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PERSPECTIVE

Production of isotopically labeled heterologous proteins in non-*E. coli* prokaryotic and eukaryotic cells

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Abstract The preparation of stable isotope-labeled proteins is necessary for the application of a wide variety of NMR methods, to study the structures and dynamics of proteins and protein complexes. The *E. coli* expression system is generally used for the production of isotope-labeled proteins, because of the advantages of ease of handling, rapid growth, high-level protein production, and low cost for isotope-labeling. However, many eukaryotic proteins are not functionally expressed in *E. coli*, due to problems related to disulfide bond formation, post-translational modifications, and folding. In such cases, other expression systems are required for producing proteins for biomolecular NMR analyses. In this paper, we review the recent advances in expression systems for isotopically labeled heterologous proteins, utilizing non-*E. coli* prokaryotic and eukaryotic cells.

Keywords Stable isotope-labeling · Prokaryotic cell · Eukaryotic cell · Heterologous proteins

Introduction

The preparation of large amounts of stable isotope-labeled recombinant proteins is currently a crucial step for structural analyses of the proteins by NMR spectroscopy. Due to obvious advantage such as ease of genetic manipulation, rapid cell growth, and low costs for isotope-labeling, the *Escherichia coli* expression system is frequently used in structural genomics (Gräslund et al. 2008). However, heterologous proteins, especially many eukaryotic proteins, cannot be folded correctly and are expressed insolubly as inclusion bodies in *E. coli*. To overcome this limitation, several strategies, such as decreasing the temperature of the cell culture (Schein and Noteborn 1988; Qing et al. 2004) and/or fusing highly soluble tags (GST, TrxA, NusA, etc.) to target proteins (Esposito and Chatterjee 2006), have been proposed to facilitate soluble expression in *E. coli*. Nevertheless, in a significant number of cases, it is impossible to express correctly folded proteins in a soluble form using *E. coli* expression systems. For example, the expression of eukaryotic proteins with complex disulfide-bonds frequently causes inclusion body formation in *E. coli*. In such cases, the use of other expression hosts should be considered, as alternatives for the preparation of stable isotope-labeled proteins. In this review, we present recent developments in the expression systems for isotope-labeling of heterologous proteins utilizing non-*E. coli* prokaryotic and eukaryotic cells.

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Stable isotope-labeling of heterologous proteins by non-*E. coli* prokaryotic cells

In order to facilitate the correct folding of expressed heterologous proteins, especially when disulfide-bond formation is involved, the secretion from the bacterial cytoplasm is occasionally employed (Georgiou and Segatori 2005). Secretion of heterologous proteins has several advantages: (1) fewer contaminants and simpler purification, (2) higher

yield, as compared with cytoplasmic expression, and (3) potentially toxic cytosolic proteins can be overexpressed without injuring the host cells.

Shinagawa et al. (2005) recently developed an isotope-labeling procedure utilizing *Corynebacterium glutamicum*, a Gram-positive bacterium. By using the *C. glutamicum* system, the authors successfully expressed soluble *Streptoverticillium mobaraense* transglutaminase (MTG), which was expressed in inclusion bodies in the *E. coli* expression system. *C. glutamicum* can be adapted for high-density cell culture, and minimal medium (1.2 g glucose, 0.6 g NH_4Cl, 20 g $MgSO_4{\cdot}7H_2O$, 0.2 g $FeSO_4{\cdot}7H_2O$, 0.2 g $MnSO_4{\cdot}7H_2O$, 30 g KH_2PO_4, 9 g thiamine hydrochloride, 9 g biotin, and 1 g $CaCO_3$ per 20 ml of distilled water, adjusted pH 7.5), in which glucose and ammonium chloride are the sole carbon and nitrogen sources, respectively, can be used for the $^{13}C/^{15}N$-labeling of target proteins. Although the concentrations of glucose and ammonium chloride in the medium are much higher than those of the standard M9 minimal medium for *E. coli*, a high expression level of MTG with *C. glutamicum* can be attained by using a culture volume of 20–100 ml. Furthermore, deuterium labeling of MTG can also be achieved by using deuterium oxide medium.

