Chapter 9
Cell-Free Protein Synthesis Using *E. coli* Cell Extract for NMR Studies

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Abstract The use of cell-free protein production systems for producing isotope labeled proteins generates new opportunities to perform unprecedented NMR studies. As compared with conventional cellular expression systems, the scrambling and dilution of amino acids are highly suppressed in the cell-free reaction, allowing the production of proteins with a wide variety of residue and site-specific isotope labeling patterns. In this chapter, the procedure for cell-free protein synthesis for NMR studies, using an *E. coli* extract, is introduced.

9.1 Introduction

The production of isotopically labeled proteins is an initial step in the analysis of a protein by NMR. Cell-free protein synthesis is a practical method for protein production, along with cell-based expression systems. In the *in vitro* expression system, a target protein is produced in a vessel, where the protein synthesis machinery is reconstituted. As compared with conventional *in vivo* expression, amino acid metabolic conversion is suppressed in the *in vitro* expression system, thus providing an opportunity to produce proteins with a wide variety of residue- and site-specific isotope labeling patterns [1]. In addition, the open nature of the *in vitro* expression enables various modifications of the reaction conditions, such as supplementation with chaperones, inhibitors, and detergents/lipids.

Historically, the *in vitro* expression of proteins has utilized an extract from *E. coli* cells [2–7]. The *E. coli* extract contains a large number of proteins needed for protein synthesis (e.g., ribosomal proteins and initiation factors). When energy sources and template DNA are added to the extract, the encoded protein is synthesized in the vessel. However, the protein synthesis soon ceases, due to the depletion of energy and substrate. Over the past decade, many efforts have been directed toward improving its productivity. Now, the *E. coli* cell-free expression method has become a practical means

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for producing proteins on a preparative scale. Furthermore, new types of cell-free synthesis systems, such as the wheat germ system [8, 9] and one exclusively composed of recombinant proteins [10, 11], have appeared as alternative cell-free methods. Especially, wheat germ cell-free protein synthesis has become a practical method for producing proteins for NMR studies, along with the *E. coli* cell-free expression system.

The advent of cell-free protein synthesis systems provides opportunities to perform NMR approaches that are not amenable to cellular-based expression system. For example, a wider range of isotope labeled amino acids can be incorporated into a target protein by using cell-free synthesis, due to the suppressed inter-conversion of amino acids [1, 12, 13]. In addition, the incorporation of unnatural amino acids can be accomplished using *E. coli* cell-free synthesis [14–16]. The cell-free protein synthesis system has also been used for the production of membrane proteins [17–19]. In general, membrane proteins need proper membrane-mimicking components (e.g., detergents and lipids) to exist in a folded state in an aqueous solution. The merit of cell-free protein synthesis is that the membrane protein can be expressed in the presence of a wide variety of detergents/lipids, owing to the open nature of the reaction, thereby increasing the chance of expressing the membrane protein in the properly folded state. Another important application of cell-free protein synthesis is the production of SAIL proteins [20, 21]. The SAIL protein is composed of chemically synthesized amino acids that are stereo- and region-selectively isotope labeled. Therefore, the SAIL amino acids must be incorporated into a target protein efficiently, without metabolic scrambling. The details of the method are described in another chapter in this book. In our laboratory, several SAIL proteins were produced by using an *E. coli* cell-free system [22–24].

This chapter provides an overview of the procedure for *E. coli* cell-free protein production, with a focus on NMR study. In the early version of the *E. coli* cell-free reaction system, the protein synthesis soon ceased, and this problem remained unsolved. By virtue of extensive efforts to improve the productivity over the past few decades, however, it has become possible to produce recombinant proteins in milli-gram quantities by using an *E. coli* cell-free system [3, 5, 25]. Another issue in the *E. coli* cell-free protein synthesis is the low amino acid labeling efficiency. The *E. coli* extract prepared by the previous protocol contained a large amount of endogenous, unlabeled amino acids, which dilute the added isotope labeled amino acid in the resulting proteins. Furthermore, in some cases, the processing of the N-terminal formyl methionine by a peptide deformylase does not occur completely in the *in vitro* expression system [26, 27]. The presence of the formyl moiety causes large chemical shift changes in nearby residues, giving rise to doubled peaks corresponding to the formylated and deformylated forms. It is also somewhat problematic that isotope scrambling and dilution still occur even in the *E. coli* cell-free system due to residual enzymatic activity, although they are relatively suppressed as compared to cellular expression.

