Journal of Biomolecular NMR, 7 (1996) 131–141
ESCOM

J-Bio NMR 327

High-level production of uniformly ^{15}N- and ^{13}C-enriched fusion proteins in *Escherichia coli*

Magnus Jansson[a,b], Yu-Chin Li[c], Lena Jendeberg[a,b], Stephen Anderson[c,d], Gaetano T. Montelione[c,d,*] and Björn Nilsson[b,*]

[a]*Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden*
[b]*Department of Structural Biochemistry, Biopharmaceuticals, Pharmacia AB, S-112 87 Stockholm, Sweden*
[c]*Center for Advanced Biotechnology and Medicine and* [d]*Department of Molecular Biology and Biochemistry, Rutgers University, 679 Hoes Lane, Piscataway, NJ 08854-5638, U.S.A.*

Received 8 July 1995
Accepted 28 November 1995

Keywords: Biosynthetic enrichment; Insulin-like growth factor; Staphylococcal protein A; Bovine pancreatic trypsin inhibitor; Triple-resonance NMR

Summary

An approach to produce ^{13}C- and ^{15}N-enriched proteins is described. The concept is based on intracellular production of the recombinant proteins in *Escherichia coli* as fusions to an IgG-binding domain, Z, derived from staphylococcal protein A. The production method provides yields of 40–200 mg/l of isotope-enriched fusion proteins in defined minimal media. In addition, the Z fusion partner facilitates the first purification step by IgG affinity chromatography. The production system is applied to isotope enrichment of human insulin-like growth factor II (IGF-II), bovine pancreatic trypsin inhibitor (BPTI), and Z itself. High levels of protein production are achieved in shaker flasks using totally defined minimal medium supplemented with $^{13}C_6$-glucose and $(^{15}NH_4)_2SO_4$ as the only carbon and nitrogen sources. Growth conditions were optimized to obtain high protein production levels and high levels of isotope incorporation, while minimizing $^{13}C_6$-glucose usage. Incorporation levels of ^{13}C and/or ^{15}N isotopes in purified IGF-II, BPTI, and Z were confirmed using mass spectrometry and NMR spectroscopy. More than 99% of total isotope enrichment was obtained using a defined isotope-enriched minimal medium. The optimized systems provide reliable, high-level production of isotope-enriched fusion proteins. They can be used to produce 20–40 mg/l of properly folded Z and BPTI proteins. The production system of recombinant BPTI is state-of-the-art and provides the highest known yield of native refolded BPTI.

Introduction

Uniform biosynthetic enrichment with ^{15}N and ^{13}C isotopes facilitates analysis of macromolecular structures by NMR spectroscopy (Fesik et al., 1990,1991; Clore and Gronenborn, 1991,1994; Ikura et al., 1992; Grzesiek and Bax, 1993; Qian et al., 1993; Wagner, 1993). The recent availability of uniformly isotope-enriched protein samples has allowed NMR spectroscopy to be applied to larger proteins and to produce more precise structure determinations than is possible using proton NMR exclusively. Isotope-enriched protein samples are a prerequisite for carrying out many modern protein NMR experiments, including multidimensional heteronuclear NMR (Clore and Gronenborn, 1991,1994), isotope-edited studies of macromolecular complexes (Fesik et al., 1990,1991; Ikura et al., 1992; Qian et al., 1993; Clore and Gronenborn, 1994), ^{15}N-relaxation studies of internal motions in proteins (Kay et al., 1989; Peng and Wagner, 1992), measurements of conformation-dependent coupling constants (Montelione and Wagner, 1989; Montelione et al., 1989, 1992a; Griesinger and Eggenberger, 1992; Grzesiek and

*To whom correspondence should be addressed.

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; Gdn-HCl, guanidinium hydrochloride; IAA, β-indole acrylic acid; IGF-II, insulin-like growth factor II; PBS, phosphate-buffered saline; PDMS, plasma desorption mass spectrometry; PFPA, pentafluoro propionic acid; RP-HPLC, reversed-phase high performance liquid chromatography; Z, IgG-binding protein domain derived from staphylococcal protein A.

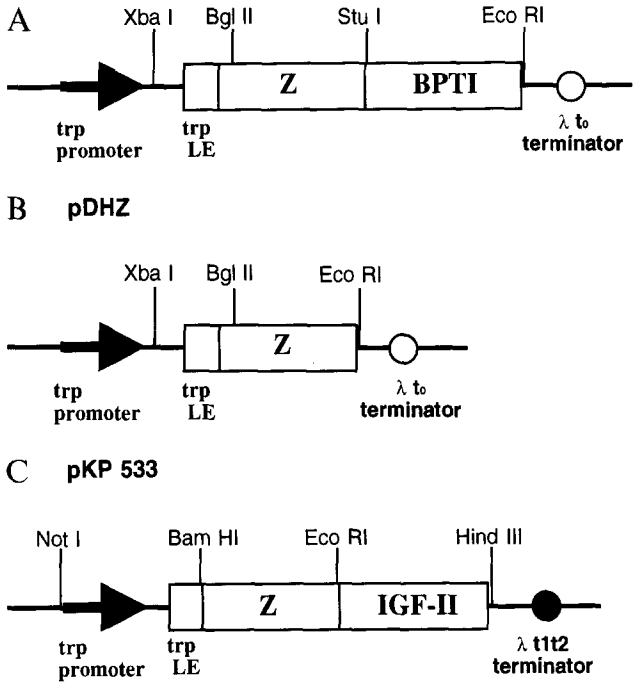

Fig. 1. Structural genes of the plasmids used for production of isotope-enriched recombinant Z proteins under control of the *trp* promoter. The following abbreviations are used in the figure: trp LE = the portion of the structural gene encoding a peptide sequence from the TrpLE gene product of *E. coli*; λ t_0 terminator = the t_0 transcriptional terminator derived from bacteriophage λ; and λ t1t2 terminator = the t1t2 transcriptional terminator derived from bacteriophage λ. Origins and features of the production plasmid vectors are described in the Materials and Methods section. (A) The expression cassette in plasmid pHAZY:BPTI used for producing TrpLE-Z-BPTI. (B) The expression cassette in plasmid pDHZ used for producing Z. (C) The expression cassette in plasmid pKP 533 used for producing TrpLE-Z-IGF-II.

Bax, 1993; Vuister et al., 1993), and triple-resonance experiments for determining sequence-specific resonance assignments (Montelione and Wagner, 1989, 1990; Ikura et al., 1990; Clore and Gronenborn, 1991,1994; Boucher et al., 1992; Logan et al., 1992; Montelione et al., 1992b; Clowes et al., 1993; Grzesiek and Bax, 1993; Lyons et al., 1993; Brutscher et al., 1994; Logan et al., 1994). The resulting ^{13}C resonance assignments are useful for characterizing secondary backbone structure (Spera and Bax, 1991; Wishart et al., 1991) and for protein structure refinement (de Dios et al., 1993; Laws et al., 1993). Multidimensional NMR experiments using ^{15}N and/or ^{13}C frequency labeling also provide crucial techniques for resolving complex NOESY spectra (Kay et al., 1990; Clore and Gronenborn, 1991,1994; Muhandiram et al., 1993) and for identifying bound water molecules in proteins (Clore et al., 1990; Kriwacki et al., 1993).

