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Stable-Isotope Labeling Using an Inducible Viral Infection System in Suspension-Cultured Plant Cells

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Summary

We established a novel strategy for preparing uniformly stable isotope-labeled proteins by using suspension-cultured plant cells and an inducible virus vector encoding the research target. By using this new method, we demonstrated the expression of three proteins, namely, *Escherichia coli* dihydrofolate reductase (DHFR), chicken calmodulin (CaM), and porcine protein kinase C-dependent protein phosphatase-1 inhibitor with a molecular mass of 17-kDa (CPI-17). In addition, we successfully expressed bovine pancreatic trypsin inhibitor (BPTI), which contains three pairs of disulfide bonds, as the soluble form. In the most efficient case, as little as 50 mL culture yielded 3–4 mg ^{15}N-labeled protein suitable for NMR experiments. The ^{1}H-^{15}N HSQC spectra of all of these proteins clearly indicated that their structures were identical to those of their counterparts reported previously. Thus, the present results suggest that our novel protocol is a potential method for sample preparation.

Abbreviations

BPTI, bovine pancreatic trypsin inhibitor; BY-2, *Nicotiana tabacum* cv. Bright Yellow 2; CPI-17(22-120), functional domain of 17-kDa protein phosphatase-1 inhibitor protein; CaM, calmodulin; DHFR, dihydrofolate reductase; ER, endoplasmic reticulum; HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; ToMV, tomato mosaic virus; XVE, chimeric LexA-VP16-ER transactivator

Key words

Inducible virus vector, BY-2, stable-isotope labeling

Running title

Sample preparation using an inducible virus vector

Introduction

Solution nuclear magnetic resonance (NMR) is used to study protein structures, dynamics, interactions, conformational changes, *etc.,* at atomic resolution (Wüthrich 1986). Until the end of the `80s, structure determination of proteins using ^{1}H-NMR experiments was limited to small molecules (< 10 kDa). In the past 20 years, however, the situation has totally changed. The uniform ^{13}C and/or ^{15}N labeling method and modern multidimensional NMR techniques dramatically pushed up the limitation of molecular weight to 20-25 kDa (Rule and Hitchens 2006). In particular, sophisticated stable-isotope labeling strategies using ^{2}H, ^{13}C, and ^{15}N have been continuously proposed (Tugarinov et al. 2005; Kainosho et al. 2006; Sprangers and Kay 2007) for studying larger proteins and their complexes (> 25 kDa) with NMR. So far, stable isotope-labeling is still a key methodology in the protein NMR field.

Calmodulin (CaM), a uniformly double ^{13}C- and ^{15}N-labeled Ca^{2+}-binding protein expressed in *Escherichia coli* systems was employed as the sample for the first demonstration of triple-resonance three-dimensional NMR experiments (Ikura et al. 1990; Kay et al. 1990). Since then, various modified and improved sample preparation methods employing *E. coli* have been proposed in parallel with the development of novel NMR experiments (Lian and Middleton 2001). Because the *E. coli* system is easy to handle, numerous vectors and hosts have been tested. As a result, many of them are now commercially available. Although the *E. coli* system has become extremely popular for NMR sample preparations, it poses some limitations for the synthesis of foreign proteins. In particular, many eukaryotic proteins expressed in *E. coli* cannot be folded accurately and processed by authentic posttranslational modifications. Therefore, the application of other living hosts, such as *Pichia*, SF9, and CHO cells, have been considered as alternatives for the preparation of labeled NMR samples (Patzelet et al. 2002). Further, cell-free protein synthesis systems have also been examined for stable isotope-labeling (Kigawa et al. 1995; Morita et al. 2003; Torizawa et al. 2004). The cell-free systems yield target proteins *in vitro* and are therefore believed effective for the expression of toxic proteins.

