-DBD (Ala124–Asp181), and WRKY4-C (Val399–Ala469) were subcloned into the pCR2.1 TOPO vector (Invitrogen, USA) by the 2-step PCR method (Yabuki et al., personal communication) from the RIKEN *Arabidopsis* full-length (RAFL) cDNA collection [17]: clone IDs RAFL15-05-B10 (MIPS code: at1g53160), RAFL09-11-N01 (MIPS code: at5g18830), RAFL04-13-I11 (MIPS code: at2g42200), RAFL09-41-M16 (MIPS code: at3g60030), and RAFL03-05-E05 (MIPS code: at1g13960), respectively. By the 2-step PCR method, the T7 promoter, the ribosome binding site, a Histidine Affinity Tag (HAT) (Clontech, USA), and a protease cleavage site were attached at the 5′ end, while the T7 terminator was attached at the 3′ end of the coding sequence. The thrombin protease cleavage site was used for SPL7-DBD, SPL9-DBD, and SPL12-DBD. The tobacco etch virus (TEV) protease cleavage site was used for SPL4-DBD and WRKY4-C. For chloramphenicol acetyltransferase (CAT) synthesis, the pK7-CAT plasmid was used [18].

Cell-free protein synthesis

The dialysis-mode cell-free protein synthesis method [19] was used in this study. The composition of the cell-free protein synthesis reaction was previously described [20]. The internal solution is composed of the buffers, substrates, template DNA, and enzymes required for transcription and translation. The external solution is composed of buffers and substrates. The S30 extract was prepared from the *E. coli* BL21 codon-plus RIL strain (Stratagene, USA), as described [20]. We developed a small-scale dialysis unit consisting of a Slide-A-Lyzer MINI Dialysis Unit, with a molecular-weight cut off (MWCO) of 10 kDa (Pierce, USA), and a Cryogenic Vial (NALGENE, USA) as a reservoir, and 30 μl of the internal solution was dialyzed against

300 μl of the external solution. The external solution was gently mixed with a small stirring bar during the incubation. The reaction was carried out at 30°C for 16 h. For the synthesis of zinc-binding proteins, 100 μM of $ZnSO_4$ (WAKO Pure Chemicals, Japan) was added to both the internal and external solutions except that 1 mM of $ZnSO_4$ was added for the synthesis of WRKY4-C. Puromycin was purchased from WAKO Pure Chemicals (Japan).

Preparation of ^{15}N-labeled proteins

The unlabeled amino acids in the internal and external solutions were substituted with 4 mg/ml of a ^{15}N-labeled algal amino acid mixture (Chlorella Industries Inc., Japan) supplemented with 1 mM each of L-[^{15}N]cysteine, L-[^{15}N]tryptophan, L-[^{15}N]glutamine, and L-[^{15}N]asparagine (TAIYO NIPPON SANSO, Japan). The internal solution (3 ml), in a dialysis tube (Spectra/Por 7, MWCO 15 kDa, Spectrum, USA), was dialyzed against the external solution (30 ml) at 30°C for 14 h with gentle shaking.

Purification of proteins

Parallel purification of the zinc-binding proteins from small-scale dialysis reactions was carried out as follows. The internal solution was recovered from the dialysis unit and centrifuged at 4,500 × *g* for 5 min, and the supernatant was transferred to a MultiScreen-HV 96-well filter unit (Millipore, USA) and mixed with 40 μl of buffer A [20 mM Tris–HCl buffer (pH 8.0), containing 750 mM NaCl, and 50 μM $ZnSO_4$] and 40 μl of a 50% TALON superflow resin slurry (Clontech, USA) equilibrated with buffer A. The filter unit was centrifuged at 400 × *g* for 1 min. Then, 150 μl of buffer A was added to the TALON resin, and the unit was centrifuged at 400 × *g* for 1 min. This wash step was repeated. Proteins were eluted with 100 μl of buffer B [20 mM Tris–HCl buffer (pH 8.0), containing 300 mM NaCl, 500 mM imidazole, and 50 μM $ZnSO_4$]. The yields of the purified proteins were determined by the Bradford method, using a Bio-Rad Protein Assay kit (Bio-Rad, USA). Bovine serum albumin was used as the protein standard.