While significant progress has been made in recent years in developing multidimensional NMR experiments that exploit heteronuclear scalar coupling interactions and frequency labeling, practical applications of these techniques are limited by the requirement to first produce and purify large amounts (5–50 mg) of isotopically enriched protein. The main obstacle for isotope-enriched protein production in most recombinant production systems in general use today is the high cost of the labeled media components, e.g., ^{13}C-glucose, ^{13}C-acetate, isotope-enriched amino acids or hydrolysates of isotope-enriched yeast and algae, limiting possibilities for upscaling to controlled multi-liter fermenters. The less well controlled conditions of shaker flask cultivations, combined with the requirement to use fully ^{15}N- and/or ^{13}C-enriched media components, often results in low protein production levels. Reduced yields in defined minimal media are sometimes observed even for systems that are highly efficient under optimal fermenter conditions. The production of ^{15}N-,^{13}C-enriched proteins therefore requires an efficient system, able to provide high-level production of the desired protein using isotopically enriched minimal growth media in small-scale bioreactors.

In this paper we describe a bacterial production system for ^{15}N-,^{13}C-enriched recombinant proteins, which is extensively used in our, as well as many other, laboratories. The system is based on intracellular production of the recombinant protein in *Escherichia coli* as a fusion to an IgG-binding domain analogue, Z, derived from staphylococcal protein A (Nilsson et al., 1987; Altman et al., 1991). In this production system, the product gene is fused at the 3' end of the gene encoding Z and transcription is initiated from the efficient promoter of the *E. coli trp* operon (Yansura, 1990). This allows for high-level intracellular production of fusion protein, which can be purified by IgG affinity chromatography (for reviews, see Nilsson and Abrahmsén (1990) and Nilsson et al. (1991)). Using this approach we have achieved high-level (40–200 mg/l) production in defined minimal media of three isotope-enriched proteins: the Z-domain, Z-insulin-like growth factor II (Z-IGF-II), and Z-bovine pancreatic trypsin inhibitor (Z-BPTI). The Z-domain is a small, proteolytically stable protein (Nilsson and Abrahmsén, 1990) of 72 amino acid residues, without disulfide bonds. Z exhibits high solubility in aqueous buffers and can be reversibly unfolded (Samuelsson et al., 1994). IGF-II contains 67 amino acid residues, is extremely unstable in *E. coli* (Hammarberg et al., 1989,1991), and is very hard to refold with high yields (Yamashiro and Li, 1985). BPTI is a well-studied protein of 58 amino acid residues comprising three disulfide bonds. In spite of its high conformational stability (Makhatadze et al., 1993), the protein has been hard to produce efficiently in *E. coli* (Altman et al., 1991).

Several recombinant systems for the production of isotope-enriched proteins have been described previously, including prokaryotic systems in *E. coli* (Venters et al., 1991; Abeygunawardana et al., 1993; Reilly and Fairbrother, 1994) and eukaryotic systems based on Chinese hamster ovary cells (Hansen et al., 1992; Archer et al.,

1993). The fusion system presented in this paper provides another robust system for production of isotopically enriched proteins. In general, different recombinant proteins behave differently in the various production systems available for isotope enrichment, and it is important that a set of such systems is available to the NMR community. The major advantage of the Z fusion system described here is the high production level obtained. The system also provides a simple approach for purification of the resulting fusion protein using IgG affinity chromatography, and supports the production of a correct and native N-terminus by the choice of an appropriate cleavage method.

Materials and Methods

Materials

$(^{15}NH_4)_2SO_4$ [99% ^{15}N] and $^{13}C_6$-glucose [99% ^{13}C] were products of Cambridge Isotope Laboratories (Woburn, MA) or Isotec, Inc. (Miamisburg, OH). The antibiotics ampicillin, tetracycline and kanamycine were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade.

Strains and plasmids

E. coli strains RV308 (Maurer et al., 1980), RV308 *ompT* (L. Isaksson, personal communication), and MM294 (Neidhart, 1987) were used as bacterial hosts for DNA constructions and protein production. Z-BPTI was produced using the vector pHAZY:BPTI (Altman et al., 1991; see Fig. 1A), modified to provide a clean chymotryptic cleavage site at the N-terminus of the BPTI sequence. For the production of Z, the vector pDHZ (Cedergren et al., 1993; see Fig. 1B) was used. Both pDHZ and pHAZY:BPTI are based on pBR322 (reviewed in Balbás et al. (1986)) and encode ampicillin- and tetracycline-resistant phenotypes in *E. coli*. For the production of Z-IGF-II, the vector pKP533 (Fig. 1C) was used. This vector has a pUC-based origin of replication with a high copy number (Balbás et al., 1986), and kanamycine resistance as its phenotypic selectable marker. In these three similar production vectors, transcription is initiated from derivatives of the efficient *E. coli trp* promoter (Yansura, 1990).

Production and purification of Z-IGF-II

A culture of *E. coli* RV308 *ompT* containing plasmid pKP533 was grown in a totally defined medium using $^{13}C_6$-glucose as the only carbon source and $(^{15}NH_4)_2SO_4$ as the sole nitrogen source. The growth medium utilized, 1×MJ, is a variant of a previously published defined minimal medium (Lundström et al., 1990). The medium contained: 2.5 g/l $(^{15}NH_4)_2SO_4$, 9 g/l KH_2PO_4, 6 g/l K_2HPO_4, 0.5 g/l sodium citrate, and 1 g/l $MgSO_4$. The pH was adjusted to 6.6 before autoclaving, and the $MgSO_4$ was autoclaved separately. Subsequently, 1 ml trace element solution (see Table 1, solution A), 0.65 ml vitamin solution (see Table 1, solution B) and 0.07 g of thiamine were added. After cooling to room temperature, 2 to 5 g sterile-filtered $^{13}C_6$-glucose (amounts according to optimization) dissolved in water and 60 mg/l kanamycine were added. The citrate is used as a metal chelator and is known not to be transported into *E. coli* (Neidhart, 1987).

Starter cultures were inoculated from frozen cultures, stored at −80 °C, and grown overnight at 30 °C in 2×YT medium (Miller, 1972) supplemented with 60 mg/l of kanamycine. A 50 ml preculture of 1×MJ containing the enriched precursor components was inoculated with 1 ml of the culture from the 2×YT medium and allowed to grow overnight before using the complete preculture to inoculate the final 1 l growth volume. Cultures of 500 ml were grown in 2 l baffled shaker flasks at 33 °C for 24–26 h with 300 rpm aeration.

The purification was performed using IgG affinity chromatography as the first step. Harvested cells were resuspended in 6 M Gdn-HCl, pH 6.5, at ambient temperature and were shaken for 4 h in order to disrupt the cell membranes and dissolve inclusion bodies. The Gdn-HCl cell suspension was diluted to a Gdn-HCl concentration of 1 M, using 1×TST (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and centrifuged before passing the supernatant over an IgG-Sepharose-6FF affinity chromatography column (Pharmacia Biotech, Uppsala, Sweden), 5×8 cm (id×h), at a flow rate of 300 ml/h. The column was washed with 10 column volumes of 1×TST buffer to remove unspecifically bound proteins. Then the

TABLE 1
TRACE ELEMENTS AND VITAMIN SOLUTIONS FOR 1×MJ MEDIUM

Component	Amount
Trace elements: Solution A (autoclaved)	
$FeCl_3 \times 6H_2O$	16.2 g
$ZnSO_4 \times 7H_2O$	2.4 g
$CoCl_2 \times 6H_2O$	4.2 g
$Na_2MoO_4 \times 2H_2O$	4.2 g
$CuSO_4 \times 5H_2O$	4.8 g
H_3BO_3	1.2 g
$MnSO_4$	3.0 g
HCl	30 ml
H_2O	570 ml
Vitamins: Solution B (adjusted to pH 7.2, sterile filtered)	
Pantothenic acid, calcium salt	0.4 g
Choline chloride	0.4 g
Folic acid	0.4 g
Myo-inositol	0.8 g
Nicotinamide	0.4 g
Pyridoxal hydrochloride	0.4 g
Riboflavin	0.04 g
Thiamine hydrochloride	0.4 g
H_2O	800 ml

pH was lowered and the buffer capacity was decreased by washing the column with 2 column volumes of 5 mM ammonium acetate, pH 5. Finally, fusion protein was eluted by 0.2 M acetic acid, pH 2.8, and lyophilized.

