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Periplasmic production via the pET expression system of soluble, bioactive human growth hormone

Jonathan T. Sockolosky, Francis C. Szoka *

Pharmaceutical Sciences and Pharmacogenomics Graduate Program, Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, 513 Parnassus Ave., Box 0912, San Francisco, CA 94143, USA

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ABSTRACT

A pET based expression system for the production of recombinant human growth hormone (hGH) directed to the *Escherichia coli* periplasmic space was developed. The pET22b plasmid was used as a template for creating vectors that encode hGH fused to either a pelB or ompA secretion signal under control of the strong bacteriophage T7 promoter. The pelB- and ompA-hGH constructs expressed in BL21 (λDE3)-RIPL *E. coli* are secreted into the periplasm which facilitates isolation of soluble hGH by selective disruption of the outer membrane. A carboxy-terminal poly-histidine tag enabled purification by Ni^{2+} affinity chromatography with an average yield of 1.4 mg/L culture of purified hGH, independent of secretion signal. Purified pelB- and ompA-hGH are monomeric based on size exclusion chromatography with an intact mass corresponding to mature hGH indicating proper cleavage of the signal peptide and folding in the periplasm. Both pelB- and ompA-hGH bind the hGH receptor with high affinity and potently stimulate Nb2 cell growth. These results demonstrate that the pET expression system is suitable for the rapid and simple isolation of bioactive, soluble hGH from *E. coli*.

Introduction

Genetic engineering has enabled the production of numerous recombinant proteins in large quantities in effect transforming the biotechnology industry by enabling the cost effective manufacture of human proteins [1–3]. The pET expression system pioneered by Studier and Moffat [4] and commercialized by Novagen is one of the most widely used systems for recombinant protein production in *Escherichia coli*. The multitude of commercially available vectors, *E. coli* strains, and related products enables expression and purification of a wide variety of foreign proteins. Of particular interest for the expression of disulfide bonded proteins is a family of pET vectors containing the N-terminal pelB secretion signal, which directs synthesized polypeptides to the *E. coli* periplasm [5]. Disulfide oxidoreductases and isomerases located in the *E. coli* periplasm catalyze the formation of disulfide bonds enabling the accumulation of properly folded, soluble protein making the periplasm an ideal compartment for expression of certain therapeutic proteins [6].

Human growth hormone (hGH) is a 191 amino acid, disulfide-linked, pituitary-derived protein that regulates a number of metabolic processes involved in growth and development [7]. *E. coli* derived recombinant hGH is approved for the treatment of multiple human diseases and new indications, treatment modalities, and novel delivery systems represent an active area of research in growth hormone based therapy [8–15]. The lack of glycosylation makes *E. coli* an ideal host for hGH production, which can be achieved in the cytoplasm [16–19] or periplasm [20–26]. Over-expression of hGH in the cytoplasm results in the formation of insoluble aggregates, or inclusion bodies. The isolation of hGH from inclusion bodies requires a re-folding step to obtain soluble protein that adds process complexity and contributes to reduced yields. Recombinant hGH directed to the periplasm can be easily isolated in its native state by selective disruption of the *E. coli* outer membrane resulting in a reduction in processing steps, complexity, and time.

There are a number of reports on the periplasmic expression of recombinant human growth hormone [20–26]; however, each uses custom prepared vectors, a range of *E. coli* strains, expression conditions, and purification schemes making replication difficult for the academic laboratory. We describe simple methods for the expression, purification, and characterization of recombinant hGH produced at the shake flask level using the pET expression system. All components necessary for cloning, expression, and purification are commercially available. This process results in an average yield of 1.4 mg/L culture of purified protein from ~10 to 15 g wet cells. The recombinant hGH isolated using this system is soluble, monomeric, binds the hGH receptor with high affinity,

* Corresponding author. Address: School of Pharmacy, University of California, 513 Parnassus Ave., Box 0912, San Francisco, CA 94143, USA.
E-mail addresses: jonathan.sockolosky@ucsf.edu (J.T. Sockolosky), szoka@cgl.ucsf.edu (F.C. Szoka).

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and potently stimulates cell growth comparable to pharmaceutical grade hGH. Thus, the pET expression system provides a rapid and economical method for production of recombinant human growth hormone in *E. coli*.

Materials and methods

Materials

Ampicillin, Terrific Broth (TB), 3,3′,5,5′-tetramethylbenzidine (TMB), and all buffer salts were purchased from Sigma–Aldrich (St. Louis, MO). Nickel Sepharose high performance resin prepacked in 5 mL HiTrap columns (HisTrap FF), PD-10 desalting columns, and Superdex 75 size exclusion chromatography (SEC) column were purchased from GE Healthcare (Piscataway, NJ). Complete EDTA-free protease inhibitor cocktail tablets and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Roche Diagnostics (Indianapolis, IN). Steri-cup 0.45 μm vacuum filters and Amicon 10 kDa MWCO spin filters were from Millipore (Billerica, MA). TEV-TROPIN (Teva Pharmaceuticals; North Wales, PA) was obtained from the UCSF pharmacy. Anti-human growth hormone antibodies and human growth hormone receptor Fc fusion (hGHR-Fc) were purchased from R&D systems (Minneapolis, MN). The bacterial expression vector pET22b was purchased from Novagen (San Diego, CA). All restriction enzymes and buffers used for cloning were purchased from New England Biolabs (Beverly, MA) and all primers were purchased from IDT (San Diego, CA).