From 3 ml of the internal solution, the ^{15}N-labeled protein was purified as follows. The internal solution was centrifuged at 8,000 × *g* for 5 min, and the supernatant was mixed with 2.6 ml of buffer A and 1.6 ml of a 50% TALON superflow resin slurry. By using the TurboFilter and a vacuum manifold (QIAGEN, Germany), the resins were washed with 9.6 ml of buffer A and the proteins were eluted with 4 ml of buffer B. TEV protease was added to the eluted fraction of the SPL4-DBD protein at a final concentration of 10 μg/ml, while thrombin was added to those of the SPL7-DBD, SPL9-DBD, and SPL12-DBD proteins at a final concentration of 7.5 unit/ml. The solutions were incubated at 30°C for 3 h to cleave the HAT tag.

Measurement of ^{1}H–^{15}N HSQC spectra

For NMR analysis, the ^{15}N-labeled WRKY4-C protein was concentrated and dissolved in buffer C [20 mM *d*-Tris–HCl buffer (pH 7.5) containing 100 mM NaCl, 50 μM $ZnCl_2$, 1 mM *d*-DTT, and 10% $^{2}H_2O$/90% $^{1}H_2O$]. As the solubilities of other proteins in buffer C were lower than that of the WRKY4-C protein, the same buffers as buffer C except for the NaCl concentration (200 mM for the ^{15}N-labeled SPL12-DBD protein and 300 mM for the ^{15}N-labeled SPL4-DBD, SPL7-DBD, and SPL9-DBD proteins) were used to achieve higher solubilities. The concentrations of the SPL4-DBD, SPL7-DBD, SPL9-DBD, SPL12-DBD, and WRKY4-C proteins were 0.30, 0.16, 0.37, 0.39, and 0.31 mM, respectively. The ^{1}H–^{15}N HSQC spectra of the ^{15}N-labeled proteins were acquired with eight scans per increment, 128 complex points in t_1 (^{15}N), and 512 complex points in t_2 (^{1}H), on Bruker AVANCE 600 and DRX 600 spectrometers equipped with triple-resonance CryoProbes.

Results and discussion

Effect of $ZnSO_4$ addition on the cell-free protein synthesis

As the $ZnSO_4$ concentration was increased, the synthesis of CAT was inhibited and its solubility was decreased (Fig. 1F), indicating that $ZnSO_4$ essentially has an inhibitory effect on the cell-free protein synthesis. In contrast, the addition of 0.05–0.25 mM $ZnSO_4$ significantly increased the solubilities of SPL4-DBD, SPL7-DBD, and SPL12-DBD (Fig. 1A, B, D). Interestingly, the productivities of SPL9-DBD and WRKY4-C were increased by the addition of $ZnSO_4$ (Fig. 1C, E). Unlike other zinc-binding proteins, the productivity of WRKY4-C linearly increased from 0 mM to 1 mM $ZnSO_4$. Therefore, for the synthesis of the zinc-binding proteins, the optimization of $ZnSO_4$ concentration is recommended to obtain best yield. In the case of the $ZnSO_4$ concentration is not optimized, 50–100 μM $ZnSO_4$ was added to the reaction, which is