The Gram-positive bacterium *Brevibacillus choshinensis* has been used to produce several heterologous proteins (Udaka and Yamagata 1993), and the prokaryotic cell has recently become commercially available (Takara Bio, Shiga, Japan). *B. choshinensis* is usually cultivated in rich medium, and the yield of the heterologous protein (12 kDa human FK506 binding protein: FKBP12) expression in M9 minimal medium was lower and insufficient for NMR measurements. However, Tanio et al. (2008) demonstrated that the protein expression using ^{15}N-labeled rich medium (C. H. L. medium: Chlorella Industry, Tokyo, Japan) improves the yield of the expressed FKBP12, thus facilitating NMR measurement. The use of commercially available rich media also reportedly improves the protein/peptide expression for other types of *Brevibacillus* strains (Vogt et al. 2003). Furthermore, the amino acid-type selective (AATS) ^{15}N-labeling of a target protein secreted by *B. choshinensis* was extensively examined, and revealed that nine amino acids (alanine, arginine, asparagine, cysteine, glutamine, histidine, lysine, methionine, and valine) were available for ^{15}N selective labeling (Tanio et al. 2009).

Stable isotope-labeling of heterologous proteins by yeast expression system

Although some of the non-*E. coli* prokaryotic expression systems has enabled the successful expression of heterologous proteins that cannot be expressed in *E. coli*, several fundamental difficulties still exist for heterologous protein expression in prokaryotic cells: (1) lack of intracellular organelles, (2) a limited number of molecular chaperones, and (3) absence pf post-translational protein modification mechanisms. The utilization of eukaryotic cells for the protein expression should overcome these issues.

The methylotrophic yeast *Pichia pastoris* is a powerful tool for the heterologous expression of proteins, and the expression system includes several advantages of both the prokaryotic and eukaryotic expression systems (Cregg et al. 2000): (1) *P. pastoris* is a single-cell microorganism that is easily grown to high cell densities, and high yields of secreted protein (typically 100–500 mg/l) can be expected when expression is carried out in a fermenter, (2) the molecular genetic manipulation techniques for *P. pastoris* are not difficult, and are similar to the well-established methods for *Saccharomyces cerevisiae*, and (3) *P. pastoris* is capable of post-translational modifications, including proteolytic processing, disulfide-bond formation, and glycosylation. Another advantage of the expression system is the stable isotope-labeling of expressed proteins at a reasonable cost, because protein production by *P. pastoris* is possible in simple minimal defined media.

The basal media for the stable isotope-labeling of proteins contain a number of salts and defined nitrogen and carbon sources. As for the carbon sources, two carbon sources are typically utilized for protein production by *P. pastoris*. Methanol is necessary for the induction of protein production, but alternate carbon sources (glycerol or glucose) are used for the growth phase of *P. pastoris*, prior to the induction. In order to obtain the maximum incorporation of the ^{13}C-isotope, isotopically labeled carbon sources (^{13}C-labeled glycerol or glucose) must be used during the growth phase of the *P. pastoris* culture (Laroche et al. 1994; Wood and Komives 1999), while ^{13}C-methanol is included during the expression phase. However, Rodriguez and Krishna demonstrated that the isotopes are not continuously required during the growth phase, and the addition of small amounts of labeled compounds 6 h prior to the induction achieved sufficient isotope incorporation (Rodriguez and Krishna 2001), thereby facilitating cost-effective isotope-labeling.

For the structural analysis of large molecular weight ($Mw > 25K$) proteins by NMR, deuterium isotope-labeling of expressed proteins is a critical requirement, since deuterium labeling simplified NMR spectra and also enhanced the relaxation properties of attached or adjacent atoms (Kay and Gardner 1997). Although bacterial expression systems are usually used to produce deuterated proteins, *P. pastoris* can also be adapted to growth in a deuterated environment to produce deuterated heterologous proteins

