J Biomol NMR (2008) 42:159–162
DOI 10.1007/s10858-008-9276-9

COMMUNICATION

Stable isotope labeling of protein by *Kluyveromyces lactis* for NMR study

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Received: 4 August 2008 / Accepted: 9 September 2008 / Published online: 1 October 2008

Abstract Stable isotope labeling for proteins of interest is an important technique in structural analyses of proteins by NMR spectroscopy. *Escherichia coli* is one of the most useful protein expression systems for stable isotope labeling because of its high-level protein expression and low costs for isotope-labeling. However, for the expression of proteins with numerous disulfide-bonds and/or post-translational modifications, *E. coli* systems are not necessarily appropriate. Instead, eukaryotic cells, such as yeast *Pichia pastoris*, have great potential for successful production of these proteins. The hemiascomycete yeast *Kluyveromyces lactis* is superior to the methylotrophic yeast *P. pastoris* in some respects: simple and rapid transformation, good reproducibility of protein expression induction and easy scale-up of culture. In the present study, we established a protein expression system using *K. lactis*, which enabled the preparation of labeled proteins using glucose and ammonium chloride as a stable isotope source.

Keywords Yeast · *Kluyveromyces lactis* · Isotope labeling · Secretary protein expression

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Abbreviations

K. lactis	*Kluyveromyces lactis*
MBP	Maltose-binding protein
YNB	Yeast nitrogen base
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
HSQC	Heteronuclear single quantum coherence

For structural analysis of proteins by NMR spectroscopy, preparation of large amounts of stable isotope-labeled recombinant protein is important. Due to its advantages, including ease of use, high-speed cell growth, high-level protein expression, and low costs for isotope labeling, *E. coli* protein expression systems are typically used in structural genomics. However, heterologous proteins with complex disulfide-bonds often form inclusion bodies when expressed in *E. coli* or other prokaryotic cells. In some cases, it is fundamentally impossible to express the correctly folded proteins using prokaryotic cells, as there are several differences when compared with eukaryotic cells: (1) intracellular redox state is lower, (2) there are no intracellular organelles in prokaryotic cells, (3) the number of molecular chaperones is insufficient, and (4) prokaryotic cells have no post-translational protein modification mechanisms (Lilie et al. 1998). Therefore, the use of eukaryotic cells is necessary to overcome these issues.

In eukaryotic cell expression systems, *P. pastoris* is widely used to express large amounts of stable isotope-labeled proteins in a cost-effective manner, as it shows high-density cell growth and high-level protein expression in comparison with other eukaryotic cells (Wood and Komives 1999). *P. pastoris* secretes heterologous proteins into culture medium. Protein secretion has useful

advantages: (1) fewer contaminants and simpler purification, (2) greater yield when compared with expression in cytoplasm, and (3) potentially toxic cytosolic proteins can be over-expressed without damaging the host cells.

Recently, an expression system using the hemiascomycete yeast *Kluyveromyces lactis* was established and its host–vector system was made commercially available (Colussi and Taron 2005). *K. lactis* also efficiently secretes heterologous proteins into culture medium. One of the major differences between *K. lactis* and *P. pastoris* is the promoters utilized for expression of the target gene. *P. pastoris* and *K. lactis* use *AOX1* (Alcohol oxidase 1) and *LAC4* promoters, respectively (Cregg et al. 1989; Colussi and Taron 2005). Therefore, the expression of target genes is induced by adding methanol and galactose to culture medium with *P. pastoris* and *K. lactis*, respectively. Furthermore, methanol and galactose act not only as inducers of target gene expression, but also as carbon sources for *P. pastoris* and *K. lactis*, respectively. In the case of *P. pastoris*, due to the toxicity of methanol, the methods for addition of methanol (manual periodic addition or consecutive 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.

In this study, we established a culture method that enables high-efficiency and low-cost ^{13}C and ^{15}N labeling of a protein produced by *K. lactis* for structural biological analysis using NMR spectroscopy.

