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An Economical Method for $^{15}N/^{13}C$ Isotopic Labeling of Proteins Expressed in *Pichia pastoris*[1]

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We report a new and cost-effective approach to prepare $^{15}N/^{13}C$ labeled proteins for NMR using the *Pichia pastoris* expression system. Four protocols (P1 to P4) were defined and compared using recombinant Ovine interferon-tau (rOvIFN-τ). Our results demonstrate that in order to get full incorporation of ^{15}N and ^{13}C, the isotopes are not totally required during the initial growth phase of *P. pastoris* culture. The addition of small amounts of ^{15}N and ^{13}C compounds 6 h prior to the methanol induction phase is sufficient to obtain 99% incorporation of heavy isotopes into the protein. Our optimized protocol P4 is two-thirds less costly than the classical method using ^{15}N and ^{13}C isotopes during the entire growth phase.

Key words: NMR, $^{15}N/^{13}C$ isotopic labeling, ovine interferon-tau, *Pichia pastoris*, protein expression.

During the last 10 years, the methylotrophic yeast *Pichia pastoris* has been developed into a highly successful system for the production of large quantities of a variety of heterologous proteins in their correctly folded native states (*1*). For *P. pastoris*, isotopic enrichment of proteins will never be as cost effective as with *Escherichia coli* because of the high cell density required for the yeast system. Nevertheless, *P. pastoris* can be advantageous since the protein is secreted directly into the medium, thus simplifying considerably the subsequent purification of the protein. Previous studies have reported the need to include ^{13}C and ^{15}N during both the growth and methanol-inducing phase (*2, 3*). Thus, most of the isotope ends up in the cell mass and the yield of the desired protein per gram of labeling reagents is much lower.

The purpose of this study was to find a new, effective and economical method to label proteins expressed in *P. pastoris*. Here, we report a comparison of four $^{15}N/^{13}C$ isotopic labeling protocols. These include methods developed by Laroche *et al.* (*2*) and by Wood and Komives (*3*), with minor modifications. We demonstrate the methods using the recombinant protein Ovine interferon-tau (rOvIFN-τ). Ovine interferon-τ is a 20 kDa protein with anti-viral (*4*) and anti-proliferative (*5*) properties as shown by major classes of IFNs (*6*). The crystallographic structure of a cleaved form of the protein (cleaved during the crystallization process) has recently been reported (*7*). Our laboratory is determining independently the NMR solution structure of the intact protein to determine some of the key structure-function relationships. In order to solve the 3D NMR structure, cost effective ^{15}N and $^{15}N/^{13}C$ isotopic labeling methods are critical for expressing the needed labeled protein.

MATERIALS AND METHODS

Expression of the Recombinant Ovine Interferon-τ in Pichia pastoris—rOvIFN-τ was expressed in *P. pastoris* as previously described with slight modifications (*8*). Transformants were grown as recommended by the manufacturer (Invitrogen, Carlsbad, CA) on BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% Yeast Nitrogen Base, 4×10^{-5} % Biotin, 1% glycerol). The cells were further centrifuged and induced in BMMY medium (same as BMGY but containing 0.5% methanol in place of glycerol) for protein expression.

Protein Purification—The recombinant rOvIFN-τ was expressed and purified in a two step process. The proteins were first passed through a Q-Sepharose column (Pharmacia, Peapack, NJ) followed by gel filtration using an HS100-Sephacryl column (Sigma). The proteins were finally dialyzed extensively for 36 h against 10 mM Tris-HCl, pH 7.6, then 1 mM Tris-d_{11}, lyophilized, and stored at −80°C in 0.02% sodium-azide.

Electrophoretic Analysis—Protein concentration was measured by the Bradford method (*9*). The purified rOvIFN-τ was subjected to PAGE on a 15% gel using Tricine buffer (*10*). Proteins were stained with Coomassie Brillant Blue R-250 (Sigma).

MS Analysis—Uniform isotopic labeling of purified rOvIFN-τ was confirmed by MALDI-TOF MS (PerSeptive Biosystems, Framingham, MA) analysis. The acceleration voltage was set to 25 kV and apomyoglobin was used to standardize the mass spectrometer.