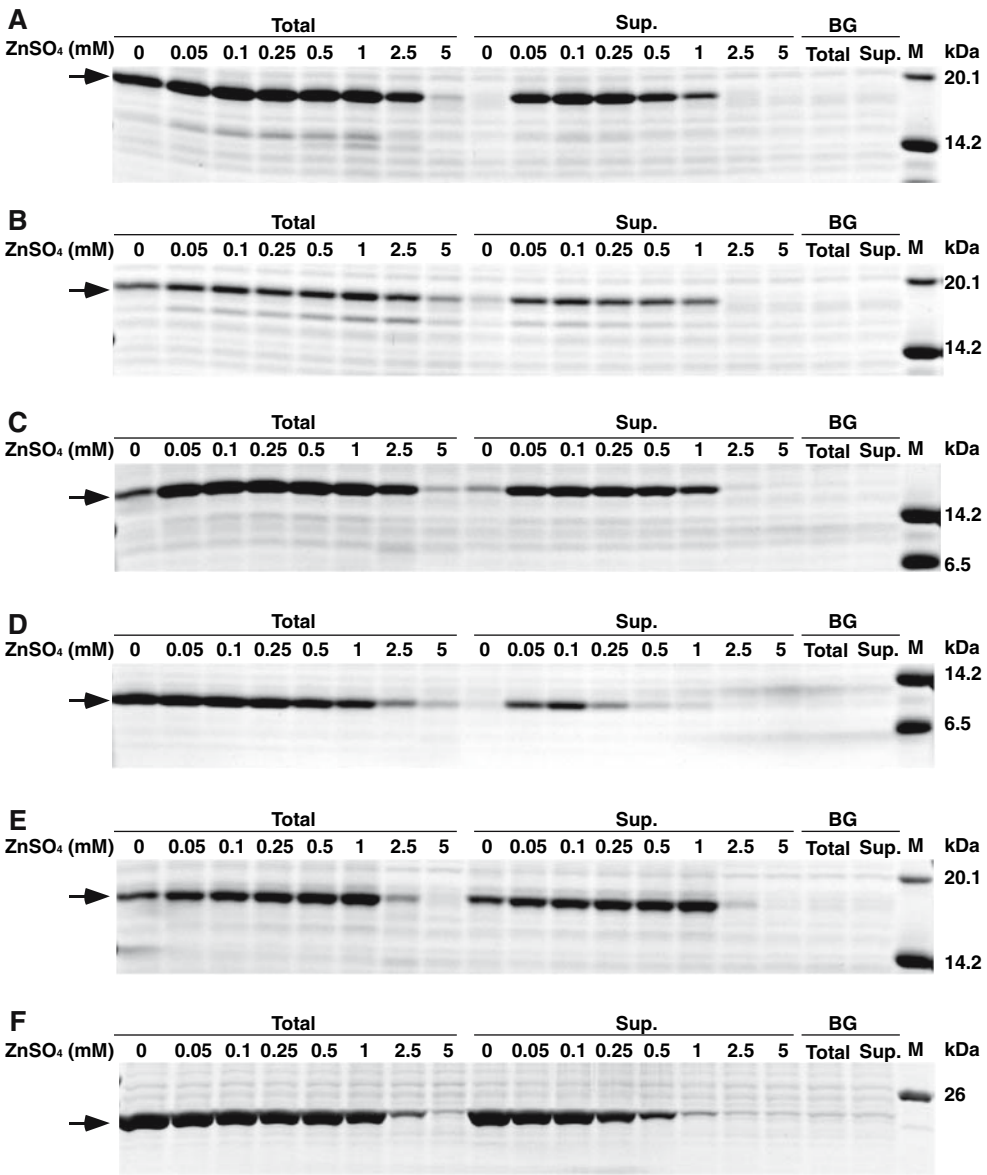

Fig. 1 SDS-PAGE analysis of synthesized zinc-binding proteins. SPL4-DBD (**A**), SPL7-DBD (**B**), SPL9-DBD (**C**), SPL12-DBD (**D**), WRKY4-C (**E**), and CAT (**F**) were synthesized in the presence of 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM $ZnSO_4$. Total and supernatant (Sup.) fractions (1 μl) of the internal solution were loaded on the gel. BG denotes the cell-free synthesis reaction in the absence of the template DNA and $ZnSO_4$

our standard condition for zinc-binding protein synthesis. The zinc-binding proteins were purified from 30 μl of the internal solution by His-tag affinity chromatography, and the yields were determined by the Bradford method. When 0.1 mM $ZnSO_4$ was added, the yields were 7.78 nmol of SPL4-DBD, 2.88 nmol of SPL7-DBD, 5.38 nmol of SPL9-DBD, 3.5 nmol of SPL12-DBD, and 4.04 nmol of WRKY4-C. The SPL4-DBD, SPL7-DBD, and SPL9-DBD proteins are indicated to bind two Zn^{2+} ions per molecule, while SPL12-DBD and WRKY4-C are indicated to bind one Zn^{2+} ion per molecule [14–16]. We added 33 nmol of $ZnSO_4$ to the cell-free protein synthesis reaction, which is a sufficient amount of Zn^{2+} ions for the protein products.

Degradation of apo-SPL9-DBD and apo-WRKY4-C

As shown above, the productivities of SPL9-DBD and WRKY4-C were significantly increased in the presence of $ZnSO_4$ at the proper concentration. However, it seems unlikely that Zn^{2+} ions enhance the cell-free protein synthesis reaction. When synthesized in the presence of 100 μM $ZnSO_4$, SPL9-DBD was observed as a thick band, while the band was weak in the absence of $ZnSO_4$ (Fig. 2A). To investigate the stability of SPL9-DBD, the translation reaction was stopped after 2 h of incubation by the addition of puromycin, an antibiotic that specifically inhibits translation, to 100 μg/ml. The reaction mixture was further incubated at 30°C, and the stability of SPL9-DBD in the internal