(Massou et al. 1999; Morgan et al. 2000; Tomida et al. 2003). Morgan et al. (2000) reported that the use of 95% D_2O media for protein expression achieves effective and uniform deuteration at the C_α positions of proteins and causes a significant reduction in the linewidth of the amide proton signals, regardless of whether a protonated or deuterated carbon source (methanol) is used for the induction (Fig. 1). However, in their study, several resonances from methyl groups as well as Lys δ/ε groups still existed in the [^{1}H, ^{13}C]-HSQC spectrum, even though deuterated methanol was used during the induction phase (Fig. 1c). If further deuteration is necessary for some applications, for example, a cross-saturation experiment for identifying a molecular interface (Takahashi et al. 2000; Nakanishi et al. 2002), the deuterated carbon sources (glycerol or glucose) should also be used during the growth phase. Ichikawa et al. successfully prepared the highly deuterated discoidin domain of DDR2 in this manner (Fig. 2), and its deuteration ratio was more than 95%, as judged from the MS analysis. Consequently, a transferred cross-saturation experiment, which requires a high level of deuteration, successfully identified the collagen-binding site of DDR2 (Ichikawa et al. 2007).

Amino acid-type selective isotope-labeling of proteins is also useful to simplify the NMR spectra and to facilitate the NMR assignment of large molecular weight proteins. Chen et al. (2006) optimized the culture conditions of *P. pastoris* to overcome a scrambling problem, and successfully performed four types (Cys, Leu, Lys and Met) of AATS ^{15}N-labeling, in which the incorporation rates were greater than 50%.

Quite recently, we established a cost-effective isotope-labeling procedure by utilizing the yeast *Kluyveromyces lactis* (Sugiki et al. 2008). Similar to *P. pastoris*, *K. lactis* also efficiently secretes heterologous proteins into the culture medium (Colussi and Taron 2005). One of the major differences between *K. lactis* and *P. pastoris* is the promoters utilized for the expression of the target gene. *P. pastoris* and *K. lactis* use the AOX1 (Alcohol oxidase 1) and LAC4 promoters, respectively (Cregg et al. 1989; Colussi and Taron 2005). In the case of *K. lactis*, the expression of target genes is induced by adding galactose to the culture medium, while methanol is used for *P. pastoris*. Galactose acts not only as an inducer of target gene expression, but also as a carbon source for *K. lactis*. In the case of *P. pastoris*, due to the toxicity of methanol, the methods for methanol addition (manual periodic addition or constant supply using a peristaltic pump), dose, feed rate, and timing must be strictly controlled during the period of cultivation, in order to achieve maximum cell growth and expression levels of protein with high reproducibility. On the other hand, in *K. lactis*, high-level expression of the target protein is continuously induced by constitutive cultivation with media containing galactose. Thus, the procedures for culture and induction of protein expression by *K. lactis* are simple, easily scaled-up, and offer high reproducibility.