Labeling Experiments for NMR Analysis—In support of NMR studies aimed at determining the structure of rOvIFN-τ, we uniformly labeled the protein with heavy isotopes of nitrogen (^{15}N) and carbon (^{13}C). The enriched

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chemicals used were: ^{15}N-ammonium chloride (99% ^{15}N, Cambridge Isotope Laboratories, MA), $^{13}C_6$-D-glucose (99% ^{13}C, Isotec, Miamisburg, OH) and ^{13}C-methanol (99.2% ^{13}C, Isotec). A 5-ml culture was grown overnight at 30°C and transferred in the morning to 100-ml culture. Two protocols, P3 and P4, were designed and compared with slight modification of protocols P1 and P2 previously described by Wood *et al.* (*3*) and Laroche *et al.* (*2*), respectively. All four protocols are described in Table I. Protocols P3 and P4 consist of culturing transformants in YNB without amino acids (0.8%), biotin (2 mg/liter), $(NH_4)_2SO_4$ (1.2%) or $^{15}NH_4Cl$ (1.2%), K_2HPO_4 (0.3%), KH_2PO_4 (0.28%), 2 ml/liter of PTM1 salts (Invitrogen), glucose (3%) or ^{13}C-glucose (3%) during the growth phase. Additional glucose (2%) was added after 24 h of culture. Since the recombinant proteins in *P. pastoris* are only expressed during the methanol-induction phase, we examined whether it is necessary to have the heavy isotopes present during the entire growth-phase. For protocol P4, ^{15}N-ammonium chloride (0.02%) and $^{13}C_6$-D-glucose (0.1%) were added 6 h prior to methanol induction, whereas P3 did not contain any isotope-containing materials during the entire growth phase. Cells were then centrifuged briefly, washed with a 0.2% glycerol solution, pelleted and suspended in protein induction media. Methanol induction either for P3 or P4 was performed as shown in Fig. 1. During the adaptation phase, a low concentration of ^{13}C-methanol (0.2%) was supplied and incrementally supplemented (0.4%) every 12 h to 1.4% from 72 to 96 h of the time-course (Fig. 1). Since the culture of *P. pastoris* at high concentration can induce cell lysis, cells were then shifted to 0.8% ^{13}C-methanol during the last 12 h. During all experiments, the pH was controlled and adjusted under agitation, when necessary, to 5.4 with a sterilized base solution of 4.4 M KOH and 7.4 M NaOH. Throughout the study, *P. pastoris* cultures were agitated at 250 rpm at 30°C.

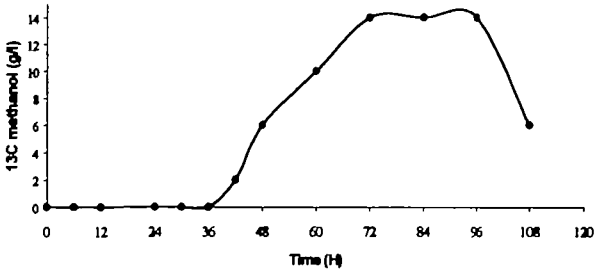

Fig. 1. **Profile of $^{13}C_6$-methanol induction for protocols P3 and P4.** *Pichia pastoris* were induced with $^{13}C_6$-methanol as described in "MATERIALS AND METHODS."

NMR Analysis—Purified $^{15}N/^{13}C$-labeled rOvIFN-τ was used for 1D proton and 2D-NMR experiments. Lyophilized protein (1 mM) was resuspended in 1 mM Tris-d_{11}, pH7.2, with 10% D_2O. All NMR experiments were performed at 25°C on a Bruker AM-600 MHz NMR.

Antiviral Assay—The antiviral activity of rOvIFN-τ was determined using a standard viral cytopathic assay on Madin-Darby Bovine kidney (MDBK) cells challenged with a virus, such as stomatitis virus, as described by Pontzer *et al.* (*4*).