Lyophilized Z-IGF-II fusion protein was redissolved at a protein concentration of 0.1 mg/ml in buffer A (0.1 M acetic acid, 0.12 M Tris, 2 M Gdn-HCl, 10% ethanol, pH 7.5) supplemented with 30 mM reduced DTT. After a 1 h incubation at 37 °C, the DTT was removed by desalting the protein on a Sephadex G25 column (Pharmacia Biotech) equilibrated in buffer A. After desalting, the pH was adjusted to 8.3 using solid Tris base, followed by a 16 h air oxidation performed in a 5 l Erlenmeyer flask with continuous mixing by a magnetic stirrer. Subsequent to the oxidation step, the pH was adjusted to 1.6 using concentrated HCl. The acidified protein solution was then desalted into solution C (0.1 M HCl and 10% ethanol, pH 1.6) and lyophilized to reduce the volume before CNBr cleavage. The cleavage was performed at room temperature in solution C and at a final CNBr concentration of 150 mM. The reaction was quenched after 3 h by rapid freezing and residual CNBr and cyanide were removed by lyophilization.

The isotope-enriched native IGF-II was separated from the remaining Z fusion partner by again using IgG affinity chromatography. The lyophilized cleavage mixture was dissolved in 0.2 M acetic acid, pH 2.8. The solution was adjusted to pH 8.5 prior to the IgG affinity step, using NaOH and solid Tris base. Final purification of IGF-II was performed by reversed phase HPLC on a 4.6×250 mm Kromasil C_8 column (Eka Nobel, Surte, Sweden), using a linear gradient from 35 to 40% acetonitrile in 0.25% PFPA over 25 min, at a flow rate of 1 ml/min.

Production and purification of Z

Uniformly ^{15}N- and ^{15}N-,^{13}C-enriched Z were produced using an approach similar to that employed in the Z-IGF-II production. A similar two-step inoculum procedure was used to grow the *E. coli* strain RV308, transformed with production plasmid pDHZ (Fig. 1B). Cell cultures were grown as described for the Z-IGF-II production, except that 15 mg/l tetracycline was used as the selective agent. Induction was performed by addition of 25 mg/l β-indole acrylic acid at mid logarithmic growth phase (OD ≈ 0.5–1 at 600 nm). Cells were grown for an additional 12 h and then harvested by centrifugation at 4000×g for 10 min. The cell pellet was resuspended in 1×TST and subsequently disrupted by sonication for 5×30 s using a Sonifer Cell Disruptor B15 (Branson, St. Louis, MO) equipped with a microtip. After centrifugation at 10 000×g for 10 min, the supernatant was passed over an IgG Sepharose-6 FF column equilibrated with TST. The column was washed with 10 column volumes of TST and 2 column volumes of 5 mM ammonium acetate, pH 5.5. The protein was then eluted with 0.3 M acetic acid, titrated to pH 3.2 using ammonium acetate.

Production and purification of Z-BPTI

Uniformly ^{15}N-enriched BPTI and [C30V,C51A]-BPTI were produced as Z-BPTI fusion proteins. *E. coli* strains RV308 were transformed with production plasmid pHAZY:BPTI (Fig. 1A) containing the BPTI gene (Hurle et al., 1990; Altman et al., 1991) and were grown overnight in 10 ml 2×YT medium. Cells were harvested by centrifugation and used to inoculate 1×MJ minimal media containing 15 mg/l tetracycline, 1 ml/l trace element solution A, and 1 ml/l vitamin solution B, using 250 ml cultivations in 1 l baffled shaker flasks. Cells were

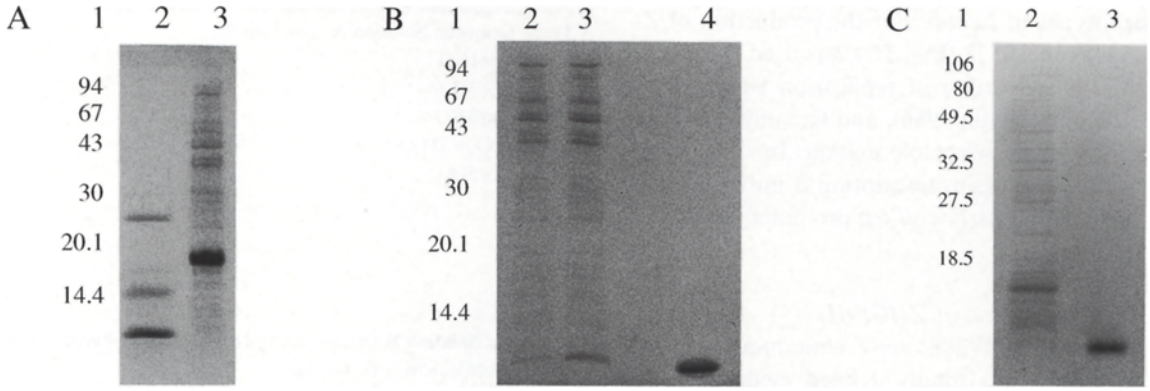

Fig. 2. SDS-PAGE analysis of isotope-enriched recombinant proteins. Marker proteins are shown with their respective sizes as molecular weight × 10^{-3}. (A) Z-IGF-II production. Lane 1: sizes of position of marker proteins (data not shown); lane 2: a marker consisting of ZZ-insulin-like growth factor I cleaved with hydroxylamine (Moks et al., 1987), where the bands correspond to ZZ-IGF-I (22.1 kDa), ZZ (14.5 kDa) and IGF-I (7.6 kDa); lane 3: crude extract of the production of ^{13}C-,^{15}N-Z-IGF-II, where the major protein band corresponds to the product. (B) Z-production before and after induction. Lane 1: sizes of molecular weight marker proteins (data not shown); lane 2: crude extract of ^{13}C-,^{15}N-Z before induction; lane 3: crude extract of ^{13}C-,^{15}N-Z after induction; lane 4: purified ^{13}C-,^{15}N-Z. (C) Z-BPTI production. Lane 1: sizes of position of molecular weight marker proteins (data not shown); lane 2: whole cell lysate showing high-level expression of 15 kDa Z-BPTI fusion protein; lane 3: purified ^{15}N-enriched BPTI.

grown at 37 °C using 3 g/l of glucose. The protein production was induced by adding 20 mg/l of β-indole acrylic acid at mid log phase, $OD_{600} = 1.0$.

The purification of Z-BPTI and Z-[C30V,C51A]-BPTI fusion proteins was carried out using a scheme derived from the one described by Altman et al. (1991). Cells from 250 ml flask cultivations were harvested 6–7 h after induction by centrifugation at 5000 × g for 30 min. The cells were lysed by resuspension in 25 ml 6 M Gdn-HCl in Pi buffer (50 mM KH_2PO_4, 150 mM NaCl, 1 mM EDTA, pH 6.5), and by sonication. The sonicated cell suspension was then diluted to 150 ml with ice-cold Pi buffer. The cell debris and precipitations were removed by centrifugation at 20 000 × g. Next, the supernatant was loaded directly onto a 10 ml (bed volume) IgG-Sepharose column, pre-equilibrated at 4 °C with 1 × TST buffer. The column was washed with 10 column volumes of TST buffer, followed by 2 column volumes of 5 mM ammonium acetate at pH 4.8. Finally, the Z-BPTI (or Z-[C30V, C51A]-BPTI) fusion protein was eluted with 0.5 M acetic acid at pH 3.3.