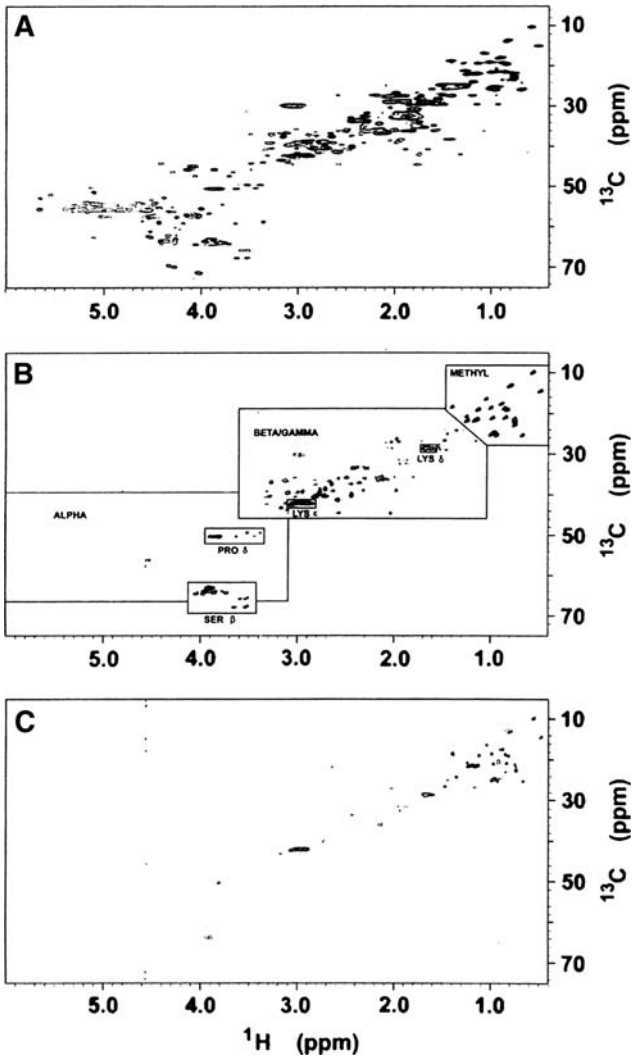

Fig. 1 Two-dimensional [^{13}C, ^{1}H]-HSQC spectra of MSP1-19 protein expressed in the yeast *Pichia pastoris* (aliphatic region). **a** ^{13}C/^{15}N-labeled (non-deuterated) protein [control]. **b** ^{2}H/^{13}C-labeled protein (inducing protein production by growing the cells in 95% D_2O with protonated ^{13}C-methanol). **c** ^{2}H/^{13}C/^{15}N-labeled protein (inducing protein production by growing the cells in 95% D_2O with deuterated ^{13}C-methanol). Specific amino acid signals that remained relatively intense in the deuterated samples (**b**, **c**) are indicated in **b**. (Reprinted with permission from Morgan et al. 2000)

In the *K. lactis* expression system, 20 g/l galactose is usually required as a carbon and energy source for cell growth, and as an inducer for the expression of target proteins. However, for uniform ^{13}C-labeling of target proteins, 20 g/l carbon source may be economically unfeasible. Merico et al. (2004) reported that heterologous protein can be induced by 20 g/l glucose in *K. lactis*, while its potential as an inducer of protein expression is lower.

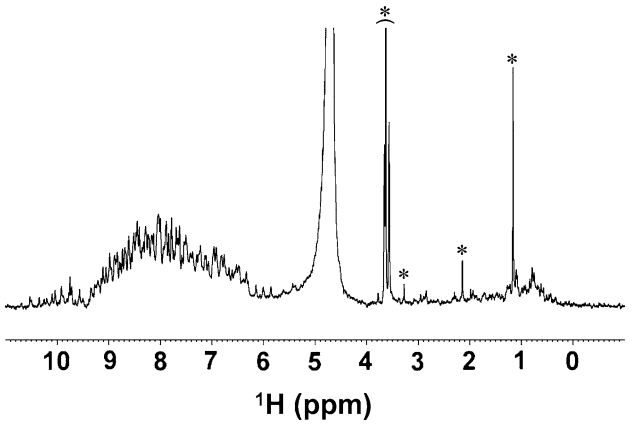

Fig. 2 One-dimensional proton NMR spectrum of perdeuterated DDR2 expressed in the yeast *Pichia pastoris* (initially by growing the cells in 99% D_2O with deuterated glucose and then inducing protein production by growing the cells in 99% D_2O with deuterated methanol. Impurity peaks are marked with *asterisks*

Therefore, we investigated culture conditions that enable sufficient production of isotope-labeled protein by *K. lactis* using a minimal amount of glucose.

The 41-kDa maltose-binding protein (MBP) was used as a model protein in the *K. lactis* protein expression system. We explored the minimal dose of glucose that enables sufficient cell growth and induction of MBP expression was observed with 4 g/l glucose, but the yield of the MBP was 1.4 mg/l, which is approximately 20-fold lower than the yield at 20 g/l glucose (23.8 mg/l) (Fig. 3a). To overcome this problem, we utilized a "Fed-batch" cultivation strategy (Sugiki et al. 2008). With this strategy it is possible to obtain 10.1 mg/l of expressed MBP with final concentration of 5 g/l glucose, which is eightfold higher than batch culture using 4 g/l glucose (Fig. 3a). NMR experiments for uniformly $^{13}C/^{15}N$-labeled MBP in the presence of β-cyclodextrin were performed and the well-dispersed chemical shift of the ^1H and ^{15}N in the [^{15}N, ^1H] HSQC spectrum indicates that the uniformly $^{13}C/^{15}N$-labeled MBP secreted by *K. lactis* is correctly folded (Fig. 3b). Furthermore, the assignment of NMR signals of the uniformly $^{13}C/^{15}N$-labeled MBP was successfully performed using HNCA spectrum (Fig. 3c), and the results were entirely consistent with previous reports (Gardner et al. 1998). This indicates that highly efficient isotope-labeling was achieved by the *K. lactis* expression system. Thus, the Fed-batch culture method enables larger amounts of proteins expressed by *K. lactis*, despite using smaller amount of glucose, which reduces costs to a level comparable to *E. coli* isotope-labeling systems. Using this culture method, the cost of isotope($^{13}C/^{15}N$)-labeling of target proteins is 6–7 times lower than that of the