Fig. 2 Degradation of apo-SPL9-DBD and apo-WRKY4-C. SPL9-DBD was synthesized in the presence or absence of 100 μM $ZnSO_4$ (**A**), and WRKY4-C was synthesized in the presence or absence of 1 mM $ZnSO_4$ (**B**). Puromycin was added to stop the protein synthesis reaction after 2 h of incubation (''+Puromycin''). Total fractions (0.5 μl) of the internal solution were analyzed by SDS-PAGE

solution was analyzed. When synthesized in the presence of $ZnSO_4$, the thickness of the band did not change from 2 h to 8 h, as determined by an SDS-PAGE analysis. Meanwhile, the band of SPL9-DBD synthesized in the absence of $ZnSO_4$ (apo-SPL9-DBD) disappeared during the incubation. This result indicates that apo-SPL9-DBD was degraded by endogenous proteases. The same result was obtained for WRKY4-C, although the degradation was slower than SPL9-DBD (Fig. 2B). We prepared an *E. coli* cell extract from the BL21 strain, which lacks the major proteases Lon and OmpT. The protease activity of this extract would be lower than those of other *E. coli* strains. Nevertheless, apo-SPL9-DBD and

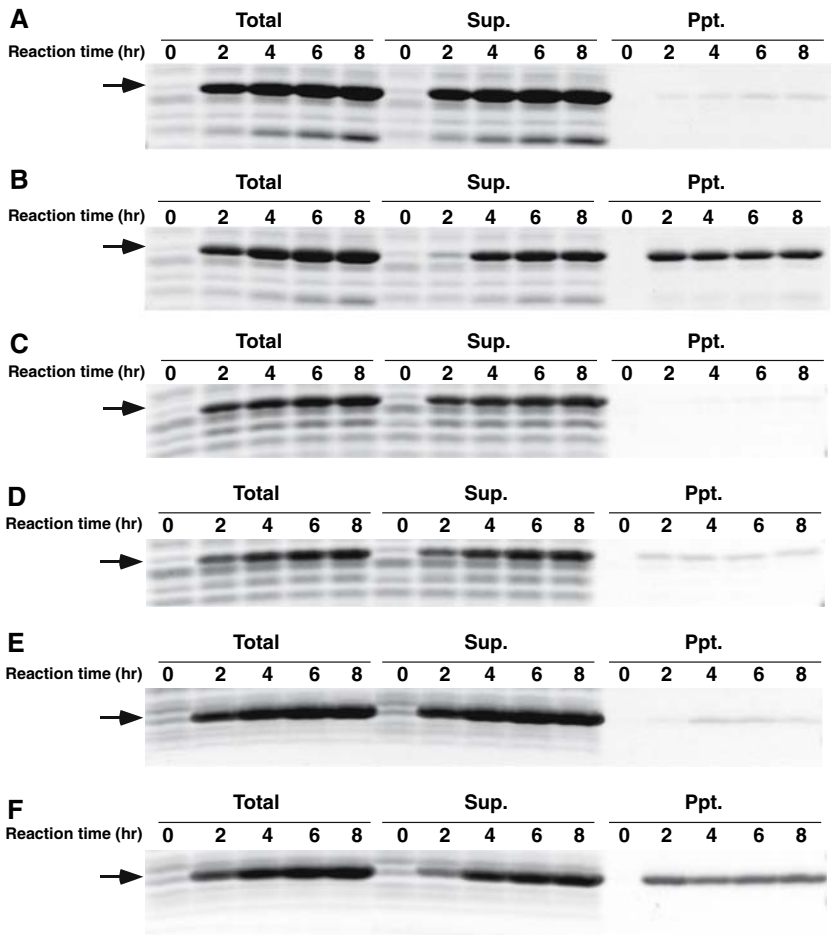

Fig. 3 Requirement of Zn^{2+} ions during protein synthesis. Requirement of Zn^{2+} ions during synthesis of SPL4-DBD (**A**, **B**), SPL7-DBD (**C**, **D**), and SPL12-DBD (**E**, **F**) were analyzed. $ZnSO_4$ (100 μM) was added at the beginning of the reaction (**A**, **C**, **E**) or after 2 h of incubation (**B**, **D**, **F**). Total (Total), supernatant (Sup.), and precipitate (Ppt.) fractions (1 μl) of the internal solution were analyzed by SDS-PAGE. Each fraction was sampled after 0, 2, 4, 6, and 8 h of incubation