Although numerous protein expression systems have been developed, as briefly described above, a universal strategy for NMR sample preparation has not been established to date. Hence, the selection, adaptation, and optimization of expression systems are still required for each research target. In this context, the increased

availability of various sample preparation methods undoubtedly provides further opportunities for NMR studies. Here, we report a novel labeling strategy using suspension-cultured plant cells. Tobacco BY-2 cells (*Nicotiana tabacum* cv. Bright Yellow 2) were used in our experiments.

Tobacco BY-2 suspension-cultured cells are among the most popular plant cell lines used in basic and applied plant sciences. The properties of BY-2 suspension-cultured cells have been extensively exploited, and appear suitable for NMR sample preparation. Similar to the M9 culture medium for *E. coli,* the Linsmaier-Skoog medium for BY-2 (Nagata et al. 1992) is easy to prepare and is considerably more economical than the media used for animal cells and cell-free extracts. Moreover, , BY-2 cells show a similar ability to animal cells to synthesize foreign proteins of eukaryotic origin, and to introduce eukaryotic post-translational modifications including glycosylation, phosphorylation, *etc*. (Doran et al. 2000; Giddings 2001; Hellwig et al. 2004; Gomord & Faye 2004). However, the known protein expression protocols employing BY-2 pose a serious problem with regard to protein productivity. The poor expression levels of foreign proteins obtained by BY-2 cells restrict their use for NMR sample preparation.

Recently, we significantly improved the protein productivity of suspension-cultured BY-2 cells by using an inducible virus vector (Dohi et al. 2006). In this novel system, laborious procedures for virus inoculation are not required. Instead, the simple addition of estradiol into the culture successfully induces the accumulation of viral RNA. Following induction, the recombinant protein is efficiently synthesized in suspension-cultured BY-2 cells. Here, we describe the preparation of ^{15}N-labeled proteins by using this novel protocol. Additionally, we report that this system can express a protein containing disulfide bonds in a soluble and authentic form. We also show the ^{1}H-^{15}N HSQC spectra of the proteins, suggesting their structural quality. Finally, we discuss the potential of this method for NMR studies. To our best knowledge, this is the first report on the preparation of stable isotope-labeled foreign proteins in cultured plant cells.

Materials and Methods

Construction of Ti plasmids

Three model proteins, originating from different biological sources, namely, *E. coli* DHFR, chicken CaM, and porcine CPI-17(22-120) were used in this study. BPTI, which contains three disulfide bonds, was also tested. DHFR and CaM were prepared without tagging. The third, CPI-17(22-120), was prepared as a fusion protein, wherein the N-terminal end was conjugated to a precision protease digestion sequence located after the His6-S tag (Terpe 2003). For the fourth, BPTI, one of the ER-targeting signal sequences (Lys-Thr-Asn-Leu-Phe-Leu-Phe-Leu-Ile-Phe-Ser-Leu-Leu-Leu-Ser-Leu-Ser-Ser-Ala) and His6 sequence followed by factor Xa digestion sequence were connected onto the N-terminal end. A Ti plasmid encoding DHFR (pBICER8-ToMV-MP-DHFR-SRz) has been reported previously (Dohi and Mori 2007). The DNA fragments encoding CaM, CPI-17(22-120), and BPTI were inserted into a Ti plasmid based on pBICER8-ToMVerG3(SF3)Srz, according to a previously reported method (Dohi et al. 2006; Dohi and Mori 2007). The Ti plasmid represents the key component in our novel protein production system and encodes the estradiol-inducible expression cassette of a modified tomato mosaic virus (ToMV). The detailed structure of the Ti plasmid is described later in the text.

Culture conditions employed for the BY-2 cells

Tobacco BY-2 suspension-cultured cells were grown in a modified Linsmaier-Skoog medium (Nagata et al. 1992) containing unlabeled KNO_3 and unlabeled NH_4NO_3 as nitrogen sources with constant shaking at 135 rpm and 26 ^oC in the dark. The cells were maintained in a subculture of fresh medium at a dilution of 1:100 every 7 days.