In the following, the procedures for the preparation of the *E. coli* cell-free extract and the cell-free protein synthesis will be described.

9.2 Preparation of the *E. coli* S30 Extract for NMR Samples

The *E. coli* cell-free expression system is commonly used in a wide variety of fields, and thus *E. coli* extracts are commercially available. However, such extracts are not necessarily optimized for the production of NMR samples. One potential problem is that they may contain large amounts of endogenous amino acids, which dilute the added isotope labeled amino acids. Therefore, we developed an *E. coli* extract preparation method. The protocol for the preparation of the *E. coli* extract consists of culturing the *E. coli* cells, washing the cells, a run-off reaction, dialysis, gel-filtration and condensation. This procedure was established by modifying pre-existing protocols [2, 5, 27–32]. The preparation of the *E. coli* extract takes about 2 days for one person.

9.2.1 Protocol for Amino Acid-Free S30 Extract Preparation

1. Inoculate *E. coli* cells (e.g., A19, BL21 Star (DE3) strains) into 10 ml of LB medium and grow the cells overnight at 37°C with shaking.
2. Use the 10 ml culture to inoculate 1 l of incomplete rich medium (5.6 g/L KH_2PO_4, 28.9 g/L K_2HPO_4, 1 g/L Bacto yeast extract, 1.5 mg/L thiamine, 20 g/L D-glucose and 1 mM $Mg(OAc)_2$) in a 2-l flask.
3. Culture the cells at 37°C with shaking, until an OD_{650} of 0.7 is attained.
4. Centrifuge the cells (5,000 g, 4°C, 10 min) and wash them three times with 200 ml of ice-cold S30 buffer (10 mM Tris-acetate (pH 8.2), 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM DTT) containing 0.05% 2-mercaptoethanol.
5. Gently resuspend the cell pellet in 200 ml of ice-cold S30 buffer containing 0.05% (v/v) 2-mercaptoethanol. Centrifuge the suspension (5,000 g, 4°C, 10 min) and weigh the *E. coli* pellet. Resuspend the pellet in 1.27 ml of S30 buffer per gram of *E. coli*.
6. Disrupt the cells with a French Press at 20,000 psi (1,400 kg cm^{-2}). Add 30 μl of 1 M DTT to the lysate immediately after the disruption of the cells. Centrifuge the lysate (30,000 g, 4°C, 30 min) using RNase-free centrifuge tubes. Carefully remove approximately 1.4 ml of the supernatant per gram of *E. coli*, without mixing with the precipitate.
7. Transfer the supernatant to RNase-free tubes. Centrifuge them (30,000 g, 4°C, 30 min) and remove approximately 1.0 ml of the supernatant per gram of *E. coli* into a 50 ml tube.
8. Shake the tube at 37°C for 80 min.
9. Dialyze the solution at 4°C for 45 min against 2 l of S30 buffer, using a dialysis tube with a molecular weight cut off of 6,000–8,000. Repeat the dialysis twice, and then centrifuge the solution (15,000 g, 10 min, 4°C) and collect the supernatant.
10. Uniformly fill an open column (Econo-column chromatography column, 2.5 × 20 cm) with Sephadex G25 resin (GE Healthcare), and place the column vertically in a cold space (4°C). Attach an Econo-column funnel to the top end of the column. Pour 500 ml of S30 buffer through the funnel into the column.
11. Apply the supernatant from step 9 to the column that was pre-equilibrated at 4°C in step 10. After loading the supernatant, continue to supply the funnel with S30 buffer, to maintain the flow in the column. When the first fraction reaches the bottom, start to collect 1.4 times the volume of the applied extract. The first fraction is determined by its color (yellow) and turbidity.
12. Dialyze the eluate at 4°C against 700 ml of an equal weight mixture of polyethyelene glycol (PEG)-8000 and S30 buffer. Before use, the PEG-S30 buffer (at 4°C) should always be stirred to avoid PEG deposition. Adjust the dialysis time so as to concentrate the extract up to 0.86 times the volume. Dialyze the concentrated extract at 4°C for 60 min against 2 l of S30 buffer.
13. Transfer the extract to 1.5 ml tubes. Freeze the tubes in liquid nitrogen. Store them at 80°C.