RESULTS

Expression and Yield of $^{13}C/^{15}N$ rOvIFN-τ—The $^{13}C/^{15}N$ double-labeled rOvIFN-τ was successfully expressed and secreted into the culture media (Fig. 2). The results of protein expression after 4 days of methanol induction were the following: 98, 226, 85, and 260 mg of $^{13}C/^{15}N$ double-labeled rOvIFN-τ per liter of initial culture were obtained using the P1, P2, P3, and P4 protocols, respectively (Table I).

Growth Curves and $^{15}N/^{13}C$ rOvIFN-τ Labeling—Cells were induced with ^{13}C-methanol when early starvation was observed (Fig. 3). Protocol P2 containing 5% ^{13}C-glucose at the early exponential phase ensured a much higher rate of cell growth in culture compared with the remaining 3 protocols, P1, P3, and P4 (Fig. 3). However, supplementation with 2% glucose in P4 during the exponential growth phase significantly increased cell growth in this phase. The re-

TABLE I. **Different $^{15}N/^{13}C$ isotopic labeling protocols.**

	P1	P2	P3	P4
Growth phase (0–30 h)	FM21 medium[a] $^{15}NH_4Cl$ (1%) ^{13}C-glucose (2%)	FM22 medium[b] $^{15}NH_4Cl$ (0.5%) ^{13}C-glucose (5%)	FM23 medium[c] $^{15}NH_4Cl$(1.2%) ^{13}C-glucose (3%)	FM23 medium[c] $(NH_4)2SO_4$ (1.2%) D-glucose (3%)
Growth phase (at 24h)				Addition of D-glucose (2%)
Growth phase (30–36h)	FM21 medium[a] $^{15}NH_4Cl$ (1%) ^{13}C-glucose (2%)	FM22 medium[b] $^{15}NH_4Cl$ (0.5%) ^{13}C-glucose (5%)	FM23 medium[c] $^{15}NH_4Cl$ (1.2%) ^{13}C-glucose (3%)	FM23 medium[c] $^{15}NH_4Cl$ (0.02%) ^{13}C-glucose (0.1%)
Induction phase (4 days)	FM21 medium[a] $^{15}NH_4Cl$ (1%) ^{13}C-methanol (2%)	FM22 medium[b] $^{15}NH_4Cl$ (0.5%) Addition of ^{13}C-methanol (0.5%) every 12 h	FM23 medium[c] $^{15}NH_4Cl$ (1.2%) Addition of ^{13}C-methanol as described in Fig. 1	FM23 medium[c] $^{15}NH_4Cl$ (1.2%) Addition of ^{13}C-methanol as described in Fig. 1
rOvIFN-τ (mg/ml)	98	226	85	260

YNB w/o aa and AS, Yeast nitrogen base without amino acids and ammonium sulfate; AS, ammonium sulfate; P1 to P4 represent the four protocols compared. Media were prepared in a 100 ml final volume and filtered through a 0.22 μm membrane. [a]FM21 medium (*3*): YNB w/o aa and AS (0.34%), Casamino acids (1%), biotin (2 mg/liter); [b]FM22 medium (*2*): 1 g/liter $CaSO_4 \cdot 2H_2O$, 14.28 g/liter K_2SO_4, 11.7 g/liter $MgSO_4 \cdot 7H_2O$, biotin (400 μg/liter), PTM1 salts (1 ml/liter) (Invitrogen)); [c]FM23 medium: YNB w/o aa and AS (0.8%), biotin (2 mg/liter), $(NH_4)_2SO_4$ (1.2%), K_2HPO_4 (0.3%), KH_2PO_4 (0.28%), PTM1 salts (2 ml/liter). The pH was adjusted to 5.5 with a base solution (KOH 4.4 M, NaOH 7.4 M). After 30 h of grow at 30°C, each culture was centrifuged (4,000 × rpm, 5 min) and the cells were resuspending in the corresponding second step growth-phase pre-warmed media. The growth phase was pursued 6 h and stopped by centrifugation (4,000 × rpm, 5 min). Yeast were washed with a 0.2% glycerol solution, centrifuged, then suspended and cultured according to the respective induction protocol. For protocols P3 and P4 ^{13}C-methanol (0.2%) was applied at a low concentration during the adaptation phase and incrementally supplemented (0.4%) every 12 h up to 1.4% from 72 to 96 h of the time-course (Fig. 1).