In the *K. lactis* expression system, according to the manufacturer's instructions, 20 g/l galactose is required as a carbon and energy source for cell growth, and as an inducer for expression of target proteins. However, for isotope 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 D-glucose in *K. lactis*, while its potential as an inducer of protein expression is low. 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 (Gardner et al. 1998; Kainosho et al. 2006; Xu et al. 2006). The complementary DNA encoding MBP (*malE*) was genetically inserted into an expression vector, pKLAC1 (New England Biolabs, Beverly, MA, USA). The plasmid pKLAC1/*malE* was linearized by digestion with *Sac*II (New England Biolabs, Beverly, MA, USA) and purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). This purified fragment (10 μg) was transformed into competent cells of the *K. lactis* GG799 strain (New England Biolabs, Beverly, MA, USA) by the heat-shock method according to the manufacturer's instructions. Protein expressed in the *K. lactis* cells undergoes processing of the signal peptide, resulting in the secretion of MBP recombinant protein into the culture medium.

We explored the minimal dose of glucose that enables sufficient cell growth and production of target proteins. *K. lactis* stably expressing MBP was precultivated in 14 ml of YPGlu medium containing 2% glucose, 1% Yeast Extract (Becton Dickinson) and 2% Bacto™ Peptone (Becton Dickinson) at 30°C for 1.5 days. The precultivated solution was diluted 100-fold with 1,400 ml of YNB medium without ammonium sulfate and amino acids (Becton Dickinson) containing 5 g/l ammonium chloride and 4–20 g/l D-glucose. The medium was then transferred into 2-l bioreactor vessels (Takasugi Co. Ltd., Tokyo, Japan) with 0.5 ml antifoam PE-M (Wako, Osaka, Japan), and was cultured with the Mini-fermentation unit TS-M (Takasugi Co. Ltd., Tokyo, Japan) at 30°C, with constant air supply using a pump at 1 l/min and agitation at 650 rpm, for 60 h. Induction of MBP expression was observed with 4 g/l D-glucose (Fig. 1, lane 2). However, the yield of the MBP was 1.4 mg/l, which is approximately 20-fold lower than the yield at 20 g/l D-glucose (23.8 mg/l; Fig. 1, lanes 1, 2).

The depletion of glucose appears to result in low cell density and protein expression. To overcome this problem,

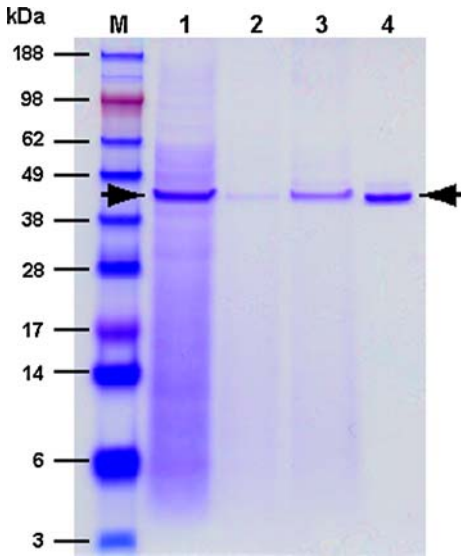

Fig. 1 SDS-PAGE of MBP secreted into culture supernatants (15 μl) after 60 h of batch culture using minimal media containing 20 g/l D-glucose (lane 1) and 4 g/l D-glucose (lane 2), or Fed-batch cultivation that continuously supplied D-glucose (See text; lane 3). SDS-PAGE of purified MBP is shown in lane 4

we tested a "Fed-batch" cultivation strategy. The Fed-batch method supplies nutrients into the culture media in a controlled manner. With this strategy it is possible to reach higher cell densities and protein expression levels than in normal batch culture (Cai et al. 1998). Fermentation was started with 700 ml of minimal media containing 4 g/l D-glucose, as described above. During the culture period (60 h), a further 700 ml of fresh minimal media containing 6 g/l D-glucose was added at a constant flow rate of 8.3 ml/h with peristaltic pump. The final concentration of D-glucose was 5 g/l and the total liquid volume of media reached 1,400 ml. The yield of expressed MBP was 10.1 mg/l, which is 8-fold higher than batch culture using 4 g/l D-glucose (Fig. 1, lane 3). Thus, the Fed-batch culture method enables larger amounts of proteins expressed by *K. lactis*, despite using smaller amount of D-glucose (5 g/l), which reduces costs to a level comparable to *E. coli* isotope labeling systems.