Transformation of BY-2 cells

BY-2 cells were transformed by using a method mediated by the *Agrobacterium tumefaciens* strain LBA4404 harboring the Ti plasmid, as described previously (Dohi et al. 2006). Transgenic BY-2 lines were selected on agar medium containing 100 mg/L kanamycin with 500 mg/L carbenicillin. The suspension culture cells were grown in 3 mL liquid medium in 6-well culture plates during primary screening, and were then transferred to 30 mL liquid medium in 100-mL flasks with constant shaking at 135 rpm. Following an initial culture for 2 to 3 weeks, the suspension cells were maintained in the absence of antibiotics. Northern blotting and

SDS-PAGE analyses were performed to assess the efficiency of the inducible viral infection, as described previously (Dohi et al. 2006; Dohi and Mori 2007).

Protein synthesis

To prepare uniform ^{15}N-labeled NMR samples, stable isotope-labeled $K^{15}NO_3$ and $^{15}NH_4{}^{15}NO_3$ were dissolved into the medium as nitrogen sources, at concentrations of 1,900 and 1,650 mg/L, respectively. The transgenic BY-2 cells encoding the target protein were preincubated in 30 mL of this medium for 7 days and subsequently resubcultured in 50–100 mL fresh medium at a ratio of 1:20. Forty-eight hours after the resubculture, 17β-estradiol was added to the medium at a final concentration of 10 μM for initiating protein synthesis. The culture medium was then incubated at 26 °C and maintained by shaking at 135 rpm for synthesis of the foreign protein. Finally, the BY-2 cells were harvested 72 h after induction.

Protein purification

The precipitated cells were resuspended in TSE buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA) and disrupted by sonication. The lysate was centrifuged at 15,000 *g* for 10 min to remove debris. Up until this step, all samples were treated in an identical manner. After the soluble fraction was obtained, the three proteins without disulfide bonds were purified according to their intrinsic isolation protocol, as reported previously (Ikura et al. 1990; Ohki et al. 1997; Kitahara et al. 2000; Ohki et al. 2003). With respect to BPTI, a Ni^{2+} affinity column and factor Xa were employed for purification and tag sequence digestion, respectively. The purity of the protein samples was assessed by SDS-PAGE. For the NMR experiments, all samples were concentrated in a buffer (10 mM Tris-HCl (pH 7.0), 100 mM KCl, and 0.02 % NaN_3) containing 10 % D_2O for the NMR internal lock. $CaCl_2$ (5 mM) and folate (3 mM) were added into the CaM and DHFR sample solutions, respectively. The final sample concentration was approximately 0.2–0.9 mM, as determined by measuring the absorption at 280 nm. Each sample solution was then transferred to a Shigemi microtube (Shigemi Co., Ltd., Tokyo, Japan) for NMR measurements.

NMR experiments

All NMR experiments were performed on a Varian INOVA750 with a ^{1}H

resonance frequency of 750 MHz. A triple resonance probe equipped with a z-axis PFG coil was used, and the sample temperature was maintained at 25 ºC duringNMR measurements. All FID data obtained in the ^{1}H-^{15}N HSQC experiments were recorded as a matrix of 512 x 128 (t_2 x t_1) complex points, and were processed and displayed on a linux-PC using NMRPipe and NMRDraw (Delaglio et al. 1995).

