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A novel isotope labeling protocol for bacterially expressed proteins

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SUMMARY

A novel protocol for isotopically labeling bacterially expressed proteins is presented. This method circumvents problems related to poor cell growth, commonly associated with the use of minimal labeled media, and problems with protein induction encountered, less commonly, when using enriched labeled media. The method involves initially growing the bacterial cells to high optical density in a commercially available enriched labeled medium. Following a suitable growth period, the cells are transferred to a different (minimal) labeled medium, appropriate for induction. The method is demonstrated using the protein melanoma growth stimulating activity (MGSA).

Recent advances in NMR spectroscopy have made it possible to determine the solution conformations of medium-sized proteins up to molecular masses of about 30 kDa (Clore and Gronenborn, 1991a,b; Bax and Grzesiek, 1993; Wagner, 1993). The new methods involve implementation of a number of 3D and 4D heteronuclear NMR techniques and require that the protein of interest be uniformly labeled with the NMR-observable isotopes ^{15}N and/or ^{13}C. Uniform isotopic labeling of proteins is most commonly achieved by expressing the protein in a microbial host, typically *Escherichia coli*, grown in a minimal medium in which the only nitrogen source is ^{15}N-labeled (e.g. $(^{15}NH_4)_2SO_4$ or $^{15}NH_4Cl$) and the only carbon source is ^{13}C-labeled (e.g. $^{13}C_6$-glucose) (McIntosh and Dahlquist, 1990). Economic considerations require that the expression system be reasonably efficient under these growth conditions. Generally, however, bacteria grow and secrete more efficiently in enriched or supplemented media, rather than minimal media. An alternative approach to the use of ^{15}N- and/or ^{13}C-labeled minimal media therefore is to use commercially available media, composed of uniformly ^{15}N- and/or ^{13}C-enriched hydrolysates extracted from algae, grown with simple isotopically labeled raw materials (e.g. $K^{15}NO_2$, and $^{13}CO_2$).

In some circumstances, however, neither of the above approaches will yield amounts of uni-

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formly labeled protein sufficient for NMR studies. In particular, certain bacterial strains may grow poorly in minimal media, leading to a significant reduction in final protein yield compared to bacteria grown in enriched media. Whilst not affecting cell growth, some components (known or unknown) present in commercially available isotopically labeled enriched media may have a negative effect on protein expression. We have recently encountered both of these problems during efforts to produce uniformly ^{15}N-/^{13}C-labeled melanoma growth stimulating activity (MGSA). The present communication gives details of a relatively simple labeling protocol which allowed us to circumvent these problems.

The MGSA expression vector, pMG34 (Horuk et al., 1993), was originally designed to give secretion of the protein to the *E. coli* periplasm. The plasmid directs MGSA transcription from a tightly controlled alkaline phosphatase (AP) promoter (Kikuchi et al., 1981); low-phosphate conditions are therefore a requirement for induction (less than ~ 0.2 mM (Torriani, 1960)). Tandem Shine–Dalgarno sequences from the tryptophan leader and heat-stable enterotoxin II (STII) are present at the translation initiation site. The STII signal sequence is used for efficient bacterial secretion of the protein.

E. coli K12 cells, transformed with pMG34, were grown in shake flask cultures. Trial experiments using unlabeled minimal media resulted in unacceptably low cell growth and consequently poor yields. Further experiments to test the feasibility of using the double-labeled enriched medium CELTONE-CN liquid (Martek Corp.) for uniform ^{15}N-/^{13}C-labeling of MGSA were carried out using the analogous unlabeled medium, CELTONE-U liquid. The CELTONE liquid medium proved unsuitable for expression of MGSA, due to the high levels of phosphate present (~ 22 mM). This concentration is approximately 100-fold greater than the maximum for optimal induction; significant phosphate depletion is not expected, since the overnight shake flask cultures only reached an OD_{550} of 5–6. A third set of trial experiments was therefore performed, in which overnight cultures were grown in either LB broth or CELTONE-U liquid media. The cultures were centrifuged and the cell pellets resuspended at an OD_{550} of 3–5 in a low-phosphate shake flask medium containing 120 mM triethanolamine (pH 7.4), 1.6 mM $MgSO_4$, 20 mM NH_4Cl, 50 mM KCl, 20 mM NaCl, and 0.4% (4 g/l) glucose. Some of the pellets from the CELTONE-U liquid overnight cultures were washed with 120 mM triethanolamine, pH 7.4, prior to resuspension in the low-phosphate medium. In addition, CELTONE-U powder was added to some of the low-phosphate cultures to a level of 0.12% (1.2 g/l), resulting in an initial phosphate concentration of approximately 2 mM; secretion and accumulation of heterologous proteins by *E. coli* is sometimes enhanced by the addition of a complex nitrogen source (Reilly, D., unpublished observations). The secondary cultures were then incubated at 37 °C, with shaking, for 24 h. All the cultures initially grown overnight in LB broth were observed to accumulate MGSA in the low-phosphate medium, either with or without addition of 0.12% CELTONE-U powder. Variable results were obtained, however, with the secondary cultures derived from the unwashed CELTONE-U liquid overnight cultures (Fig. 1). Inclusion of the wash step resulted in consistent accumulation of MGSA in the secondary cultures (both the minimal low-phosphate media and the media containing 0.12% CELTONE-U powder), indicating that the variability observed with the unwashed cultures was due to varying amounts of CELTONE-U liquid carry-over, and therefore presumably phosphate carry-over, from the initial overnight culture. The phosphate concentration in LB broth was determined to be ~ 2.7 mM; carry-over of residual phosphate from the LB broth overnight cultures does not appear to significantly inhibit induction of the AP promoter.