Isotope labeling of MBP by *K. lactis* for NMR measurement was performed by the established Fed-batch culture method using 1,400 ml of minimal media containing ^{13}C-D-glucose (>99% U-$^{13}C_6$, Cambridge Isotope Laboratories, Inc., Cambridge, MA, USA) and 5 g/l ^{15}N-ammonium chloride (>97% ^{15}N, Wako, Osaka, Japan). After the Fed-batch culture was finished, the supernatant of the media containing MBP, which was secreted by *K. lactis*, was collected by centrifugation at 7,000*g* for 30 min, and residual cells and the other debris were eliminated by microfiltration with a membrane filter (0.22 μm pore size) (Millipore, Billerica, MA, USA). The supernatant was dialyzed against buffer containing 20 mM Tris–HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA, and was then loaded onto amylose resin (5 ml, New England Biolabs, Beverly, MA, USA), which was equilibrated with the buffer described above. After washing the resin, MBP was eluted with the buffer described above containing 10 mM maltose.

SDS-PAGE analysis of the eluted solution showed a major band for MBP (>90% of total protein) (Fig. 1, lane 4). For NMR measurement, the MBP solution was dialyzed against buffer containing 10 mM HEPES-NaOH (pH 7.4), 1 mM EDTA, 1 mM β-cyclodextrin and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and was then concentrated to 0.3 mM with Amicon Ultra (Millipore, Billerica, MA, USA). The yield of the purified MBP under these conditions was approximately 14.0 mg. Unlabeled MBP was also expressed and purified with the same procedures as described above.

The unlabeled and ^{13}C, ^{15}N-labeled MBP were analyzed by MALDI-TOF mass spectrometry. The mass of the unlabeled MBP was 40547.7 Da, which is consistent with the calculated mass of MBP at natural abundance (40584.0 Da). Molecular mass of the ^{15}N- and ^{13}C, ^{15}N-labeled MBP were 41017.5 Da and 42876.2 Da, respectively, which are consistent with predicted molecular mass of ^{15}N-labeled MBP (41050.0 Da) and ^{13}C, ^{15}N-labeled MBP (42894.0 Da) (Data not shown). From these results, it was estimated that the efficiency of ^{13}C and ^{15}N incorporation into the MBP secreted by *K. lactis* was approximately >95%, which is sufficient for heteronuclear NMR experiments.

NMR experiments for uniformly ^{13}C, ^{15}N-labeled MBP in the presence of β-cyclodextrin (Gardner et al. 1998) were performed with a Bruker Avance 700-MHz spectrometer equipped with TXI probe at 37°C. Data were processed using TopSpin 2.1 (Bruker, Karlsruhe, Germany) and NMRPipe (Delaglio et al. 1995), and were analyzed with Sparky (Goddard and Kneller 2006).

The well-dispersed chemical shift of the ^{1}H and ^{15}N in the [^{15}N, ^{1}H] HSQC spectrum indicates that the uniformly ^{13}C, ^{15}N-labeled MBP secreted by *K. lactis* into the culture medium is correctly folded (Fig. 2a). Furthermore, the assignment of NMR signals of the uniformly ^{13}C, ^{15}N-labeled MBP was successfully performed using HNCA spectrum (Fig. 2b), 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.