Results and Discussion

Transgenic BY-2 cells for protein expression

To produce ^{15}N-labeled foreign proteins for NMR analysis, we adopted a novel inducible viral infection system in plant cells (Dohi et al. 2006). In this system, the genomic RNA of an engineered plant virus encoding a foreign protein is transcribed from stably transformed cDNA under the control of an inducible promoter. The induced cells are then self-infected with the transcribed viral RNA and begin to extensively produce the target foreign protein encoded in the engineered virus. In this study, ToMV was employed as the viral vector, because it can efficiently produce foreign proteins, as described in previous reports (Dohi et al. 2006; Dohi and Mori 2007). The Ti plasmid, which was utilized for the *Agrobacterium*-mediated transformation of BY-2, encoded the modified ToMV cDNA that contained a DNA fragment encoding the target protein in place of the virus coat protein gene. This viral genomic cDNA was fused to the estradiol-inducible promoter O_{LexA}-46 (Zuo et al. 2000), enabling the initiation of transcription at the first nucleotide of the ToMV sequence. At the 3' end of the cDNA, the ribozyme sequence of tobacco ringspot virus satellite RNA was introduced to remove the non-viral 3' extension containing a poly(A) tail, which may inhibit viral replication, from the primary viral RNA transcript via ribozyme self-cleavage (Dohi et al. 2006).

The expression cassette of estradiol-inducible ToMV was introduced into the transgenic BY-2 line expressing the chimeric LexA-VP16-ER transactivator (XVE), which is activated by estrogen (Dohi et al. 2006). Transgenic lines that were efficiently infected with the induced virus and produced target proteins were obtained by screening based on the results of the northern and SDS-PAGE analyses, in a similar manner to that described in previous reports (Dohi et al. 2006; Dohi and Mori 2007).

Expression of foreign proteins

^{15}N-labeled recombinant proteins for the NMR experiments were produced via inducible virus-mediated expression. Initially, the transgenic BY-2 cells were subcultured in medium containing $K^{15}NO_3$ and $^{15}NH_4{}^{15}NO_3$ at a ratio of 1:1000, and preincubated for one week to enable the replacement of unlabeled nitrogen in the cells with ^{15}N. During this process, XVE remained inactive, although it was expressed in the transgenic cells. Thus, the viral RNA was not transcribed until this point. Subsequently, the cells were resubcultured in 50-150 mL fresh labeling medium at a ratio of 1:20, and incubated for 48 h prior to the addition of 17β-estradiol for induction. The estradiol activated XVE and induced transcription of the modified ToMV RNA beginning at the target promoter O_{LexA}-46. The non-viral 3' extension containing a poly(A) tail was removed from the primary transcript via ribozyme self-cleavage. Viral RNA was then transported to the cytoplasm and replicated vigorously to establish the viral infection. Finally, the target protein encoded in place of the virus coat protein was produced abundantly in the cells.

Following induction, an accumulation of the target protein occurred after a lapse of 3 days. Thus, this novel method requires a relatively long period for expression. The time-scale is longer than those of the popular *E. coli* system and the cell-free system employing *E. coli* extract, but is similar to mammalian cells and wheat germ cell-free system. The slow rate appears disadvantageous for high-throughput sample preparation, however, may induce folding of proteins with complicated structures as the soluble product forms. In the present study, all samples were synthesized as soluble proteins. During the long protein expression time, degradation of the product might occur, but this is not a critical point at present because we can obtain sufficient amounts of the proteins with high purity confirmed by SDS-PAGE and NMR spectra.

The expression levels of the recombinant proteins were assessed by performing SDS-PAGE and then by measuring absorption at 280 nm (Fig. 1). The protein yield was 1-4 mg / 50 mL culture medium. The most efficient protein production was observed for DHFR. In this case, the amount of the foreign protein was estimated as approximately 10% of the total proteins in a cell. Approximately 3–4 mg DHFR was purified from 50 mL culture medium. This amount of target protein is more than sufficient for NMR measurements. Our demonstration suggests that protein productivity obtained by the inducible ToMV vector infection system in BY-2 cells is considerably

better than that of the popular *E. coli* system. This high productivity will contribute to minimizing the amount of labeling materials required in the culture medium. Protein production by this method could be improved further by tagging, silent mutation, modifying codon usage, *etc*. (Torizawa et al. 2004).