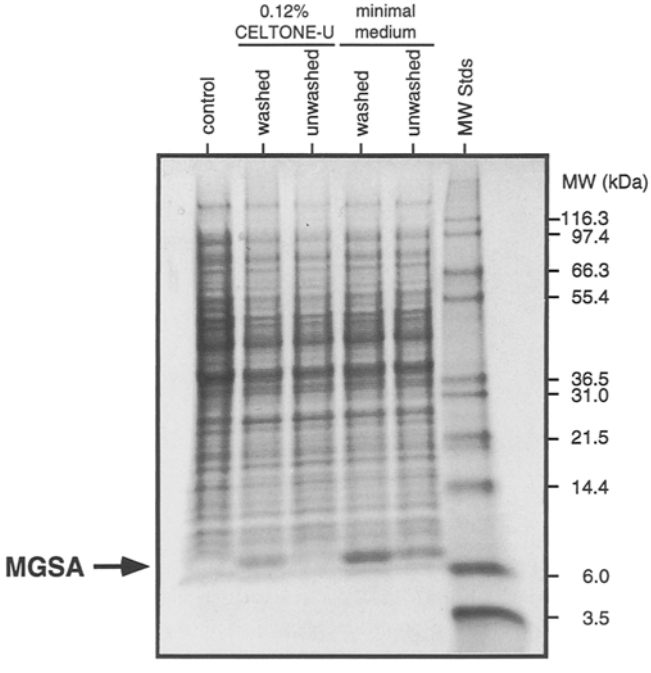

Fig. 1. A 10–20% Tricine gel (Novex, San Diego, CA), illustrating the accumulation of MGSA in the *E. coli* periplasm under various secondary culture conditions. Primary cultures were grown in CELTONE-U liquid in all cases. From left to right: lane 1, negative *E. coli* control; lane 2, minimal media plus 0.12% CELTONE-U powder, washed; lane 3, minimal media plus 0.12% CELTONE-U powder, unwashed; lane 4, minimal media only, washed; lane 5, minimal media only, unwashed; lane 6, Novex wide-range molecular weight markers. The position of the ~ 7 kDa MGSA band is indicated by an arrow.

Uniform ^{15}N-/^{13}C-labeling of MGSA was achieved by growing overnight shake flask cultures in CELTONE-CN liquid medium at 37 °C and 200 rpm. This step ensured that all the amino acid pools (both free and protein incorporated) were labeled. The overnight cultures were centrifuged and the pellets washed with 120 mM triethanolamine, pH 7.4. The washed pellets were resuspended at an OD_{550} of 3–5 in the low-phosphate medium containing 0.12% CELTONE-CN powder (we have found that addition of CELTONE powder to the secondary culture medium gives variable results and is unnecessary for MGSA expression; see Fig. 1), 20 mM $^{15}NH_4Cl$ and 0.4% ^{13}C-glucose instead of the corresponding unlabeled components used in the trial experiments. The secondary cultures were incubated at 37 °C, with shaking, for 22–26 h. Purification of the secreted MGSA from the cell culture supernatant was carried out as described (Fairbrother et al., 1993; Horuk et al., 1993), yielding ~ 22 mg of purified protein per litre of cell culture.

Isotopic labeling to a level of about 96% was achieved, as indicated by mass spectrometry and NMR spectroscopy analysis. The ^{15}N- and ^{13}C-HSQC spectra (Bax et al., 1990; Norwood et al., 1990) obtained from the double-labeled sample are illustrated in Fig. 2. A second labeling experiment, in which cells were grown overnight in unlabeled LB broth prior to induction in labeled minimal medium, yielded approximately 80% isotopic incorporation, as judged by mass spectrometry analysis of the purified protein.