We thus established a culture method for *K. lactis* using a minimal amount (5 g/l) of glucose to enable sufficient production of target protein with highly efficient isotope labeling. Using this culture method, the cost of isotope labeling of target proteins is six to seven times lower than that of the *P. pastoris* expression system, if it is assumed that equal amounts of target protein are expressed in both systems. The cultivation procedure for *K. lactis* is simple, easily scaled-up, and has high reproducibility when compared to the system using *P. pastoris*. In addition, it is possible to produce deuterated proteins using the present *K. lactis* culture system, as methods for expressing deuterated proteins by *P. pastoris* have already been established (Morgan et al. 2000; Ichikawa et al. 2007). It is feasible to express glycoproteins by using *K. lactis* expression system (Fleer et al. 1991a, b; Tokunaga et al. 1997; Wildt and Gerngross 2005) and stable isotope labeling of glycans of glycoproteins could be possible if a minimal media utilizing ammonium chloride and glucose is used for the protein expression. However, care should be taken that the glycosylation type is rather characteristic for the yeast expression (Daly and Hearn 2005 and references cited therein), therefore glycans attached to target proteins in the yeast expression are not necessarily identical to the original ones expressed in mammalian cells. Furthermore, since several membrane integral proteins are successfully expressed in yeast *P. pastoris* or *Saccharomyces cerevisiae* with their native form (Weiss et al. 1998; Tate 2001; Long et al. 2005; Jidenko et al. 2005), it could be possible to express membrane proteins by using *K. lactis*

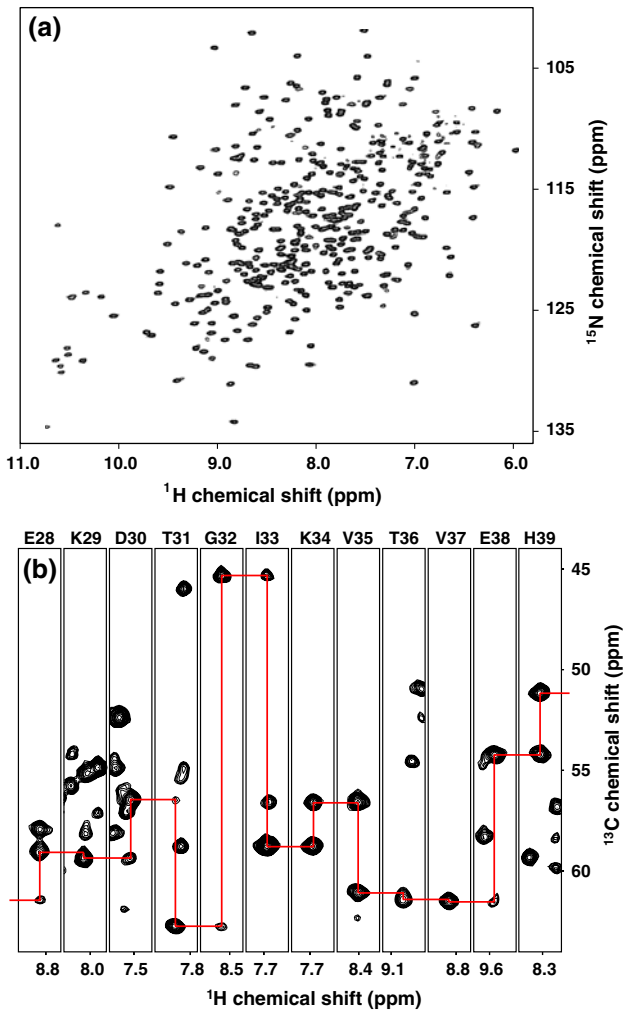

Fig. 2 **a** [^{15}N, ^{1}H] HSQC spectrum, and **b** Resonance assignment of 0.3 mM uniformly ^{13}C, ^{15}N-labeled MBP using [^{13}Cα, ^{1}H^{N}]-strips taken from 3D HNCA spectrum at each ^{15}N of residues 28–39

expression system. We believe that the *K. lactis* expression system is a powerful tool for isotope labeling of heterologous proteins for NMR study.

Acknowledgements This work was supported by the Ministry of Economy, Trade and Industry (METI) and the New Energy and Industrial Technology Development Organization (NEDO). We would like to thank to Mr. Osamu Ichikawa (Graduate School of Pharmaceutical Sciences, University of Tokyo, Japan) for assistance with the *P. pastoris* protein expression system. In addition, we are especially grateful to Naoki Yoshida, Ph.D., Hitoshi Tainaka, Ph.D. (New England Biolabs Japan, Inc.), and C. H. Taron, Ph.D. (New England Biolabs, Inc.), for technical support regarding the *K. lactis* protein expression system.

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