NMR experiments

To check the protein folding patterns, the ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled samples were observed. As shown in Figure 2, we obtained excellent NMR spectra. For the NMR data of DHFR shown in panel a, five Trp side chain NH signals were clearly observed. In panel b, four Gly residues (25, 61, 98, and 134) located at the Ca^{2+} binding loops are observed at very low field in ^{1}H dimension, suggesting CaM is in the stable Ca^{2+}-binding form. And the down-field shifted Ile 27, Ile 100, and Asn 137 suggest forming the hydrogen bonding in short anti-parallel β-sheets to connect Ca^{2+} binding loops. The peak distribution in panel c reflects the helical conformation of CPI-17(22-120). All of the ^{1}H-^{15}N HSQC obtained here are identical to the data obtained for their counterparts expressed in *E. coli* (Ikura et al. 1990; Falzone et al. 1994; Ohki et al. 2003). Thus, the results obviously indicate that proteins expressed by this system are able to fold and form appropriate three-dimensional structures. We attempted mass spectrometry after NMR experiments. The results suggested that almost all of the nitrogen atoms in the samples were labeled with ^{15}N (data not shown).

In this study, CaM and DHFR were expressed without tagging. As observed in the NMR data, these proteins retained their original conformations, indicating that the N-terminus chemical structure of protein expressed by this system is homogeneous. Thus, unlike the cell-free protein synthesis (Torizawa et al. 2004), this system may be advantageous for expressing proteins whose N-terminal ends possess some biological functions. On the other hand, CPI-17(22-120) was expressed with an S-tag and a His6 sequence at the N-terminal end, which was connected to the protease digestion sequence. The present results on CPI-17(22-120) revealed that the system could express a protein with addition of a polypeptide at the N-terminus. This may suggest the possible application of known signal peptide sequences at the N-terminus for controlling the location of a foreign protein in a cell.

Protein containing SS-bonds

To examine the workability of signal peptide sequences, we tried to express a protein containing disulfide bonds (SS-bonds). In this trial, we employed BPTI, which contains three SS-bonds, as a model protein. In this case, the endoplasmic reticulum (ER)-targeting signal sequence plus His6 tag and factor Xa digestion site were attached to the N-terminal end of the protein. Thus, the synthesized foreign protein was transported to the ER lumen in the cells; the ER is known to have the ability to fold protein to its mature form.

Figure 3 (A) shows the results of SDS-PAGE assessment of the expression of BPTI in the BY-2 system. The results clearly indicate that our BY-2 system can yield BPTI as a soluble form, although *E. coli* expresses it as an inclusion body (Yu et al. 1995). Purification using Ni^{2+} affinity resin was performed after sonication. And the buffer of the eluted protein solution was changed to the NMR buffer. Then, the sample was concentrated and subjected to NMR measurement. To check the three-dimensional structure of the BY-2 product, we recorded ^{1}H-^{15}N HSQC. After one-step simple purification, the sample gave a very clear NMR spectrum, as shown in Figure 3 (B). Resonance assignments of all peaks are illustrated within the figure. The NMR spectrum undoubtedly indicates that the BPTI expressed by the BY-2 system forms the correct three-dimensional structure including 3 SS-bonds, although the signaling peptide sequence and His6 tag are still attached to its N-terminal end. We found that addition of ~20 mM DTT into this sample solution disturbed the NMR spectrum (data not shown), thus providing evidence that the BPTI sample contains 3 SS-bonds.

The present demonstration showed that our new system has the ability to synthesis and fold proteins containing SS-bonds in the soluble form. This is a unique property and advantageous for the BY-2 system. In our demonstration, the NMR measurement was ready after simple purification without refolding processes. Of course, digestion of the tag sequence conjugated to the research target protein could be achieved by standard biochemical experiments.