apo-WRKY4-C were degraded. This indicates that apo-SPL9-DBD and apo-WRKY4-C are quite sensitive to proteolysis. Unlike properly folded proteins, unfolded proteins are easily degraded by low concentrations of protease [21]. Our results strongly suggest that Zn^{2+} ions are required for the proper folding of SPL9-DBD and WRKY4-C. We have already reported that SPL4-DBD, SPL7-DBD, and WRKY4-C were largely unfolded, when an excess amount of EDTA was added to chelate the bound Zn^{2+} ions, and that the folding processes of SPL4-DBD, SPL7-DBD, and WRKY4-C were zinc-dependent [14, 16]. The amino acid sequence of SPL9-DBD shares 60% and 47% identities to those of SPL4-DBD and SPL7-DBD, respectively, suggesting that the folding process of SPL9-DBD could be zinc-dependent, like those of SPL4-DBD and SPL7-DBD. These previous results coincide with the results obtained in this study.

Zn^{2+} ions are required during protein synthesis

The SPL4-DBD protein was mostly soluble, when 100 μM of $ZnSO_4$ was added at the beginning of protein synthesis (Fig. 3A), whereas it became insoluble in the absence of $ZnSO_4$ (Fig. 3B). Soluble SPL4-DBD was synthesized after a final concentration of 100 μM of $ZnSO_4$ was added during the reaction. The amount of insoluble SPL4-DBD did not change even after the $ZnSO_4$ addition. For the SPL12-DBD protein, the same result as SPL4-DBD