9.2.2 Considerations About the Preparation Protocol

The *E. coli* cells are grown in either complex (LB) medium or glucose minimal medium. The type of growth medium affects both the activity and composition of the resulting extract [33]. In addition, the type of *E. coli* cells affects the expression level and the residual enzymatic activity to some extent. In our laboratory, *E. coli* A19 and BL21 Star (DE3) (Invitrogen, Carlsbad, CA) strains are preferably used, for their high expression levels. When culturing *E. coli* cells, the growth of the cells is an important factor that significantly influences the activity of the extract [34]. Therefore, the growth rate of the cells should be monitored. If the growth is slower than usual, then it is likely that the resulting extract will have lower activity.

Cells within the exponential growth phase are harvested by centrifugation, and then washed four times with buffer. After the final wash, the cells are disrupted. This step also affects the activity of the resulting extract. The cells should be extensively disrupted. However, the protein synthesis machinery, such as ribosomes, should not be damaged. The solution containing the disrupted cells is centrifuged at 30,000 g to eliminate the insoluble materials. It may be noteworthy that one preparation protocol performs this centrifugation at 12,000 g [35]. According to a report by Pederson and coworkers, the use of this extract (termed S12 extract) leads to a 30% increase in the expression level [36].

A run-off reaction (or called pre-incubation) is then performed to release ribosomes/polysomes from the endogenous mRNA. In the original procedure, a solution containing amino acids and polymerases without template DNA was added and incubated for 40–80 min. However, the addition of amino acids to the extract can cause the dilution of the added isotope labeled amino acids. Therefore, we perform the run-off reaction without adding the solution. Fortunately, the run-off reaction reportedly proceeds even in the absence of the solution containing amino acids and polymerases [30].

After the run-off reaction, the solution is dialyzed, using a semi-permeable membrane with a molecular weight cut off of 6–8,000. By doing so, small molecules, (e.g., ADP, inorganic phosphate and amino acids) are eliminated from the extract solution. The elimination of the small molecules is important, as they disturb the protein synthesis and cause the dilution of isotope labeled amino acids.

In our laboratory, a gel filtration step is performed after the dialysis to minimize the amount of endogenous amino acids present in the resulting extract [27, 31, 32]. Our tests revealed that some endogenous amino acids remained even after the dialysis. The gel-filtration step separates the extract from the endogenous amino acids. For example, when [U-^{2}H, ^{13}C, ^{15}N]-calmodulin (CaM) protein was produced by using an *E. coli* S30 extract prepared by the original protocol, the labeling efficiency was only ~90%, but it was improved up to 96% by introducing the gel filtration step [27] (Fig. 9.1).

After the dialysis, the resulting extract is condensed by using polyethylene glycol (PEG). The condensation of extract reportedly increases the productivity of the extract [4, 5]. In our experience, however, excess condensation leads to the precipitation of the extract, and thus the duration of the PEG condensation should be adjusted such that the highest activity is obtained.

Finally, the extracts are retrieved and stored at −80°C. To evaluate the presence of endogenous amino acids in the prepared extract, an isotope labeled protein is expressed using the prepared sample, and then the labeling efficiency is monitored by either mass spectroscopy or a ^{13}C-filtered ^{1}H1D experiment [27].