Concluding remarks

In summary, we examined the application of a novel protocol involving inducible virus-mediated expression of a foreign protein in suspension-cultured plant cells for NMR sample preparation. Our demonstration showed that ^{15}N-labeled proteins

were successfully expressed using the new system. The ^{1}H-^{15}N HSQC spectra clearly showed that all the proteins tested here exhibited appropriate folding patterns. Thus, our present report indicates that this protein expression system is a good candidate for NMR sample preparation.

We have only reported ^{15}N-labeling using this system, but it is apparent that uniform ^{13}C-labeling can be achieved when ^{13}C-labeled sucrose, which is commercially available, is employed as the sole carbon source in the culture medium for BY-2. Further experiments are currently in progress within our research group, including ^{13}C-labeling, random fractional ^{2}H-labeling, and so on.

In the most efficient case, sufficient NMR samples were obtained from as little as 50 mL culture medium. This scale merit is particularly advantageous for laboratory handling. Furthermore, we successfully expressed a protein containing SS-bonds as the soluble form. This was achieved by using a signal peptide sequence to control the location of the products in the cell. This result may indicate that our BY-2 method using signal peptide sequences will open a new way to produce many kinds of proteins that are currently difficult to prepare in laboratories.

From another viewpoint, it is interesting to note the properties of our system. First, as described above, foreign protein expressions by this system require a relatively long time compared with the *E. coli* system. Second, the cell cycle of the BY-2 cells in the culture flask can be synchronized, if necessary. These properties may be beneficial for in-cell NMR studies on eukaryotes (Sakai et al. 2006).

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Figure Legends

Figure 1.

SDS-PAGE confirming the expression and purification of proteins. For each lane, the applied sample was made from 5 μL culture medium. The arrows indicate the target protein. The symbols - and + represent the samples before and after the addition of estradiol, respectively. The samples for the lanes – and + were prepared by the sonication of the cells. The two lanes labeled "boiled" represent the samples following heat treatment (90 ºC for 5 min). The lane marked "His" for CPI-17(22-120) represents the sample solution following purification over a Ni^{2+} affinity column. The proteins purified from the 50 mL culture medium were estimated at 3-4, 1-2, and 2-3 mg for DHFR, CaM, and CPI-17(22-120), respectively, from absorption at 222 and/or 280 nm.

Figure 2.

^{1}H-^{15}N HSQC spectra of the recombinant proteins. (a) DHFR, (b) CaM, and (c) CPI-17(22-120). The frequency range in the ^{15}N axis is not identical among these spectra because of differences in their peak dispersion. For clarity, only several peaks are labeled with one-letter amino acid codes and residue numbers [for further details, see Ikura et al. 1990; Falzone et al. 1994; Ohki et al. 2003].

Figure 3.

BPTI expressed in BY-2. (A) SDS-PAGE suggests that BPTI was expressed as the soluble form. The arrow indicates BPTI with tag. (B) ^{1}H-^{15}N HSQC of BPTI. The sample was purified only by an Ni^{2+} affinity column. The resonance assignments were according to BMRB #5359. Six Cys residues were labeled with relatively large fonts. Asterisks indicate the peaks that resonated from the signaling sequence. Unlabeled peaks resonated from side-chains, and some of them were fold-back peaks.