sults demonstrate a growth rate to a cell density similar to P2 just prior to ^{13}C-methanol induction (Fig. 3). The decreased cell density observed for P2 after 72 h may be explained by toxicity caused by the presence of 2% ^{13}C-methanol during the entire induction period. To prevent cell-death of *P. pastoris* after long-term ^{13}C-methanol exposure, the rate of ^{13}C-methanol addition was modified. During the adaptative phase (for 6-h post-induction), ^{13}C-methanol was given at a low concentration (0.2%), and the concentration was subsequently increased by 0.4% every 12 h until it reached 1.4%. After 24 h at high concentration (1.4%), the ^{13}C-methanol concentration was decreased to 0.6% for the last 12 h. Starting the ^{13}C-methanol induction period at about the same cell density, P4 resulted in a slight increase (16%) of $^{15}N/^{13}C$ labeling over protocol P2 protocols P1 and P3 resulted in about same growth curves and gave 98 and 85 mg, respectively, of purified $^{13}C/^{15}N$ double-labeled rOvIFN-τ per liter of initial culture. Protocol P3 is the only one with only 0.1% ^{13}C-glucose and 0.02% ^{15}N-ammonium chloride isotopes for 6 h at the end of the growth phase. Importantly, when comparing isotope incorporation in rOvIFN-τ by protocol P3 with the other protocols, protocal P4 resulted in a 99% incorporation of ^{15}N and ^{13}C isotopes.

Purification of the Protein and Tricine-PAGE—After the double purification step, unlabeled and $^{13}C/^{15}N$ double-labeled rOvIFN-τ were obtained with over 95% purity as observed by Tricine-PAGE electrophoresis, 1D-NMR and MS analyses (Figs. 2, 4, and 5).

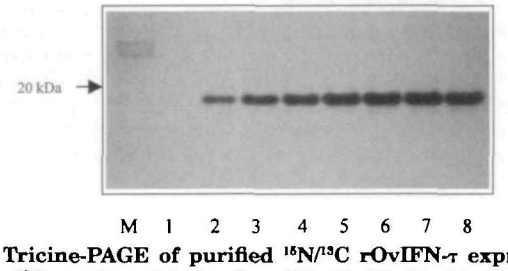

Fig. 2. **Tricine-PAGE of purified $^{15}N/^{13}C$ rOvIFN-τ expressed during $^{13}C_6$-methanol induction (42, 60, 72, 84, 96, 108, 120, and 132 h) in shake flask (protocol P4).** Lane M indicates the prestained protein molecular marker (BioRad, Hercules, CA). Lanes 1 to 8 show purified $^{15}N/^{13}C$ rOvIFN-τ expressed at 42, 60, 72, 84, 96, 108, 120, and 132 h respectively.

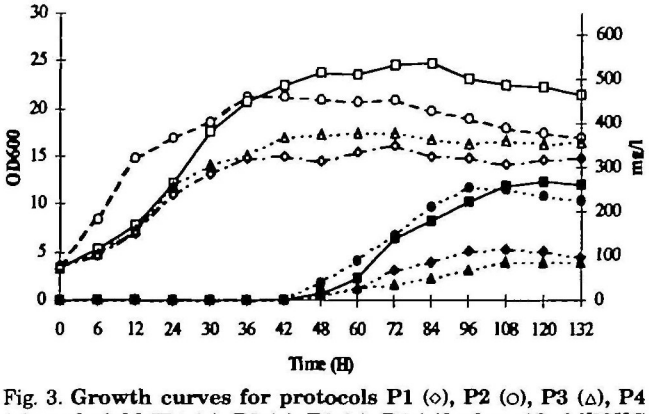

Fig. 3. **Growth curves for protocols P1 (◇), P2 (○), P3 (△), P4 (□), and yield [P1 (◆), P2 (●), P3 (▲), P4 (■)] of purified $^{15}N/^{13}C$ rOvIFN-τ.** *Pichia pastoris* were grown and induced for rOvIFN-τ expression as described in "MATERIALS AND METHODS."