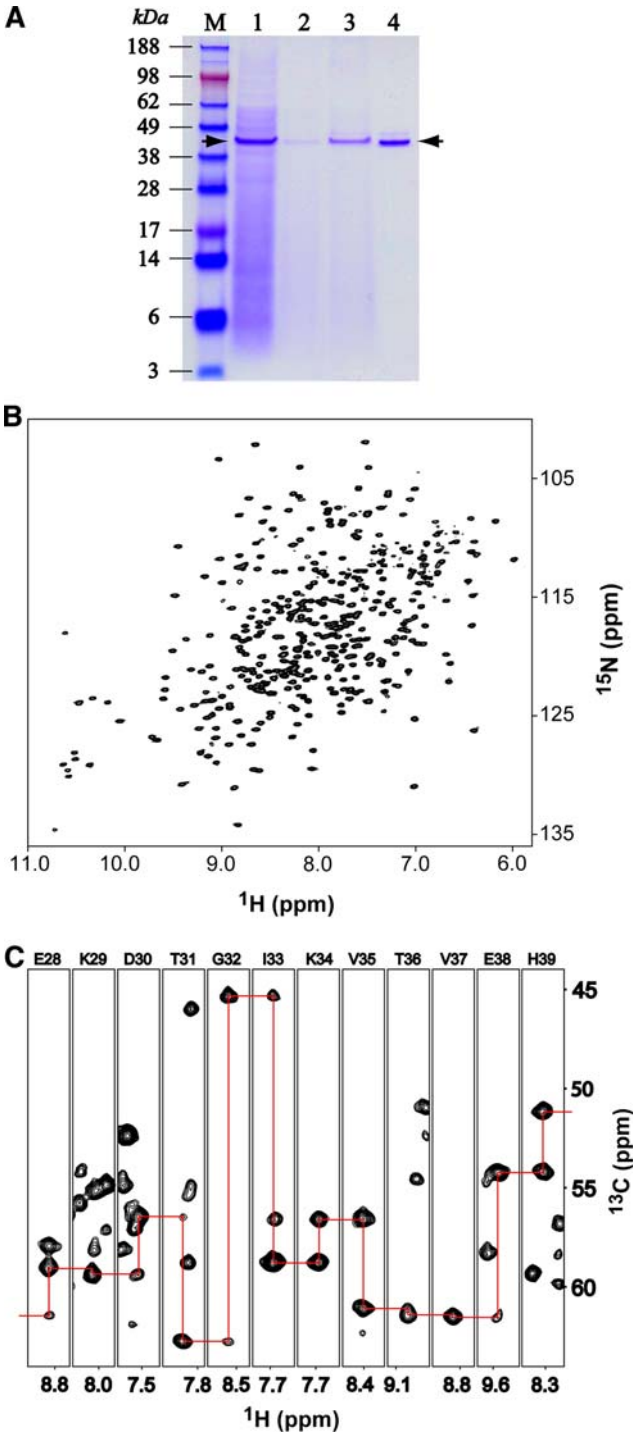

Fig. 3 **a** SDS–PAGE of MBP secreted by the yeast *K. lactis* into culture supernatants (15 µl) after 60 h of batch culture using minimal media containing 20 g/l D-glucose (*lane 1*), 4 g/l D-glucose (*lane 2*), or fed-batch cultivation with continuously supplied D-glucose (*lane 3*). SDS–PAGE of purified MBP is shown in *lane 4*. **b** [^{15}N, ^1H] HSQC spectrum of $^{13}C/^{15}N$-labeled MBP expressed in the yeast *K. lactis*. **c** Resonance assignment of 0.3 mM uniformly $^{13}C/^{15}N$-labeled MBP expressed in the yeast *K. lactis* using [$^{13}C_\alpha$, $^1H^N$]-strips taken from the 3D HNCA spectrum at each ^{15}N of residues 28–39