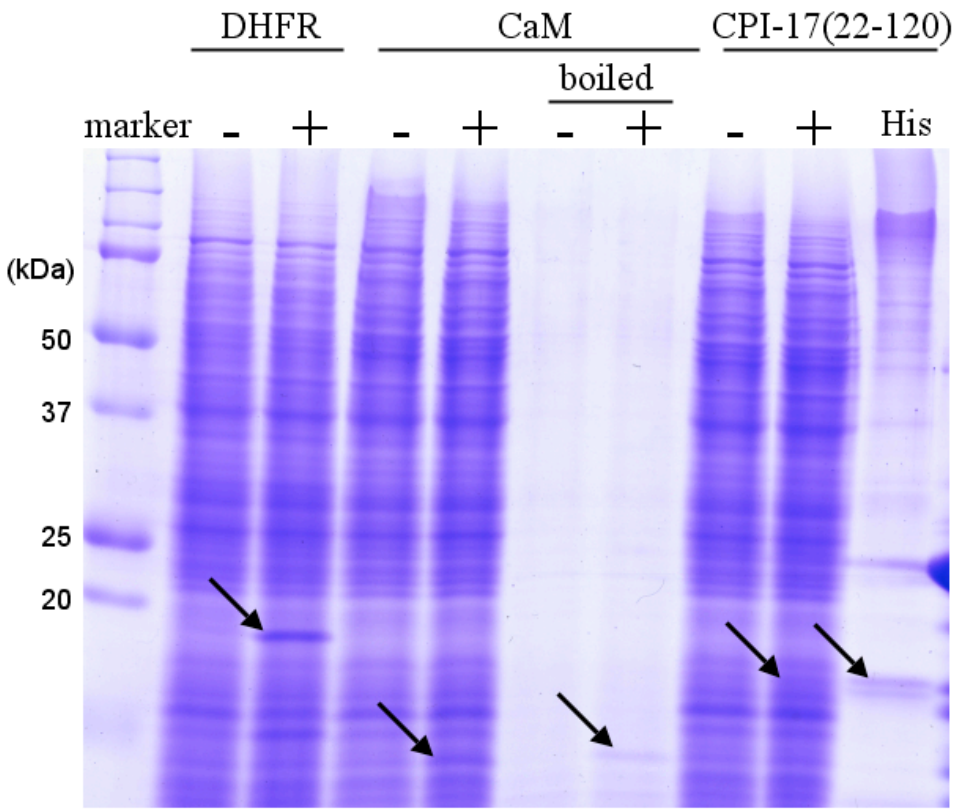


Figure 1

Figure 2

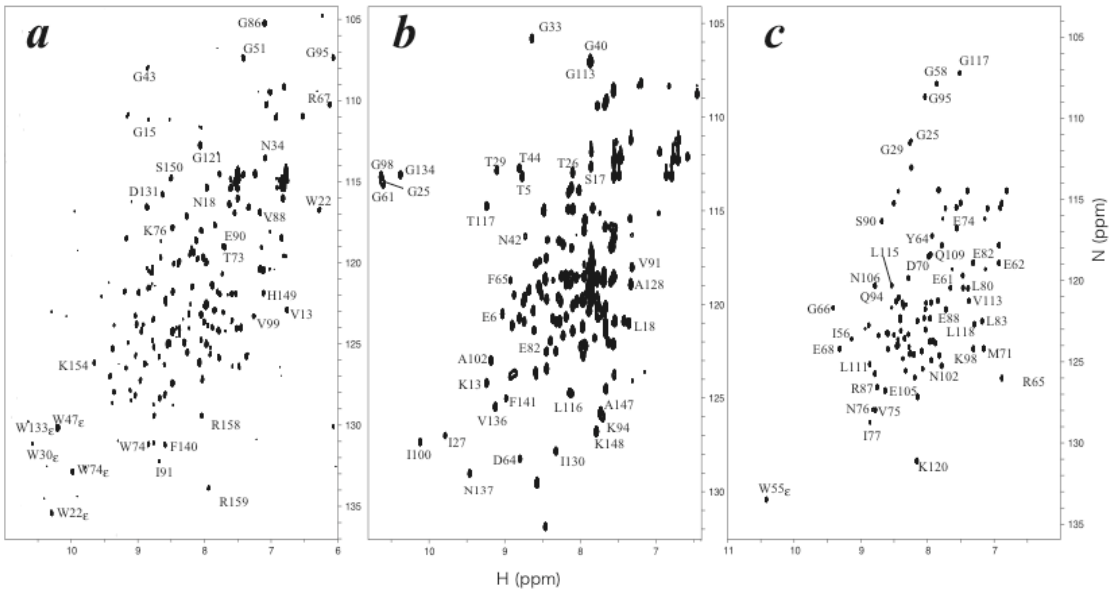


Figure 3 (A)