9.3 *E. coli* Cell-Free Protein Synthesis

9.3.1 Configuration of the E. coli Cell-Free Protein Synthesis

The cell-free systems are classified into two types: batch-type and continuous exchange cell-free (CECF) configurations. In the batch-type configuration, the experiment is performed on small-scales (~a few μl). In the early days, the protein synthesis in the batch-configuration ceased soon after the initiation of protein synthesis, probably due to the depletion of the energy sources and the accumulation of inorganic phosphate. Therefore, the batch-configuration mode is mainly used for checking the expression level, and is not used for preparative scale production. Recently, however, the productivity of the batch-mode has been dramatically increased, by improving the ATP (adenosine triphosphate) regeneration system. In the case of the CECF configuration, the reaction solution is connected to the dialysis solution via a semi-permeable membrane, such that the energy sources are continuously supplied from the dialysis solution, and the by-products generated in the reaction solution are diluted [4]. One representative of CECF is a dialysis system, where the reaction solution and the dialysis solution are partitioned by using a semi-permeable membrane (Fig. 9.2). The protein synthesis occurs in the

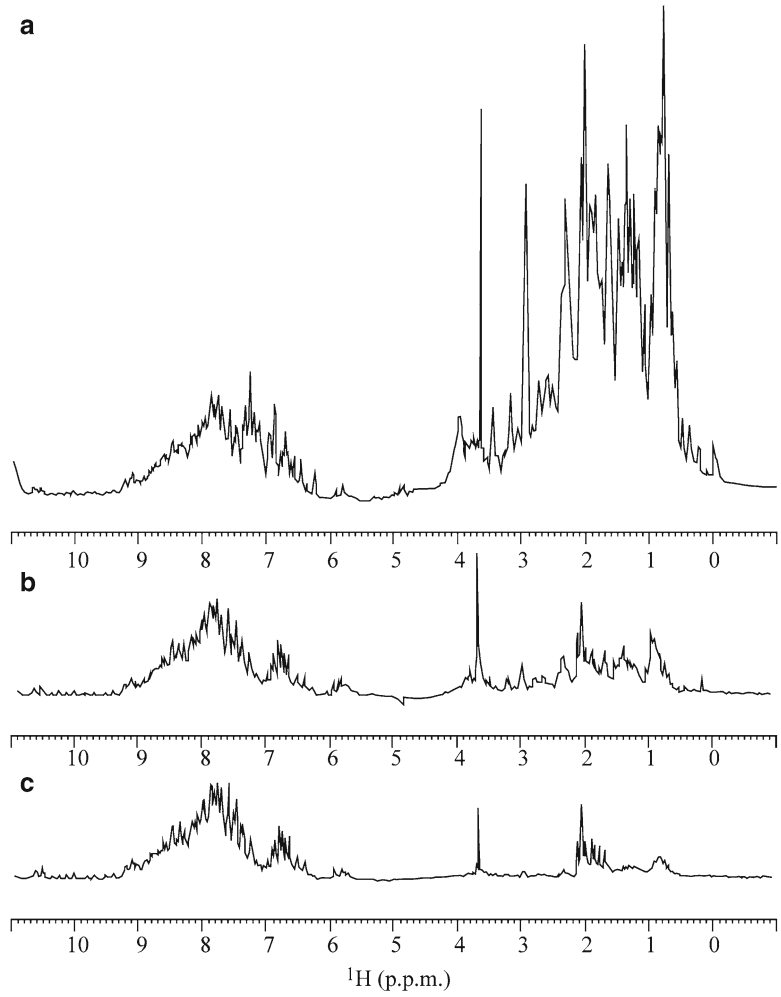

Fig. 9.1 ^{13}C-filtered ^{1}H-NMR spectra of CaM. (**a**) ^{15}N-labeled CaM synthesized with the conventional extract. (**b**) ^{2}H, ^{13}C, ^{15}N-labeled CaM synthesized with the conventional S30 extract. (**c**) ^{2}H, ^{13}C, ^{15}N-labeled CaM synthesized with the improved, dialyzed S30 extract. All of the spectra are adjusted for the intensities of the amide region. Since the peaks of the protons attached to ^{13}C are filtered, only the protons attached to ^{12}C give rise to resonances in the aliphatic region (Reproduced from Ref. [27]. With permission from Elsevier Science)

reaction solution, and the dialysis solution supplies energy sources and dilutes harmful by-products, such as adenosine diphosphate (ADP) and inorganic phosphate. In the following section, the cell-free synthesis using the dialysis system is introduced.