The purified fusion protein was refolded at pH 8.7 using a mixture of reduced and oxidized glutathione, as described by Altman et al. (1991). The refolding solution was then applied to a 10 ml (bed volume) chymotrypsin affinity column, equilibrated in TEA buffer (100 mM triethanolamine, 300 mM NaCl, 10 mM $CaCl_2$, pH 7.8). The immobilized chymotrypsin acts both to cleave the fusion protein at the junction between the Z and BPTI moieties and to bind to the BPTI protein, thereby facilitating the purification. Incorrectly folded BPTI molecules are also eliminated in this step. The mature, folded molecules were eluted from this column using 0.5 M acetic acid at pH 2.5, and were further purified by ion exchange chromatography on a MonoS HR 10/10 column (Pharmacia, Uppsala, Sweden) using a linear gradient of 0.5 to 5 M ammonium acetate at pH 4.0 (Hurle et al., 1990). Under these conditions, the Z-BPTI fusions elute in approximately 3 M ammonium acetate.

Protein analysis

Amino acid compositions were determined by acid hydrolysis, followed by analysis using a Beckman 6300 amino acid analyzer equipped with a System Gold data handling system (Beckman, Fullerton, CA). Protein homogeneity was evaluated by SDS-PAGE (Laemmli, 1970) or by RP-HPLC.

Mass spectrometry

Molecular masses of IGF-II and BPTI were determined using a ^{252}Cf plasma desorption mass spectrometer (PDMS), Bio-Ion 20 (Applied Biosystems, Foster City, CA). The proteins were bound to a nitrocellulose-coated foil, dried by spinning the foil and analyzed in the positive ion mode at an acceleration voltage of 18 kV (Forsberg et al., 1990). The molecular mass of Z was determined using a JEOL SX 102 mass spectrometer equipped with electrospray. The errors in the measured masses by both techniques are estimated to be less than 0.1%.

NMR spectroscopy

Samples for NMR spectroscopy were prepared in aqueous solutions of 0.5–2 mM protein concentration. NMR spectra were obtained with a Varian Unity 500 spectrometer.

Results

Basic concept

A general system was designed for high-level production of uniformly ^{15}N- and/or ^{13}C-enriched recombinant proteins in *E. coli*. Utilizing the efficient *E. coli trp* promoter for initiating transcription (Yansura, 1990; Altman et al., 1991), three similar Z production vectors were used to produce isotopically enriched IGF-II, BPTI and Z itself. In this system, the protein product of interest is produced as a fusion to Z, a single IgG-binding domain based on staphylococcal protein A. By utilizing a fusion protein approach, we retain the efficient *E. coli trp* LE (Yansura, 1990) translation initiation sequence (Fig. 1) in all our constructs, increasing the probability to retain high-level protein production from the transcribed mRNA of the hybrid system. It is well established that fusion to the TrpLE leader peptide also enhances the probability to form inclusion body precipitates of the product by decreasing its solubility (Yansura, 1990), allowing high-level production with minimal proteolytic degradation. However, the major influence in forming inclusion bodies is associated with the high production level from the TrpLE-Z expression vectors (Altman et al., 1991) and the solubility properties of the fused product. Indeed, two of the three examples below, TrpLE-Z-IGF-II and TrpLE-Z-BPTI, form intracellular inclusion bodies in *E. coli* and one, TrpLE-Z, does not, even though production levels are rather similar. The latter can be explained by the extremely high solubility of Z itself at neutral pH (Samuelsson et al., 1994), compensating for the inherent insolubility of the small TrpLE portion.

In order to fully exploit the general advantages described for the production system, an initial screening of some basic growth parameters must be performed. We have found the growth parameters to be product dependent, and a screening of basic parameters was performed for each of the proteins produced. The growth medium 1 × MJ is based on a medium originally developed for controlled growth reactor conditions (Lundström et al., 1990). This minimal medium is of unusual complexity compared with standard minimal media, e.g. M9. The advantage of using such a medium is that the additive of vitamins and trace elements allows cell growth in the

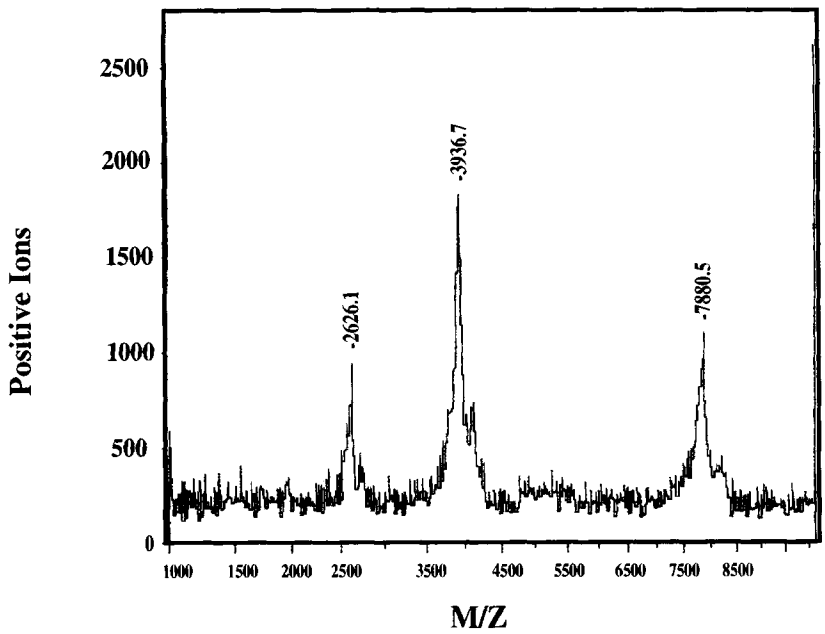

Fig. 3. Plasma desorption mass spectrometry (PDMS) analysis of ^{15}N-,^{13}C-IGF-II as described in the Materials and Methods section. From right to left, the three peaks correspond to the 1+, 2+ and 3+ ions of IGF-II, respectively. The measured molecular mass (7880.5) corresponds to >99% ^{15}N,^{13}C enrichment (see Table 2).

absence of undefined carbon-containing supplements such as yeast extract. The production system was initially optimized for high-level production with minimal glucose utilization. The parameters evaluated in each optimization were host strain, glucose usage, temperature, and aeration; where appropriate promoter induction parameters were evaluated as well. Here, we describe the optimization performed for the isotopic enrichment of Z-IGF-II in more detail. The production of the other two isotopically enriched protein products was optimized similarly.

Production of isotope-enriched IGF-II

For the production of Z-IGF-II, the optimization was based both on monitoring inclusion body formation by microscopy and on SDS-PAGE analysis of crude cell lysates (Fig. 2). Glucose concentrations were varied between 2–10 g/l. Levels of remaining glucose in the growth media were tested by glucose-sensitive BM-Glycemie test strips (Boehringer, Mannheim, Germany). Under pre-optimized growth conditions, glucose utilization is maximized at a concentration of 4 g/l, as defined by all detectable glucose being used up and no significant increase of cell density being observed on increasing the glucose content of the media. Higher glucose levels result in the presence of residual glucose in the media and production of undesired acid metabolites in the completed cultivation as detected by a lowered pH (data not shown). The temperature dependence on inclusion body formation was evaluated over the temperature range 30–37 °C. Early appearance of inclusion body formation seems to result in hampered growth rates. Formation of inclusion bodies was monitored using phase contrast microscopy (Zeiss, Oberkochen, Germany) at 1000× magnification. A compromise between a reasonable growth rate and efficient inclusion body formation was found at 33 °C. The *trp* promoter is poorly repressed in the vector-strain combination utilized to produce Z-IGF-II fusion protein, most likely due to the high copy number of the vector (data not shown). The constitutive expression of the fusion protein combined with the minimal media conditions results in long cell doubling times and relatively low cell densities. Maximum cell densities of about 2 (OD_{600} in a 1 cm cuvette) were reached after as long as 24–26 h of incubation.