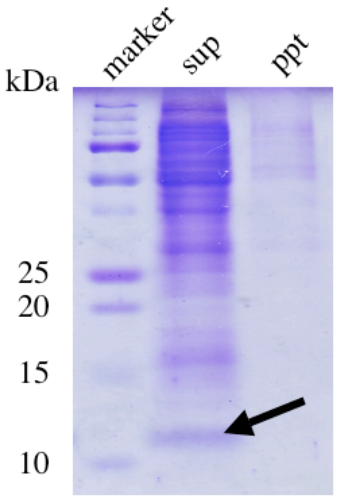
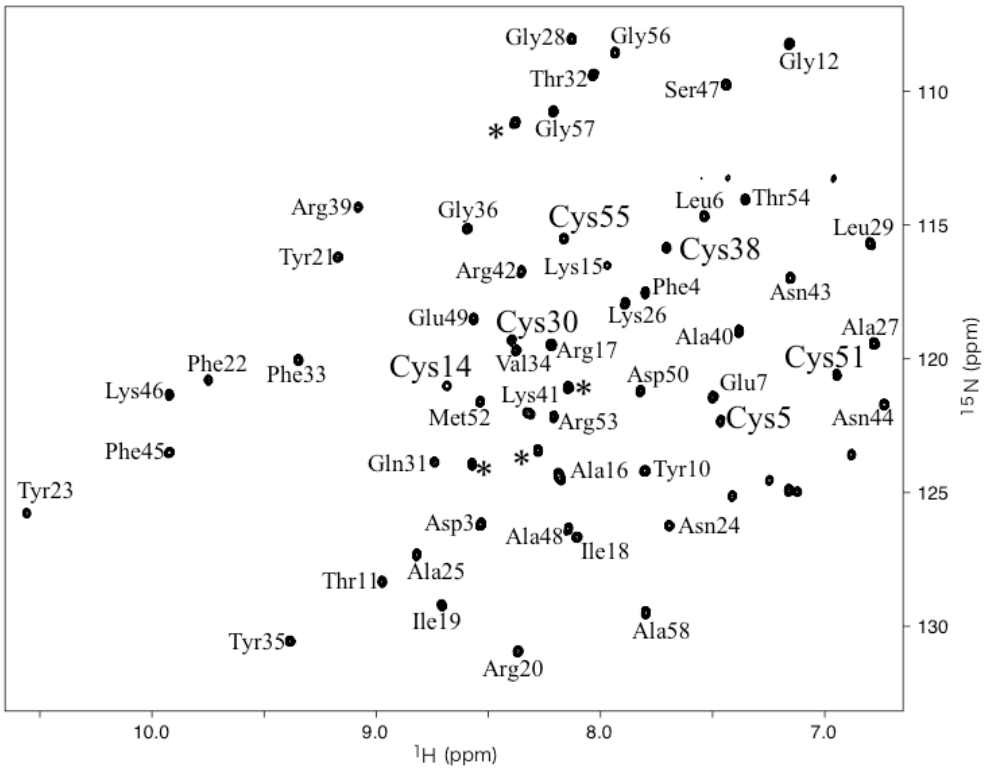


Figure 3 (B)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Lys15
Phe4
Asn43
Glu49
Cys30
Lys26
Ala40
Ala27
Arg17
Phe22
Phe33
Cys14
Val34
Asp50
Cys51
Glu7
Lys46
Lys41
Met52
Arg53
Cys5
Asn44
Phe45
Gln31
Ala16
Tyr10
Tyr23
Asp3
Ala48
Asn24
Ile18
Thr11
Ala25
Ile19
Ala58
Tyr35
Arg20
110
115
120
125
130
15N (ppm)
10.0
9.0
8.0
7.0
1H (ppm)