P. pastoris expression system, if it is assumed that equal amounts of target protein are expressed in both systems.

Stable isotope-labeling of heterologous proteins by other eukaryotic cells

There are some disadvantages of yeast protein expression systems, regarding their inability to accomplish certain complex post-translational modifications, such as prolyl hydroxylation as well as a certain type of phosphorylation and high mannose glycosylation. Therefore, the use of higher eukaryotic cells with advanced cell machinery facilitates the proper folding and post-translational modifications, essential for the biological functionality of target proteins. Among the eukaryotic expression systems, the baculovirus expression system (BVES) is one of the most popular and powerful expression systems for the expression of mammalian proteins such as kinases and membrane proteins. The BVES is based on the infection of insect cells (in many cases, Sf9 cells are used) with a recombinant baculovirus carrying a target gene, and the subsequent expression of the target protein by the insect cells (O'Reilly et al. 1994). Nowadays, gene manipulation to construct the recombinant baculovirus is facilitated by using commercially available molecular biology kits.

However, since insect cells cannot be grown in minimal medium, it is difficult to cost-effectively perform the uniform isotope-labeling of target proteins using the BVES. Instead, by optimizing the medium and culture conditions, AATS ^{15}N-labeling by BVES can be successfully performed for several amino acid types (Brüggert et al. 2003; Strauss et al. 2003). Recently, isotopically labeled expression media for BVES has become commercially available (BioExpress-2000 (Insect Cell); Cambridge Isotope Laboratories, Inc., Cambridge, MA, USA), and Strauss et al. (2005) used these media for the uniform ^{15}N- and ^{13}C/^{15}N-labeling of the catalytic domain of Abl kinase. Although the source and the concentration of the amino acids in BioExpress-2000 media have not been disclosed to the public, the expression levels of Abl with BioExpress-2000 series are comparable to those of other culture media for protein expression in insect cells, such as SF900 II or EX-CELL 420. The total label incorporation rate, as well as that for each single amino acid, is more than 90%.

A major bottleneck of uniform labeling in the BVES is the high cost for such labeled rich media. Another potential limitation of the BVES is the expression of perdeuterated proteins. Most higher eukaryotic cells are more sensitive to toxic substances than bacteria and cannot survive in deuterium oxide media, and thus cost-effective perdeuteration of target proteins is not currently possible. In the case of Abl kinase, a 32 kDa protein, CBCA-type triple resonance experiments are difficult without deuterated samples. However, the combination of AATS ^{15}N-labeling and several basic triple resonance experiments (HNCO, HNCA, HN(CO)CA, and ^{15}N-edited NOESY) allowed the assignment of 96% of all backbone ($^{1}H_N$, ^{15}N, $^{13}C_\alpha$, and ^{13}CO) resonances (Vajpai et al. 2008). On the basis of this nearly complete backbone resonance assignment, Vajpai et al. performed structural and dynamical NMR analyses of Abl kinase complexed with three clinical inhibitors, by utilizing residual dipolar coupling and ^{15}N relaxation data.

Ideally mammalian proteins should be produced by using mammalian cells, such as Chinese hamster ovary (CHO) cells, HEK293 cells, and so on. However, these mammalian cells cannot grow on the isotopically enriched-minimal media used for bacterial or yeast expression systems, and require amino acids, vitamins, and in many cases, serum. Therefore, the uniform isotope-labeling of target proteins in mammalian cells is not currently possible to perform in a cost-effective manner, for reasons similar to those discussed for insect cells.