TABLE 2
LEVELS OF ISOTOPE ENRICHMENT AS DETERMINED BY MASS SPECTROMETRY

Protein	Theoretical molecular mass (Da)		Experimental molecular mass (Da)	
	Unenriched protein	Isotope-enriched protein	Measured by mass spectrometry	Total isotope enrichment (%)
^{15}N-,^{13}C-IGF-II	7475.4	7880.4	7880.5	>99
^{15}N-,^{13}C-Z	8209.1	8668.7	8660.0	>99
^{15}N-Z	8209.1	8310.8	8313.0	>99
^{15}N-BPTI	6513.0	6595.0	6596.7	>99

The TrpLE-Z-IGF-II fusion protein is produced at very high levels (Fig. 2A), mainly in the form of inclusion bodies. *E. coli* RV308 *ompT*, lacking the outer membrane-located OmpT protease (Grodberg and Dunn, 1988), was used for the production. Earlier results concerning expression of unenriched recombinant IGF-II in *E. coli* have shown the advantages of using an OmpT protease negative strain for secreted ZZ-IGF-II (Hammarberg et al., 1991). Even though the intracellular fusion protein approach used in this work results in the formation of insoluble inclusion bodies, giving potential protection against proteolytical degradation, we found that the recovery of native IGF-II increased significantly in the RV308 *ompT* strain compared to RV308 (data not shown), which is consistent with findings showing degradation of recombinant γ-interferon by the OmpT protease during recovery, using intracellular production in *E. coli* (Sugimura and Higashi, 1988).

Production levels are expressed as milligram fusion protein per liter of growth medium (mg/l) that was recovered after the initial IgG affinity chromatography step. IGF-II was produced both as uniformly ^{15}N and ^{15}N,^{13}C doubly enriched protein. The production level of ^{15}N-enriched TrpLE-Z-IGF-II was 200 mg/l, which is similar to the production using corresponding unenriched medium (data not shown), and the production level of ^{15}N-, ^{13}C-enriched TrpLE-Z-IGF-II was 90 mg/l.

The protein purity of the ^{15}N-,^{13}C-IGF-II preparation after refolding, CNBr cleavage, and final purification was estimated by reversed phase HPLC analysis to be at least 995%. The total yield at this homogeneity was 1.4 mg/l fermentation, as determined by quantitative amino acid analysis. This indicates a relatively low recovery (i.e. ~3%) of the ^{15}N-,^{13}C-IGF-II purified at fusion protein level. This low yield is primarily attributable to the well-known difficulties in refolding and purifying the hydrophobic IGF-II molecule (Yamashiro and Li, 1985; Hammarberg et al., 1991), rather than the enrichment procedure per se. Mass spectrometric analysis (Fig. 3) confirmed >99% total isotopic enrichment in this ^{15}N-,^{13}C-IGF-II sample (Table 2).

Production of isotope-enriched Z

^{15}N- and ^{15}N-,^{13}C-enriched Z domain was produced in a shaker flask under conditions similar to those used to produce Z-IGF-II. Also for this protein product, the optimization was performed to minimize the amount of glucose necessary, and at 4 g/l the cell density was found to be maximum, no glucose was detectable upon harvest and the pH remained at 7. The effective one-step purification scheme results in material that is >95% homogeneous on SDS-PAGE (Fig. 2B), with >99% total isotopic enrichment (Table 2). The yields of purified ^{15}N- and ^{13}C-, ^{15}N-Z were approximately 40 mg/l growth volume, as determined by quantitative amino acid analysis and by absorbance measurements. The overall recovery yield of Z was estimated to be 90–95% of the protein present in the cells when harvested.

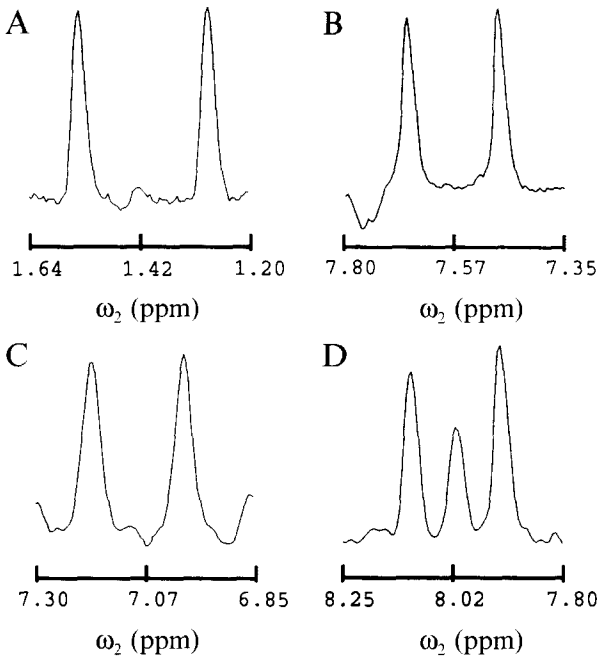

Fig. 4. Cross sections along ω_2 through NOESY and TOCSY cross peaks of isotope-enriched proteins, showing heteronuclear coupling and providing site-specific information about levels of isotopic enrichment. (A) Cross section through a NOESY cross peak of ^{13}C-,^{15}N-enriched Z at the ω_1 frequency of the Ala^{56} H^{β} resonance. The sample was dissolved at a protein concentration of 2.5 mM in 90% H_2O, 10% D_2O at pH 6.5 and 30 °C. The NOESY mixing time was 200 ms with broadband ^{13}C and ^{15}N decoupling in the ω_1 dimension. No heteronuclear decoupling was used in the ω_2 dimension. This cross peak exhibits one large ^{13}C-H^{β} splitting for the 99% of molecules that are isotope enriched in ^{13}C at this site. (B) Cross peak from the same NOESY spectrum of ^{13}C-,^{15}N-enriched Z at the ω_2 frequency of the H^N resonance of Ala^{46}. This cross peak exhibits one large ^{15}N-H^N splitting for the 99% of molecules that are isotope enriched in ^{15}N at this site. (C) Cross section through a NOESY cross peak of ^{15}N-enriched Z at the ω_2 frequency of the Ala^{56} H^N resonance. The sample was dissolved at a protein concentration of 3.0 mM in 90% H_2O, 10% D_2O at pH 6.5 and 30 °C. The NOESY mixing time was 200 ms with broadband ^{15}N decoupling in the ω_1 dimension and no heteronuclear decoupling in the ω_2 dimension. This cross peak exhibits one large ^{15}N-H^N splitting for the 99% of molecules that are isotope enriched in ^{15}N at this site. (D) Cross section through a TOCSY cross peak of ^{15}N-enriched [C30V,C51A]-BPTI at the ω_2 frequency of the Thr^{32} H^N resonance. This sample was produced using a minimal medium different from that described in the text, which included 0.8 g/l yeast extract as a vitamin source. The protein concentration was 1.0 mM in 90% H_2O, 10% D_2O at pH 6.5 and 30 °C. The proton TOCSY mixing time was 45 ms with broadband ^{15}N decoupling in the ω_1 dimension and no heteronuclear decoupling in the ω_2 dimension. This cross peak exhibits one large ^{15}N-H^N splitting for the 80% of molecules that are isotope enriched in ^{15}N, and a central singlet peak for the 20% of molecules containing ^{14}N at this site. Different residues exhibit different apparent degrees of ^{15}N enrichment in this experiment, ranging between 60–80%, which is consistent with the more precise and accurate value of 65–69% determined by mass spectrometry (data not shown).