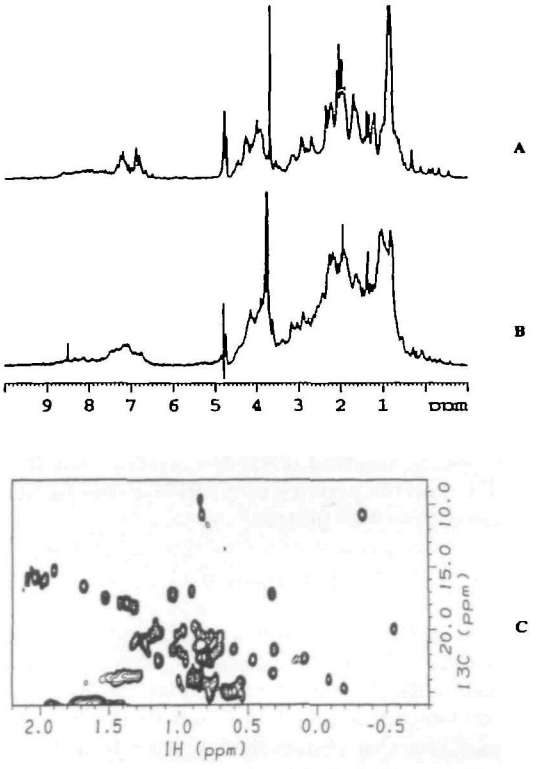

Fig. 4. **^1H 1D-NMR spectra of unlabeled (A) and $^{15}N/^{13}C$ rOvIFN-τ (B), and 2D-NMR (C) of the $^{15}N/^{13}C$ rOvIFN-τ at pH 7.2, 1 mM, 25°C.**

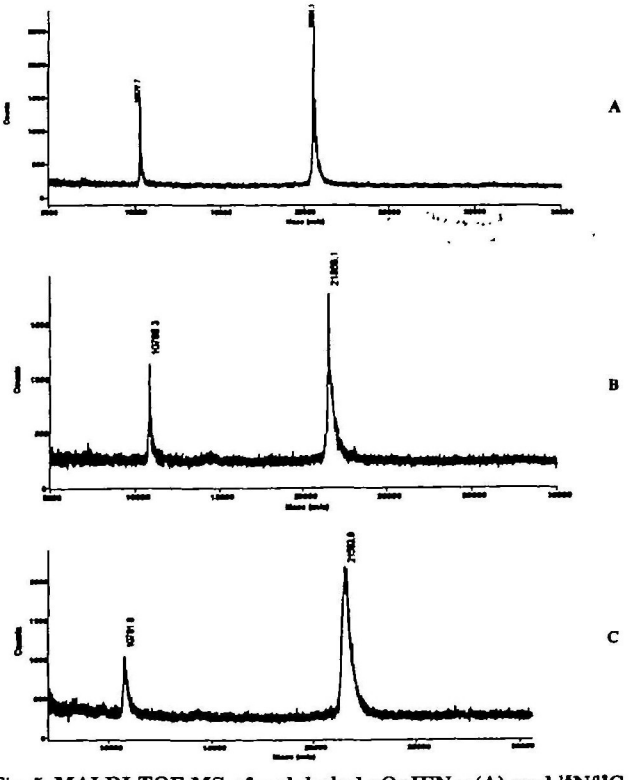

Fig. 5. **MALDI-TOF MS of unlabeled rOvIFN-τ (A) and $^{15}N/^{13}C$-labeled rOvIFN-τ (B, control using protocol P2; C, using protocol P4).**