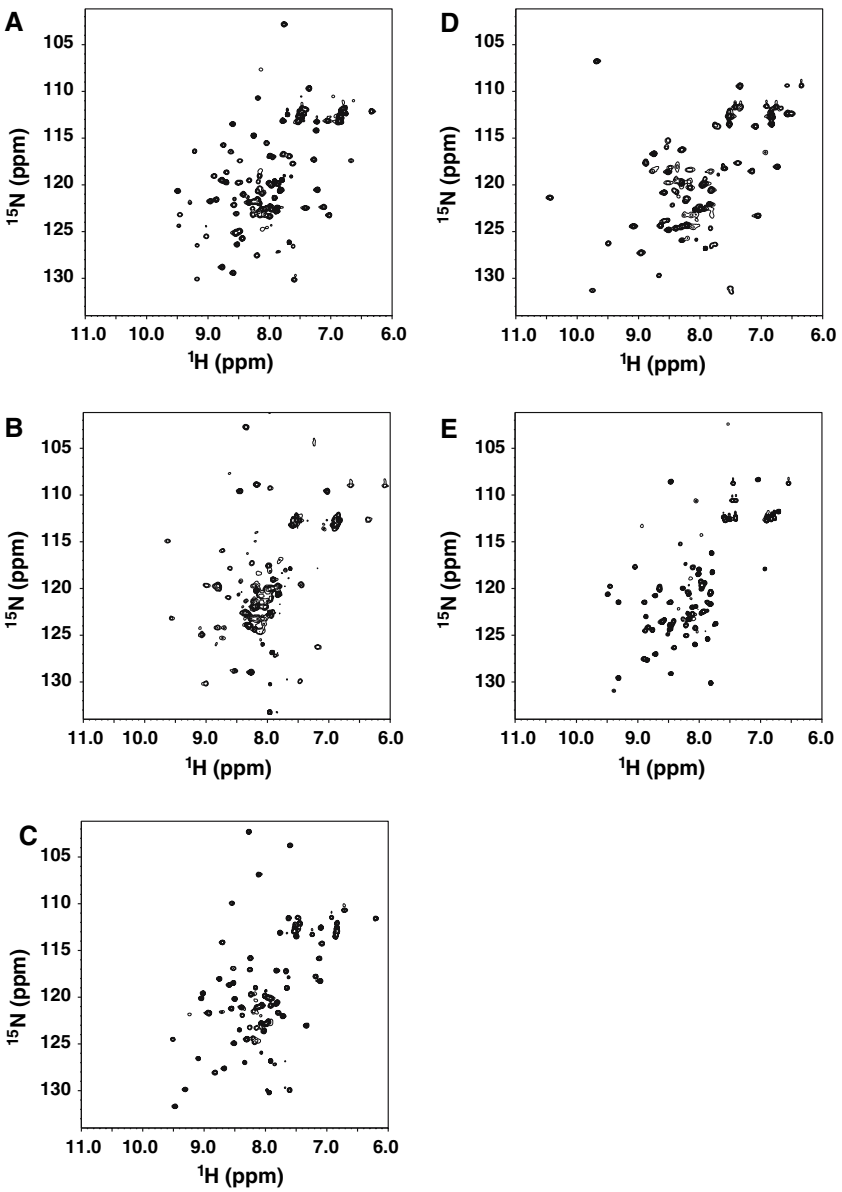

Fig. 4 ^{1}H–^{15}N-HSQC spectra of the ^{15}N-labeled SPL4-DBD (**A**), SPL7-DBD (**B**), SPL9-DBD (**C**), SPL12-DBD (**D**), and WRKY4-C (**E**) proteins. The tag sequences of the SPL4-DBD, SPL7-DBD, SPL9-DBD, and SPL12-DBD proteins were cleaved by a protease before the NMR analysis

was obtained, except that approximately half amount of SPL12-DBD was insoluble in the absence of $ZnSO_4$ (Fig. 3E, F). In the case of SPL7-DBD protein synthesized in the presence of $ZnSO_4$, most SPL7-DBD was soluble (Fig. 3C). Unlike SPL4-DBD and SPL12-DBD, only the small amount of SPL7-DBD was precipitated, which was synthesized during the absence of $ZnSO_4$ (Fig. 3D). It is likely that apo-SPL7-DBD precipitate more slowly than SPL4-DBD and SPL12-DBD, as most of SPL7-DBD precipitated during 16 h incubation in the absence of $ZnSO_4$ (Fig. 1B). These results indicate that precipitated apo-form zinc-binding proteins could not be re-solubilized again by the $ZnSO_4$ addition. To achieve a high yield, proper amount of $ZnSO_4$ should be added at the beginning of protein synthesis.

NMR spectra of zinc-binding proteins

Uniformly ^{15}N-labeled SPL4-DBD, SPL7-DBD, SPL9-DBD, SPL12-DBD, and WRKY4-C were prepared by the dialysis-mode cell-free method in the presence of $ZnSO_4$, and the ^{1}H–^{15}N HSQC spectra of these proteins were measured. The cross peaks in the ^{1}H–^{15}N HSQC spectra of SPL4-DBD, SPL7-DBD, SPL9-DBD, SPL12-DBD, and WRKY4-C were well-dispersed, which is characteristic of folded proteins (Fig. 4A, B, C, D, E, respectively). The SPL4-DBD, SPL7-DBD, SPL12-DBD, and WRKY4-C proteins synthesized by the cell-free method exhibited their specific DNA binding activities [14–16]. These results indicate that the cell-free method can be used to synthesize properly folded and functional zinc-binding proteins.

Based on the results obtained in this study, a high-throughput protein preparation pipeline for zinc-binding proteins using the cell-free synthesis method was established. We usually add 50–100 μM $ZnSO_4$ to the reaction as a standard condition, because this concentration of $ZnSO_4$ is sufficient for most zinc-binding proteins. We have investigated its suitability for the structure determinations of many kinds of zinc-binding domains from *A. thaliana*, mouse, and human proteins, and then determined the solution structures of more than 80 proteins by NMR spectroscopy (to be published elsewhere).

The cell-free synthesis method overcomes the limitation of the conventional cell-based methods, in that we can optimize the concentration of ligands and can even add ligands that are unable to cross the cell membrane. Folded zinc-binding proteins were produced by simply adding the proper amount of $ZnSO_4$ to the cell-free protein synthesis reaction, even though further optimization of the reaction might be required for some proteins. For the synthesis of Fe–S cluster-containing proteins, the addition of both iron and sulfur sources increases the productivity and maturation of the holo-enzyme [22]. Thus, the cell-free synthesis method can be widely applied to proteins that require ligand(s) for their enzymatic activity and/or structure formation and stabilization.

Acknowledgements The authors thank Yukako Miyata, Emi Nunokawa, Yukiko Fujikura, Natsuko Matsuda, Natsumi Suzuki, Yasuko Tomo, Eiko Seki, Masaomi Ikari, and Fumiko Hiroyasu at RIKEN for technical assistance. The authors also thank Takashi Yabuki for valuable suggestions and comments. The authors are grateful to Azusa Ishii, Kiyomi Yajima, and Tomoko Nakayama for expert secretarial assistance. This work was supported by the RIKEN Structural Genomics/Proteomics Initiative (RSGI), the National Project on Protein Structural and Functional Analyses, Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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