Production of isotope-enriched BPTI

^{15}N-enriched BPTI was produced using MJ media. The result of SDS-PAGE analysis of a purified isotope-enriched sample is shown in Fig. 2C. The yields of fusion protein using this production system were 70–100 mg/l of fermentation media, representing approximately 95% of the BPTI fusion protein present in the cells when harvested. The final yield of purified ^{15}N-enriched BPTI was 10–20 mg/l. Mass spectrometry analysis shows that the BPTI has >99% ^{15}N enrichment (Table 2). Similarly, the same method was used to enrich BPTI and Z-[C30V, C51A]-BPTI with ^{13}C and ^{15}N using 1 g/l $^{15}NH_4Cl$ and 3 g/l of ^{13}C-glucose (data not shown).

Analysis of isotope-enriched proteins by ω_1 heteronuclear-decoupled NOESY and by heteronuclear 2D NMR experiments

Isotope enrichment was verified for certain samples using 2D proton NOESY or TOCSY experiments, modified to provide broadband decoupling of ^{13}C and ^{15}N in the ω_1 dimension but not in the ω_2 dimension. The resulting NOESY (or TOCSY) cross peaks exhibit large one-bond heteronuclear splittings in the ω_2 dimension for the ensemble of proton spins directly connected to ^{15}N or ^{13}C and a central multiplet arising from the ensemble of protons not directly coupled to a heteronucleus. Cross sections along ω_2 through these NOESY cross peaks therefore provide estimates of the degree of isotopic enrichment of the heteroatom that is covalently bound to the directly observed proton. Examples of such measurements are shown in Fig. 4. Representative 2D NMR spectra, including ^{15}N-HSQC, ^{13}C-HSQC, and triple-resonance HCC(CO)NH-TOCSY, of these isotope-enriched proteins are presented in Fig. 5.

Discussion

Different recombinant proteins behave differently in the various protein production systems currently available, and no single production system is best for every protein. Rather, the modern protein NMR spectroscopist requires a battery of protein expression systems from which to choose in evaluating the most efficient system for any particular protein production problem. In this paper we have shown successful high-level production of uniformly isotope-enriched Z fusion proteins. The highly buffered, totally defined growth medium described here (i.e. MJ medium) allows cell growth for prolonged times, providing means of producing relatively high cell densities under the strained conditions imposed by minimal media. Both the induceable (for TrpLE-Z and TrpLE-Z-BPTI production) and the constitutive (for TrpLE-Z-IGF-II production) variants of the *trp* promoter-based expression vectors used in this study show consistent high protein production under defined media conditions. Although several other systems have been described for production of isotope-enriched proteins in *E. coli* (e.g. those described by Venters et al. (1991), Abeygunawardana et al. (1993) and Reilly and Fairbrother (1994)), the Z fusion production system is competitive with these and is one of the first tested for each new recombinant protein studied in our laboratories. Recently, this Z fusion technology has been extended to enrich BPTI mutant proteins, human insulin-like growth factor I, the Kunitz inhibitor domain of the human Alzheimer protease precursor protein (APP-KI), the albumin-binding domain of streptococcal protein G, and human α-lactalbumin with ^{15}N and ^{13}C isotopes (data not shown). The Z fusion production system and MJ growth medium together provide a general and robust system for the production of isotope-enriched recombinant proteins.

The disadvantage of Z fusions, as with any other fusion protein production technology, is the necessity to cleave the fusion product in order to release the product of interest. Even though this must be taken into consideration when selecting which production system to use for a given protein product, we do not feel that this is a major drawback. There are several readily available and well-documented cleavage methods of both chemical and enzymatic nature (for a review see Nilsson et al. (1992)). In this paper we have used cleavages with cyanogenbromide to release IGF-II and chymotrypsin to release BPTI. Both methods are simple, work with high efficiency and produce chemically homogeneous protein products.

A fusion protein approach provides many potential advantages for the expression and purification of recombinant proteins (for a review see Nilsson et al. (1992)). Even though other fusion systems may be useful for isotope enrichment of proteins for NMR analysis, these may not behave as well as the Z fusion system under defined media conditions. The Z domain, besides functioning as a handle for affinity chromatography, also serves to increase the solubility of the fusion partner during the refolding process (Samuelsson et al., 1994). The Z domain itself is extremely stable and soluble and does not form visible inclusion bodies, even at the high expression levels described here. The solubilizing action of the Z fusion partner is crucial in the IgG purification step, since both IGF-II and BPTI are not very soluble under reduced conditions. The fusion protein approach, besides being a valuable purification and analytical tool, thereby also facilitates folding of the enriched protein.

The multiple step inoculum procedure described in the Methods section is important to achieve reproducible high levels of isotope incorporation, since the isotope enrichment efficiency can drop significantly through carry-over effects from unenriched components present in starting cultures. This potential problem is overcome in our protocol by growing the seed culture on enriched media components. A first culture in unenriched media allows cell