The isotope labeling method used here for MGSA should be generally applicable to cases in which the use of minimal media is not sufficient to yield the quantities of protein required for NMR studies and in which enriched labeled media 'poison' expression (but not bacterial cell growth). In summary, the method involves initially growing the cells to a high OD in enriched labeled medium (labeled medium is used to ensure labeling of all cellular amino acid pools), followed by transfer to a minimal labeled medium for induction. To avoid carry-over of induc-

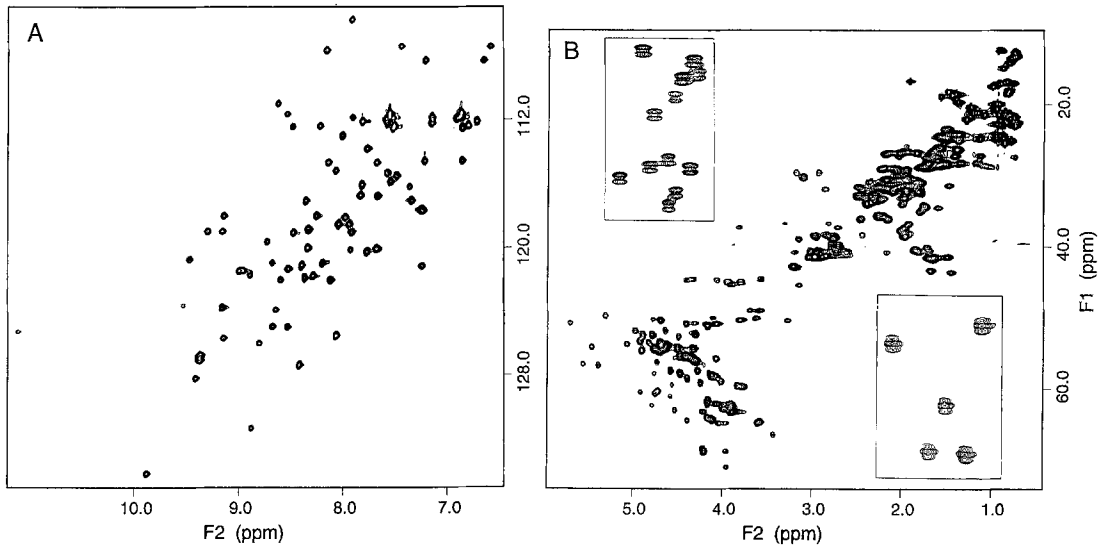

Fig. 2. (A) ^{15}N-HSQC and (B) ^{13}C-HSQC spectra of ~ 1.5 mM ^{15}N-/^{13}C-labeled MGSA in 92% H_2O/8% D_2O and 99.99% D_2O, respectively, and 50 mM KPi, pH 5.7, at 30 °C, acquired on a Bruker AMX 500 NMR spectrometer. The insets in (B) display expanded regions of a higher resolution (in F_1) ^{13}C-HSQC spectrum, showing splittings due to ^{13}C–^{13}C couplings. The upper left inset shows the isoleucine methyl region and the resolved couplings between the $C^{\gamma 2}$ and C^{δ} methyl carbons and their directly attached C^{β} and $C^{\gamma 1}$ carbons, respectively. The lower right inset shows the downfield shifted $^1H^{\alpha}$–$^{13}C^{\alpha}$ region and splittings due to both $^1J_{C^{\alpha}CO}$ and $^1J_{C^{\alpha}C^{\beta}}$ couplings.

tion-inhibiting components present in the enriched medium (phosphate in the MGSA example presented here), it was found necessary to add a washing step before adding the cells to the secondary culture medium.

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REFERENCES

Bax, A. and Grzesiek, S. (1993) *Acc. Chem. Res.*, **26,** 131–138.
Bax, A., Ikura, M., Kay, L.E., Torchia, D.A. and Tschudin, R. (1990) *J. Magn. Reson.*, **86,** 304–318.
Clore, G.M. and Gronenborn, A.M. (1991a) *Prog. NMR Spectrosc.*, **23,** 43–92.
Clore, G.M. and Gronenborn, A.M. (1991b) *Science,* **252,** 1390–1399.
Fairbrother, W.J., Reilly, D., Colby, T. and Horuk, R. (1993) *FEBS Lett.*, **330,** 302–306.
Horuk, R., Yansura, D.G., Reilly, D., Spencer, S., Bourell, J., Henzel, W., Rice, G. and Unemori, E. (1993) *J. Biol. Chem.*, **268,** 541–546.
Kikuchi, Y., Yoda, K., Yamasaki, M. and Tamura, G. (1981) *Nucleic Acids Res.*, **9,** 5671–5678.
McIntosh, L.P. and Dahlquist, F.W. (1990) *Q. Rev. Biophys.*, **23,** 1–38.
Norwood, T.J., Boyd, J., Heritage, J.E., Soffe, N. and Campbell, I.D. (1990) *J. Magn. Reson.*, **87,** 488–501.
Torriani, A. (1960) *Biochim. Biophys. Acta,* **38,** 460–479.
Wagner, G. (1993) *J. Biomol. NMR,* **3,** 375–385.