Hansen et al. (1992) first introduced a practical, cost-effective approach for obtaining uniformly ^{15}N- and ^{13}C/^{15}N-labeled proteins from mammalian cells. In this approach, a hydrolysate of bacteria or algae grown on inexpensive $^{15}NH_4Cl$ and ^{13}C-labeled glucose or $^{13}CO_2$ is first prepared, and an isotopically labeled amino acid mixture is purified from the hydrolysate. The authors utilized this prepared ^{15}N-labeled amino acid mixture along with ^{15}N-labeled glutamine, which is enzymatically synthesized from rather inexpensive ^{15}N-labeled glutamate, and commercially available ^{15}N-labeled cysteine, to produce uniformly ^{15}N-labeled urokinase in Sp2/0 cells (Hansen et al. 1992). The preparations and multinuclear NMR analyses of uniformly labeled proteins expressed in other mammalian cells, according to this strategy, were also reported (Lustbader et al. 1996; Shindo et al. 2000). Nowadays, purified algal amino acid mixtures are commercially available from manufacturers. Furthermore, mammalian cell growth media (BioExpress 6000; CIL) for the isotopic(^{15}N and ^{13}C/^{15}N)-labeling of proteins has recently become commercially available, and Werner et al. (2008) successfully produced isotopically labeled mammalian G protein-coupled receptor (GPCR), rhodopsin, in mammalian HEK293 cells by using this medium. However, the uniform isotope-labeling by using a mammalian expression system currently faces similar issues to those of the BVES, regarding its relatively high cost, as compared to the bacterial expression system, and the difficulty in achieving perdeuteration of target proteins. Therefore, NMR analyses using AATS isotope-labeling of proteins have been mainly employed to obtain the structural and dynamical information of target proteins so far (Arata et al. 1994; Klein-Seetharaman et al. 2002, 2004).

Table 1 Comparison of different expression systems

	Cell growth	Expression level	Post-translational modification			Cost for $^{13}C/^{15}N$-labeling	^{2}H-labeling
			N-linked glycosylation	Phosphorylation	Acetylation		
E. coli	Rapid	High	No	No	No	Low	Feasible
Yeast cells (*P. pastoris, K. lactis*)	Rapid	Low–high	Yes[a]	Yes	Yes	Low–medium	Feasible
Insect cells (BVES)	Slow	Low–high	Yes[b]	Yes	Yes	High	Difficult
Mammalian cells	Slow	Low–moderate	Yes	Yes	Yes	High	Difficult

[a] Mostly high mannose-type oligosaccharides

[b] Not as complex oligosaccharides as those produced in mammalian cells, e.g., the lack of terminal sialic acid residues. The table is compiled based upon that in Yin et al. (2007)

Recently, a stable isotope-labeling procedure using an inducible viral infection system in Tobacco BY-2 suspension-cultured plant cells was reported by Ohki et al. (2008). In the most efficient case, sufficient NMR samples (1–4 mg) were obtained from 50 ml cultures of BY-2 cells. In order to prepare uniformly ^{15}N-labeled proteins, $K^{15}NO_3$ and $^{15}NH_4^{15}NO_3$ were used as nitrogen sources. In principle, ^{13}C-labeling is possible if ^{13}C-labeled sucrose is employed as the sole carbon source in the culture medium. ^{15}N-labeled bovine pancreatic trypsin inhibitor (BPTI), which contains three disulfide-bonds and is expressed as an inclusion body in *E. coli*, was successfully expressed in the soluble form by using this system.

Future prospects

Characteristic features of the different expression systems are summarized in Table 1. The utilization of eukaryotic cells is attractive for the expression and stable isotope-labeling of mammalian proteins, especially human proteins, which are important targets for drug discovery. In particular, eukaryotic membrane proteins are generally not expressed in an active form in the *E. coli* expression system. As mentioned above, a GPCR, bovine rhodopsin, was successfully expressed, and AATS labeling can be performed in mammalian cells (Werner et al. 2008) as well as in the BVES (Creemers et al. 1999). Other GPCRs [CCR5 (Nisius et al. 2008) and thromboxane A2 receptor (Ruan et al. 2008)] were also expressed in the BVES and solubilized to conduct ^{1}H-NMR analyses, although isotope-labeling has not been employed yet. In the case of the yeast *P. pastoris* expression system, the systematic expression of GPCRs was performed for structural biology applications (Lundstrom et al. 2006; Lundstrom 2006). Therefore, the yeast expression system is expected to be useful for expressing partially or uniformly $^{2}H/^{13}C/^{15}N$-labeled mammalian membrane proteins, thus enabling extensive multinuclear NMR analyses. Overall, the further development of cost-effective expression systems for mammalian proteins that can be labeled with arbitrary combinations of stable isotopes will facilitate the elucidation of the structural aspects of fully functional mammalian proteins with posttranslational modifications.

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