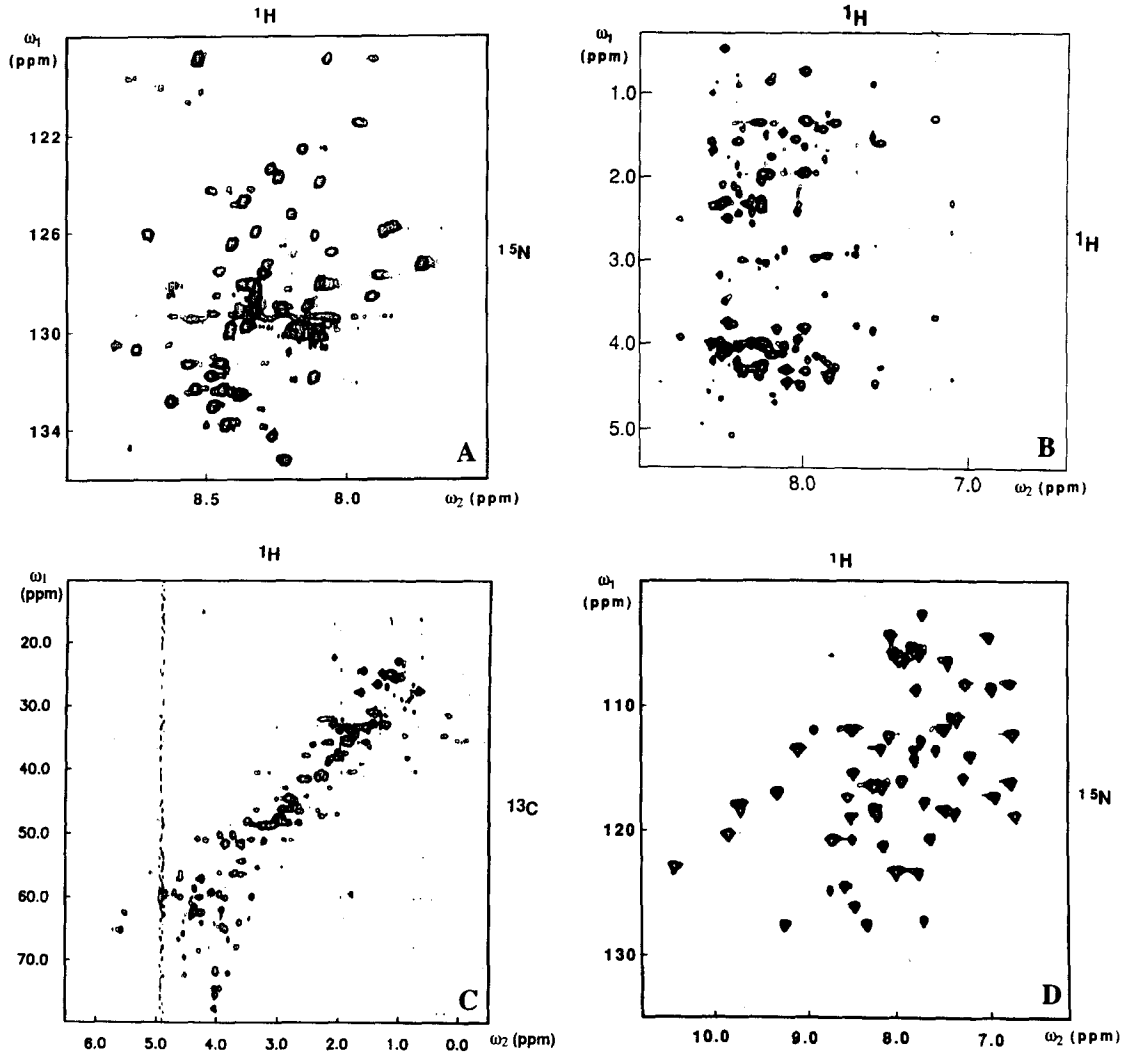

Fig. 5. Heteronuclear NMR spectra of isotope-enriched proteins. (A) ^{15}N-HSQC spectrum of 100% ^{15}N-,^{13}C-enriched IGF-II recorded at 0.5 mM protein concentration in a buffer containing 90% H_2O, 10% D_2O, 20 mM KH_2PO_4, 50 mM NaCl, 0.1 mM EDTA and 0.5 mM NaN_3 at pH 2.5 and a temperature of 55 °C. (B) HCC(CO)NH-TOCSY spectrum (Montelione et al., 1992b) of 100% ^{15}N-,^{13}C-enriched Z at 2 mM protein concentration in a buffer containing 90% H_2O, 10% D_2O, 5 mM KH_2PO_4 and 0.5 mM NaN_3 at pH 6.5 and a temperature of 30 °C. (C) ^{13}C-HSQC spectrum of ^{15}N-,^{13}C-enriched BPTI at 1 mM protein concentration in D_2O, pH 6.6, and a temperature of 10 °C. (D) ^{15}N-HSQC spectrum of 100% ^{15}N-enriched BPTI recorded at 1 mM protein concentration in a solvent containing 90% H_2O and 10% D_2O at pH 6.0 and a temperature of 36 °C.

growth without imposing the growth limitations of a defined minimal medium. Then, these cells are used to inoculate a second culture in enriched media. This second culture is used both to adapt the *E. coli* cells to minimal media conditions and to exclude carry over of unenriched nutrient components. The second culture is then grown and used to inoculate the large fermentation in MJ minimal medium. Isotopic enrichment exceeding 99% and relatively high protein production levels were achieved for Z-IGF-II, Z-BPTI, and Z itself using this procedure. An alternative inoculation strategy using a complex isotope-enriched medium (Celltone®) for the starting culture has been recently described by Reilly and Fairbrother (1994). Our two-step inoculation method has the advantage of adapting the culture to minimal medium conditions prior to large-scale fermentation, precluding the requirement for isotope-enriched complex media like Celltone.

The production level of ^{15}N-labeled Z-IGF-II was equal to that obtained for unenriched protein. In the case of ^{15}N-,^{13}C-labeled protein roughly a 50% decrease in production was observed. Even though the effect was reproducible, we do not believe that this is a ^{13}C isotope effect. Instead, the quality of ^{13}C-glucose batches seems to vary and this may show up as an apparent isotope effect. Indeed, similar yields were obtained for ^{15}N-,^{13}C- and ^{15}N-enriched Z.

The purification of IGF-II results in relatively low yields compared to the Z and BPTI purifications. We believe that these low yields reflect the inherent difficulties in refolding and purifying IGF-II and are not a property of the enrichment procedure itself. In fact, our yields of recovery of correctly folded IGF-II (~3%) are significantly higher than what was obtained in the refolding and purification of chemically synthesized IGF-II (Yamashiro

and Li, 1985). The final recovery of the much more soluble Z protein, lacking internal disulfides, is extremely high (~ 40 mg/l) in this system. While 10–20 mg/l final yields of disulfide-intact BPTI were obtained, 50–75% of the potentially recoverable mass of BPTI present in these purified fusion protein samples was lost in the refolding, fusion cleavage, and purification steps. These yields were achieved only by avoiding lyophilization of the fusion protein subsequent to IgG-Sepharose purification.

Mass spectrometry provides a rapid and accurate analysis of total isotope incorporation (Fig. 3). Since protein mass spectrometry analysis requires only small amounts of sample, it is highly recommended as a standard analytical procedure in the production of isotope-enriched proteins. The ω_1 heteronuclear decoupled NOESY and TOCSY experiments also provide useful tools for estimating isotope enrichment levels, with an accuracy of about ± 10%. These experiments have the advantage of measuring isotope enrichment at specific sites in the protein, and of determining ^{13}C- and ^{15}N-enrichment levels independently in partially ^{15}N-,^{13}C-enriched samples, which cannot be done easily by mass spectrometry. However, it is generally difficult to detect less than about 5–10% of unenriched sites in these 2D NMR spectra.

Conclusions

In summary, this paper describes Z fusion production systems and biochemical protocols useful for isotopic enrichment of proteins for NMR studies. We, and other laboratories, are already utilizing these production systems and protein samples in NMR studies of protein structure and dynamics and in NMR pulse sequence development (see for example Montelione et al. (1992b) and Lyons et al. (1993)). The Z fusion system is another high-level production system, which should be included in the set of production systems tested by spectroscopists when considering biosynthetic enrichment of a new protein product for NMR studies. The optimized systems described here provide reliable, high-level production of isotope-enriched fusion proteins. They can be used to produce 10–40 mg/l of isotope-enriched Z and BPTI proteins, and are available from the laboratories of the corresponding authors for producing samples useful for NMR methods development and biophysical studies.

Acknowledgements

This project has been supported by National Science Foundation Grants MCB-9407569 to G.T.M. and MCB-9018707 to S.A., an NSF Young Investigator Award to G.T.M., National Institutes of Health Grants RO1-GM47014 to G.T.M. and RO1-AG10462 to S.A., and by Pharmacia Biopharmaceuticals, Sweden. L.J. is supported by a research grant from NUTEK, Sweden. We are most grateful to H. Wadensten for invaluable help and expertise on the purification and HPLC analysis of IGF-II, to Maria-Jose Castro for providing data on the production and purification of isotope-enriched BPTI, and to T. Clemente for production of the ^{15}N-enriched Z samples. We also thank Drs. A. Arseniev, B. Celda, and Z. Shang for helpful comments on the manuscript. G. Palm and K. Zachrisson are acknowledged for performing the amino acid composition analysis, and G. Forsberg and S. Hober for assisting the PDMS analysis on IGF-II.

References

Abeygunawardana, C., Weber, D.J., Frick, D.N., Bessman, M.J. and Mildvan, A.S. (1993) *Biochemistry*, **32**, 13071–13080.

Altman, J.D., Henner, D., Nilsson, B., Anderson, S. and Kuntz, I.D. (1991) *Protein Eng.*, **4**, 593–600.

Archer, S.J., Bax, A., Roberts, A.B., Sporn, M.B., Ogawa, Y., Piez, K.A., Waetherbee, J.A., Tsang, M.L.-S., Lucas, R., Zheng, B.-L., Wenker, J. and Torchia, D.A. (1993) *Biochemistry*, **32**, 1152–1163.

Balbás, P., Soberón, X., Merino, E., Zurita, M., Lomeli, H., Valle, F., Flores, N. and Bolivar, F. (1986) *Gene*, **50**, 3–40.

Boucher, W., Laue, E.D., Campbell-Burk, S.L. and Domaille, P.J. (1992) *J. Am. Chem. Soc.*, **114**, 2262–2264.

Brutscher, B., Simorre, J.-P., Caffrey, M.S. and Marion, D. (1994) *J. Magn. Reson. Ser. B*, **105**, 77–82.