NMR and MS Analysis—In order to confirm the level of incorporation of both isotopes ^{15}N and ^{13}C, NMR experiments were performed on a 600 MHz Bruker instrument. Figure 4 shows the 1D-NMR spectra obtained under identical conditions for unlabeled (Fig. 4, A and B) and $^{13}C/^{15}N$ double-labeled rOvIFN-τ (Fig. 4C).When ^{15}N and ^{13}C isotopes were used during the entire growth phase (protocol P3), partial incorporation of both isotopes was observed (range 68–83%). However, when 0.1% ^{13}C-glucose and 0.02% ^{15}N ammonium chloride were added 6 h prior to the start of the methanol induction-phase (Protocol P4), nearly full incorporation was observed. Results of the MALDI-TOF spectra confirmed, within 0.05% error, the results of the 1D and 2D-NMR data, showing more than 99% incorporation of ^{15}N and ^{13}C into rOvIFN-τ (Fig. 5).

Antiviral Assay—Antiviral activity determind by cytopathic assays showed that rOvIFN-τ is a functionally active protein. The specific activity of rOvIFN-τ was found to be 10^8 units/mg of purified protein.

DISCUSSION

The purpose of this study was to find a new way to label rOvIFN-τ with ^{15}N and ^{13}C isotopes at lower cost in shake flask experiments. We have compared two protocols, P3 and P4, with previous protocols, P1 (*3*) and P2 (*2*), that we have slightly modified. Our results demonstrate that ^{15}N and ^{13}C isotopes are not required throughout the initial growth period but are necessary at low concentration a few hours prior to the protein expression period. Full incorporation (99%) was observed when 0.1% ^{13}C-glucose and 0.02% ^{15}N-ammonium chloride were added for 6 h prior to the ^{13}C-methanol induction phase. We evaluated the cost of this method and previous methods. In our experiments, a 100 ml *P. pastoris* culture yielded 26 mg of purified protein, which was sufficiently concentrated for NMR experiments. Taking the price of the isotopes into account, the cost of our method (P4) was found to be about one-third that of the classical method using ^{15}N-ammoniumchloride (0.25%) and $^{13}C_6$-D-glucose (3%) during the entire growth phase. The cost of our method would be even lower compared with methods that use $^{13}C_6$-glycerol during the growth phase, because this compound is even more expensive than $^{13}C_6$-D-glucose. It seems that cell lysis and the resulting possible protein degradation observed when using high concentrations of methanol (>1.5%) can be prevented by reducing the amount of ^{13}C-methanol during the initial adaptation growth phase (0.2 to 0.6%) and the last 12 h of the methanol induction period (0.6%). This was observed for protocol P2 when using 2% ^{13}C-methanol during the induction period. These results are in agreement with a prior study showing that initial and terminal moderate methanol induction can regulate acid phosphatase appA2 mRNA expression (*11*).

The sequential addition of a carbon source or 5% ^{13}C-glucose during the exponential growth phase was found to be beneficial for improving cell density in shake flasks (*1*, *2*). However, aeration is a main limiting factor in shake flasks and cannot be obviously controlled. One way would be to substantially increase the aeration by agitation when 2% glucose is added during the exponential growth phase. Another way would be to perform experiments in a fermentor in which parameters such as oxygenation can be controlled more accurately.

In order to get much more labeled protein at a lower cost, fermentation experiments using the P4 protocol have some benefits. First, oxygenation is controlled and much higher during fermentation than in shake flasks. Second, a fed-batch phase using D-glucose (24–36 h) during the growth phase should give a much higher cell density, thus giving a much higher level of labeled proteins expressed. Third, methanol accumulation, which is responsible for cell toxicity, can be monitored and better controlled during the induction phase.

In conclusion, we have successfully expressed and purified functionally active and labeled $^{15}N/^{13}C$-rOvIFN-τ in *P. pastoris*. Our results demonstrate that in order to obtain full incorporation of ^{15}N and ^{13}C, these isotopes are not continuously required, at least during the initial exponential growth phase of the *P. pastoris* culture. These results present a new, reliable and economical method that might be of interest for preparing new NMR labeling samples using *P. pastoris* as an expression host.

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