9.3.2 Composition of the E. coli Cell-Free Reaction Solution

The typical compositions of the solutions in the dialysis system are shown in Table 9.1. For the dialysis system, the reaction and dialysis solutions are prepared separately. The reaction solution contains components required for producing the proteins encoded by the added DNA template. On the other hand, the dialysis solution provides energy sources for the reaction solution and dilutes the generated by-products.

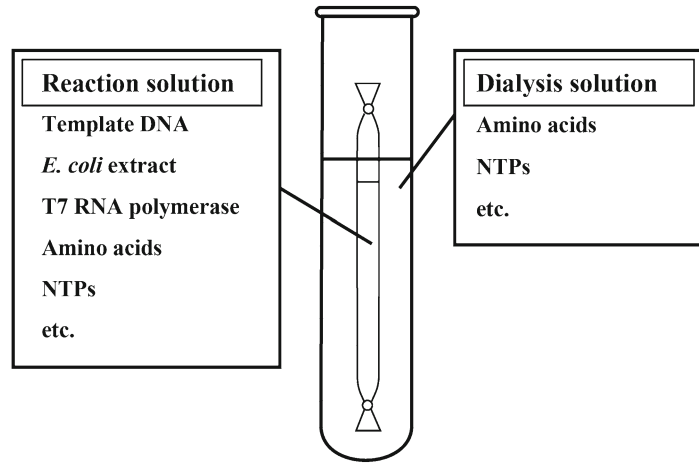

Fig. 9.2 The dialysis system for cell-free expression

Table 9.1 Composition of reaction and dialysis solutions in cell-free reactions

Stock solution	Reaction solution	Dialysis solution
RNase-free water	112 µl	1160.8 µl
1.4 M NH_4OAc	9.8 µl	39.2 µl
0.5 M $Mg(OAc)_2$	15 µl	60 µl
Amino acid mixture (25 mM each)	20 µl	80 µl
0.645 M creatine phosphate	40 µl	160 µl
Low molecular weight mixture[a]	125 µl	500 µl
1 mg/ml template DNA	10 µl	–
11 mg/ml T7 RNA polymerase	4.5 µl	–
40 units/µl RNase inhibitor	1.25 µl	–
10 mg/ml creatine kinase	12.5 µl	–
E. coli S30 extract	150 µl	–
Total volume	0.5 ml	2 ml

[a]The low molecular weight (LM) mixture is prepared by combining the following components: 22 ml of 2 *M* HEPES-KOH (pH 7.5), 33.4 ml of 6 *M* K(OAc), 210 mg of DTT, 530 mg of ATP (Sigma), 338 mg of CTP (Sigma), 335 mg of GTP (Sigma), 310 mg of UTP (Sigma), 172 mg of cAMP (Wako, Osaka, Japan), 28 mg of folinic acid (Sigma) and 140 mg of tRNA (Roche, Basel, Switzerland), and, if needed, 64 ml 50% (w/v) PEG-8000. RNase-free water is then added, to bring the volume up to 200 ml

In *E. coli* cell-free expression, the transcription and translation occur in a vessel in a coupled manner. Most of the currently employed *E. coli* cell-free synthesis systems are designed such that the mRNA is transcribed from the template DNA by T7 RNA polymerase [37]. Therefore, a plasmid encoding a DNA sequence under the control of the T7 promoter, such as the pET vectors (Novagen), can be used as-is. An advantage of the cell-free system over cellular expression is that PCR products can also be used as templates, thus accelerating the functional and structural analyses of proteins [38].