Cedergren, L., Andersson, R., Jansson, B., Uhlen, M. and Nilsson, B. (1993) *Protein Eng.*, **6**, 441–448.

Clore, G.M., Bax, A., Wingfield, P.T. and Gronenborn, A.M. (1990) *Biochemistry*, **29**, 5671–5676.

Clore, G.M. and Gronenborn, A.M. (1991) *Science*, **252**, 1390–1399.

Clore, G.M. and Gronenborn, A.M. (1994) *Protein Sci.*, **3**, 372–390.

Clowes, R.T., Boucher, W., Hardman, C.H., Domaille, P.J. and Laue, E.D. (1993) *J. Biomol. NMR*, **3**, 349–354.

de Dios, A.C., Pearson, J.G. and Oldfield, E. (1993) *Science*, **260**, 1491–1496.

Fesik, S.W., Gampe, R.T., Holzman, T.F., Egan, D.A., Edalji, R., Luly, J.R., Simmer, R., Helfrich, R., Kishore, V. and Rich, D.H. (1990) *Science*, **250**, 1406–1409.

Fesik, S.W., Gampe, R.T., Eaton, H.L., Gemmecker, G., Olejniczak, E.T., Neri, P., Holzman, T.F., Egan, D.A., Edalji, R., Simmer, R., Helfrich, R., Hochlowski, J. and Jackson, M. (1991) *Biochemistry*, **30**, 6574–6583.

Forsberg, G., Palm, G., Ekebacke, A., Josephson, S. and Hartmanis, M. (1990) *Biochem. J.*, **271**, 357–363.

Griesinger, C. and Eggenberger, U. (1992) *J. Magn. Reson.*, **97**, 426–434.

Grodberg, J. and Dunn, J.J. (1988) *J. Bacteriol.*, **170**, 1245–1253.

Grzesiek, S. and Bax, A. (1993) *Acc. Chem. Res.*, **26**, 131–138.

Hammarberg, B., Nygren, P.Å., Holmgren, E., Elmblad, A., Tally, M., Hellman, U., Moks, T. and Uhlén, M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4367–4371.

Hammarberg, B., Tally, M., Samuelsson, E., Wadensten, H., Holmgren, E., Hartmanis, M., Hall, K., Uhlén, M. and Moks, T. (1991) *J. Biol. Chem.*, **266**, 11058–11062.

Hansen, A.P., Petros, A.M., Mazar, A.P., Pederson, T.M., Rueter, A. and Fesik, S.W. (1992) *Biochemistry*, **31**, 12713–12718.

Hurle, M.R., Marks, C.B., Kosen, P.A., Anderson, S.A. and Kuntz, I.D. (1990) *Biochemistry*, **29**, 4410–4419.

Ikura, M., Kay, L.E. and Bax, A. (1990) *Biochemistry*, **29**, 4659–4667.

Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B. and Bax, A. (1992) *Science*, **256**, 632–637.

Kay, L.E., Torchia, D.A. and Bax, A. (1989) *Biochemistry*, **28**, 8972–8979.

Kay, L.E., Clore, G.M., Bax, A. and Gronenborn, A.M. (1990) *Science*, **243**, 411–414.

Kriwacki, R.W., Hill, R.B., Flanagan, J.M., Caradonna, J.P. and Prestegard, J.H. (1993) *J. Am. Chem. Soc.*, **115**, 8907–8911.

Laemmli, U.K. (1970) *Nature*, **227**, 680–685.

Laws, D.D., de Dios, A.C. and Oldfield, E. (1993) *J. Biomol. NMR*, **3**, 607–612.

Logan, T.M., Olejniczak, E.T., Xu, R.X. and Fesik, S.W. (1992) *FEBS Lett.*, **314**, 413–418.

Logan, T.M., Thériault, Y. and Fesik, S.W. (1994) *J. Mol. Biol.*, **236**, 637–648.

Lundström, H., Brobjer, M., Österlöf, B. and Moks, T. (1990) *Biotechnol. Bioeng.*, **36**, 1056–1062.

Lyons, B.A., Tashiro, M., Cedergren, L., Nilsson, B. and Montelione, G.T. (1993) *Biochemistry*, **32**, 7839–7845.

Makhatadze, G.I., Kim, K.-S., Woodward, C. and Privalov, P. (1993) *Protein Sci.*, **2**, 2028–2036.

Maurer, R., Meyer, B.J. and Ptashne, M. (1980) *J. Mol. Biol.*, **139**, 147–161.

Miller, J.H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Moks, T., Abrahmsén, L., Holmgren, E., Bilich, M., Olsson, A., Uhlén, M., Pohl, G., Sterky, C., Hultberg, H., Josephson, S., Holmberg, A., Jörnvall, H. and Nilsson, B. (1987) *Biochemistry*, **26**, 5239–5244.

Montelione, G.T. and Wagner, G. (1989) *J. Am. Chem. Soc.*, **111**, 5474–5475.

Montelione, G.T., Winkler, M.E., Rauenbuehler, P. and Wagner, G. (1989) *J. Magn. Reson.*, **82**, 198–204.

Montelione, G.T. and Wagner, G. (1990) *J. Magn. Reson.*, **83**, 183–188.

Montelione, G.T., Emerson, S.D. and Lyons, B.A. (1992a) *Biopolymers*, **32**, 327–334.

Montelione, G.T., Lyons, B.A., Emerson, S.D. and Tashiro, M. (1992b) *J. Am. Chem. Soc.*, **114**, 10974–10975.

Muhandiram, D.R., Xu, G.Y. and Kay, L.E. (1993) *J. Biomol. NMR*, **3**, 463–470.

Neidhart, F.C. (1987) *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, American Society for Microbiology, Washington, DC.

Nilsson, B., Moks, T., Jansson, B., Abrahmsén, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T.A. and Uhlén, M. (1987) *Protein Eng.*, **1**, 107–113.

Nilsson, B. and Abrahmsén, L. (1990) *Methods Enzymol.*, **185**, 144–161.

Nilsson, B., Forsberg, G. and Hartmanis, M. (1991) *Methods Enzymol.*, **198**, 3–16.

Nilsson, B., Forsberg, G., Moks, T., Hartmanis, M. and Uhlén, M. (1992) *Curr. Opin. Struct. Biol.*, **2**, 569–575.

Peng, J.W. and Wagner, G. (1992) *Biochemistry*, **31**, 8573–8586.

Qian, Y.Q., Otting, G., Billeter, M., Müller, M., Gehring, W.J. and Wüthrich, K. (1993) *J. Mol. Biol.*, **234**, 1070–1083.

Reilly, D. and Fairbrother, W.J. (1994) *J. Biomol. NMR*, **4**, 459–462.

Samuelsson, E., Moks, T., Nilsson, B. and Uhlén, M. (1994) *Biochemistry*, **33**, 4207–4211.

Spera, S. and Bax, A. (1991) *J. Am. Chem. Soc.*, **113**, 5490–5492.

Sugimura, K. and Higashi, N. (1988) *J. Bacteriol.*, **170**, 3650–3654.

Venters, R.A., Calderone, T.L., Spicer, L.D. and Fierke, C.A. (1991) *Biochemistry*, **30**, 4491–4494.

Vuister, G.W., Wang, A.C. and Bax, A. (1993) *J. Am. Chem. Soc.*, **115**, 5334–5335.

Wagner, G. (1993) *J. Biomol. NMR*, **3**, 375–385.

Wishart, D.S., Sykes, B.D. and Richards, F.M. (1991) *J. Mol. Biol.*, **222**, 311–333.

Yamashiro, D. and Li, C.H. (1985) *Int. J. Pept. Protein Res.*, **26**, 299–304.

Yansura, D.B. (1990) *Methods Enzymol.*, **185**, 161–166.