The continuous supply of ATP to the reaction system is essential for high productivity. For this purpose, a compound carrying a high-energy phosphate bond and the corresponding kinase (e.g., phospho(enol)pyruvate (PEP)/pyruvate kinase and creatine phosphate (CP)/creatine kinase) are included in the reaction solution. One problem with the energy source regeneration system is that the high-energy compound is degraded by the phosphatases present in the extract, which leads to the production of inorganic phosphate [39]. The accumulation of inorganic phosphate leads to the cession of protein synthesis. To mitigate this problem, CP is used as an energy source rather than PEP, as CP does not exist in *E. coli* cells and thus there are fewer phosphatases able to degrade CP in the *E. coli* extract [5].

The 20 amino acids are supplemented as precursors in the cell-free system. Therefore, residue-selective labeling can readily be achieved by using an amino acid bearing an intended isotope labeling pattern. For the production of uniformly ^{13}C and/or ^{15}N labeled proteins, an algal hydrolysate containing a mixture of ^{13}C and/or ^{15}N labeled amino acids is useful. However, it should be noted that the composition of each amino acid in the algal hydrolysate varies, depending on the lot and vendor. Especially, most algal hydrolysates lack Asn, Gln, Trp and Cys residues. In this regard, Asn and Gln are synthesized from Asp and Glu by transaminases present in the *E. coli* extract, and the side-chain amino groups of Asn and Gln are transferred from ammonium acetate. Therefore, by using ^{15}N ammonium acetate, the side-chain amino groups are enriched by ^{15}N. On the other hand, Trp and Cys are not synthesized, and thus they must be supplemented if the target protein contains them in its amino acid sequence.

9.3.3 Protocol for Cell-Free Expression

1. Wear sanitary gloves and thaw the LM mixture and the S30 extract.
2. Prepare the reaction and dialysis solutions, as shown in Table 9.1. In the case of a small-scale reaction for evaluating expression levels, the typical volumes of the reaction and dialysis solutions are 0.5 and 2.0 ml, respectively. For large production quantities, each volume is scaled up while maintaining the volume ratio of the reaction solution to the dialysis solution.
3. Transfer the dialysis solution to an RNase-free vessel. Tie one end of the dialysis tube firmly. Transfer the reaction solution into the dialysis tube, and tie off the other end. Fold the tubing four to six times, and place the dialysis tube into the vessel, such that the dialysis tube is completely submerged in the dialysis solution.
4. Incubate the vessel with shaking for 4–8 h at 37°C.
5. Retrieve the reaction and dialysis solutions. If the produced expressed protein has a molecular weight smaller than molecular weight cut-off of the membrane, then the produced protein can be found in both the reaction and dialysis solutions.

9.3.4 Optimization of Conditions for Small-Scale Reactions

The optimal conditions for cell-free reactions vary, depending on the kind of protein, and thus the reaction conditions should be optimized to some extent before proceeding to the expression of isotopically labeled proteins. The optimization is performed based on pilot experiments with small-scale reactions.

As the expression level of the target protein in an *E. coli* cell-free expression system is affected by the sequence of the template DNA, it is worth considering the introduction of a silent mutation to the DNA sequence. Even if they encode the same amino acid, distinct DNA sequences lead to different expression levels, and thus the introduction of a mutation is worth trying, unless the amino acid is altered. Empirically, the DNA sequence following the translation initiation site greatly affects the expression level. Unfortunately, however, there is no rational strategy to design silent mutations, and thus the optimized sequence must be found by trial and error. To systematically optimize the DNA sequence of the N-terminus, we transfer the target DNA sequence into a line of vectors with different silent mutations [27]. These vectors were constructed by introducing the DNA sequence encoding the N-terminal His-tag sequence of the pET15 vector (Novagen) into the multi-cloning site of the pIVEX2.3d vector (Roche). The DNA sequence encoding a target protein is introduced into the sites following the His-tag sequence in the line of vectors. While all of the vectors encode the target protein fused with the N-terminal His-tag, their expression levels differ, and thus a vector with high productivity can be identified (Fig. 9.3).

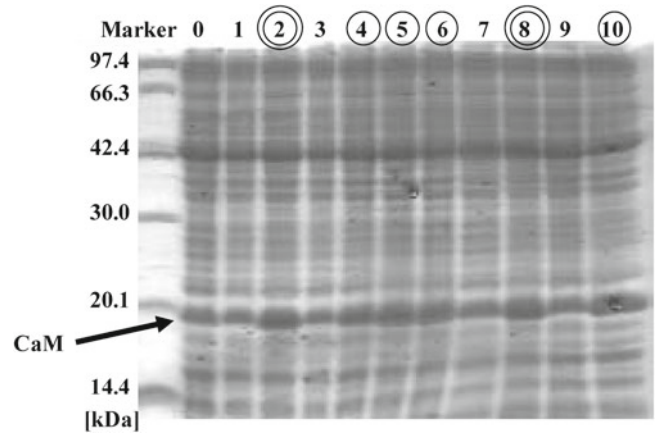

Fig. 9.3 SDS–PAGE of CaM, synthesized using plasmids without or with silent mutations predicted to enhance production. Lane 0: protein from the construct without the silent mutations. Lanes 1–10: proteins from constructs with ten different silent mutations. In the comparison of the intensities of lanes 1–10 to lane 0, the candidates with higher intensity are shown by a *circle* and those with much higher intensity by a *doubled circle* (Reproduced from Ref. [27]. With permission from Elsevier Science)

The concentration of magnesium is also an important parameter. During a cell-free reaction, phosphate accumulates due to the presence of endogenous phosphatases, and magnesium precipitates upon binding to the phosphate. This problem could be mitigated by adding a large amount of magnesium. In practical applications, however, the optimal concentration of magnesium differs depending on the target protein, and thus the best magnesium concentration should be determined in small-scale reactions.

The volume ratio of the dialysis and reaction solutions is an important parameter. By increasing the ratio, the productivity is expected to increase, due to the increased supply of energy and efficient dilution of harmful by-products. However, the isotope labeled amino acids are membrane-permeable, and thus the efficiency of amino acid usage becomes worse with a higher volume ratio, in terms of the cost of isotope labeled amino acids. In our laboratory, the ratio of the dialysis solution to the reaction solution ranges from 4 to 10.

9.3.5 Checking the NMR Spectral Quality of Proteins Produced by the Cell-Free Synthesis System

Once the conditions for achieving an acceptable expression level are established in small-scale reactions, we recommend scaling-up the reaction volume to produce a uniformly ^{15}N-labeled protein under the established conditions. A comparison of its [^{1}H, ^{15}N]-HSQC spectrum with that produced by cellular expression reveals the differences between them. As the enzyme activity is reduced in the *in vitro* expression, the property of the resulting protein may be altered. Special attention should be paid to the incomplete deformylation of the N-terminus of proteins in the cell-free reaction [26, 27]. To overcome this problem, the use of the aforementioned engineered N-terminal cleavable tag is helpful [27]. The inhomogeneous N-terminus is cleaved by a protease, and thus the homogeneous N-terminus is obtained (Fig. 9.4).

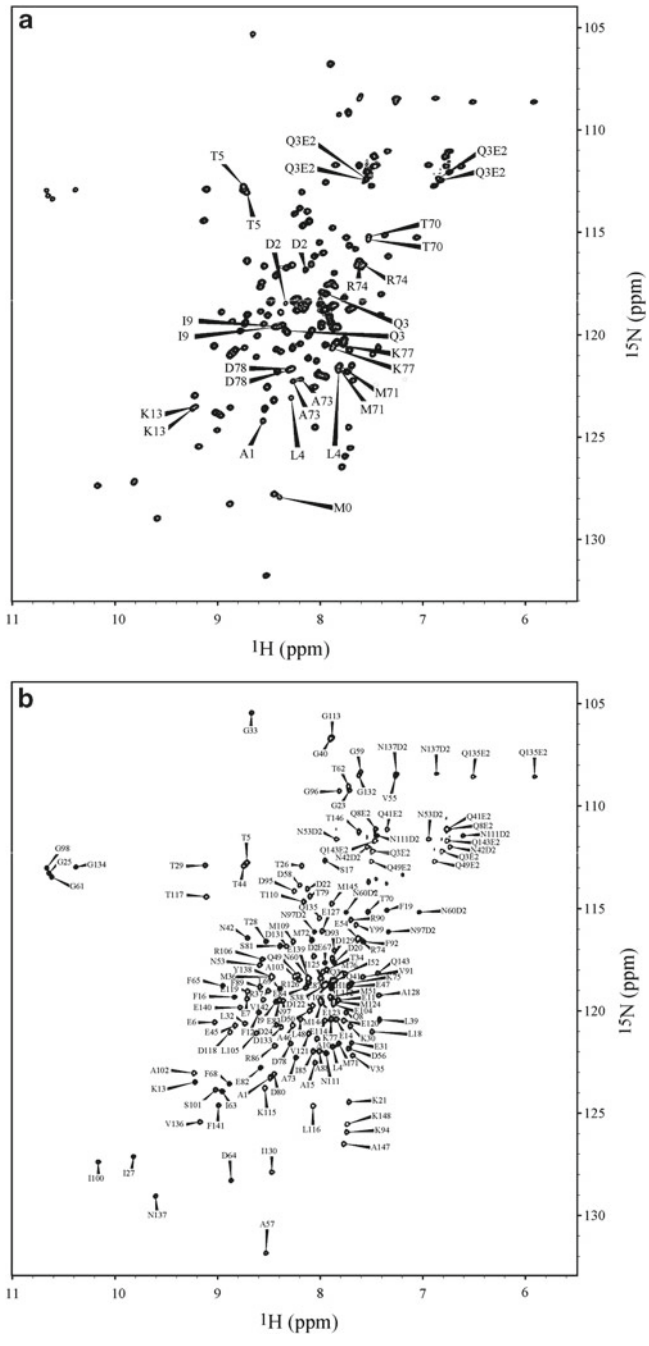

Fig. 9.4 Incompete deformylation leads to the doubling of NMR peaks. ^{1}H-^{15}N HSQC spectra of [U-^{13}C, ^{15}N] CaM synthesized by an *E. coli* cell-free reaction gives rise to doubled peaks for residues spatially proximal to its N-terminus (**a**) This peak doubling was eliminated by producing CaM as a fusion with an N-terminal cleavable tag and digestion by the protease (**b**) The extra peaks in (**a**) and all peaks in (**b**) are labeled with their assignments (Reproduced from Ref. [27]. With permission from Elsevier Science)

It should be noted that metabolic conversion is not completely suppressed even in the *E. coli* cell-free system. For example, the exchange of the α- and, to a lesser extent, β-proton(s) of an amino acid with the solvent water can occur in the *E. coli* cell-free system [40, 41]. Therefore, if the expression of a deuterated protein is intended, the reaction should be performed in a D_2O solution; otherwise, the α-protons and, to a lesser extent, β-protons of the amino acids will exchange with the solvent water. To suppress the activity of the enzyme, the use of inhibitors has been proposed [26, 41–44]. However, an excess amount of inhibitor can exert harmful effects on the expression of the target proteins. Therefore, the optimal concentration should be evaluated in terms of a compromise between the inhibition of scrambling and the expression level. In the future, the use of the PURE system, in which the protein synthesis machinery is reconstituted exclusively with recombinant proteins, will ultimately resolve this problem.

9.4 Conclusions

In this chapter, we described the procedure for *E. coli* cell-free protein synthesis. Along with *E. coli* cell-free expression, wheat-germ cell-free expression has now become a practical method for producing NMR samples. In addition, the application of the PURE system to NMR sample production has high potential, due to the ability to completely control the enzymatic activity. The continuous improvement of cell-free protein synthesis and the increasing number of NMR applications will pave the way toward unprecedented NMR studies.

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