E. coli expression vectors

Human growth hormone cDNA was purchased from OpenBiosystems (Huntsville, AL) and maintained in the vector pCR4-TOPO. The gene encoding hGH was PCR amplified from pCR4-TOPO with primers designed to incorporate 5′ NcoI and 3′ XhoI restriction sites. All PCR reactions were performed with Phusion DNA polymerase (New England BioLabs) as follows: 98 °C 1 min; 98 °C 15 s, 69 °C 30 s, 72 °C 15 s, repeat for 35 cycles; 72 °C 10 min, 4 °C hold. The resulting PCR product was purified and restriction cloned into the NcoI and XhoI sites of the bacterial expression vector pET22b (Novagen; San Diego, CA). The resulting vector, pET22b_pelB_hGH, encodes hGH containing a N-terminal pelB leader sequence to enable periplasmic secretion via the Sec translocation machinery and a C-terminal poly-histidine tag for purification by immobilized metal affinity chromatography (IMAC). The corresponding vector, pET22b_ompA_hGH was generated by PCR amplification of the gene encoding hGH from pCR4-TOPO with primers designed to incorporate a 5′ NdeI site followed by the ompA signal sequence and a 3′ XhoI site. The resulting PCR product was restriction cloned into the NdeI and XhoI sites of pET22b. The resulting vector encodes hGH containing a N-terminal ompA leader sequence to enable periplasmic secretion and a C-terminal poly-histidine tag for purification. All plasmids were confirmed by DNA sequencing. A complete list of primers used in this study is provided in Table 1.

Protein expression and purification

Expression of hGH was carried out in BL21-Codon Plus (λDE3)-RIPL *E. coli* cells (Stratagene; La Jolla, CA) harboring the pET22b-hGH vectors described above. A single colony was selected and cultured overnight at 37 °C in 10 mL of terrific broth (TB) containing 100 μg/mL ampicillin. The 10 mL overnight *E. coli* culture was used to inoculate a 1 L culture of TB containing 100 μg/mL ampicillin. Cells were cultured at 37 °C until OD_{600} = 0.7–0.9. Protein expression was induced by addition of 0.1 mM IPTG and cultured for an additional 16–18 h at 25 °C. Cells were harvested by centrifugation (7650 g for 30 min) and the periplasmic *E. coli* fraction was extracted via osmotic shock as previously described [27,28]. Briefly, harvested cells were suspended in a hypertonic solution of 30 mM Tris, 20% w/v sucrose, 1 mM ETDA, pH 8 (25 mL) and incubated for 30 min at 4 °C. Cells were centrifuged and the supernatant collected. Cells were re-suspended in a hypotonic solution of 5 mM $MgSO_4$ (25 mL) and incubated for 30 min at 4 °C followed by an additional centrifugation. The supernatant from the hypotonic solution was combined with the supernatant from the hypertonic solution, centrifuged to remove debris, and dialyzed against D-PBS overnight at 4 °C.

The periplasmic solution containing soluble hGH was clarified over a 0.45 μm filter and purified by Ni^{2+} affinity chromatography as follows. A 5 mL HisTrap FF column charged with Ni^{2+} was equilibrated with 20 mM Tris, 300 mM NaCl, 40 mM imidazole, pH 8. The clarified osmotic shock fluid was loaded onto the HisTrap FF column and washed with equilibration buffer for eight column volumes (CV). Bound protein was eluted with three CVs of 20 mM Tris, 300 mM NaCl, 500 mM imidazole, pH 8. Fractions with an absorbance at 280 nm greater than 0.05 were pooled, concentrated and buffer exchanged into D-PBS, and subject to analysis by reducing and non-reducing SDS–PAGE. Protein concentrations were measured based on absorbance at 280 nm assuming an extinction coefficient of 17670 M^{-1} m^{-1} predicted based on the mature hGH amino acid sequence using ExPASy ProtParam tool (www.expasy.org). The hGHs characterized in this study contain a carboxy terminal poly-histidine tag with the exception of TEV-TROPIN. The amino acid sequence of the tag appended to the carboxy-terminus of hGH is LVPRGSLEHHHHHH.

Matrix-assisted laser desorption and ionization (MALDI)-time of flight (TOF) mass spectrometry

The intact mass of purified hGH was determined by MALDI-TOF mass spectrometry. Purified hGHs and TEV-TROPIN were desalted using C_4 ZipTips (Millipore; Billerica, MA) per the manufactures recommended protocol and eluted with 4 μL of 75% acetonitrile, 0.1% TFA in water. Desalted proteins (1 μL) were mixed with 1 μL of a saturated solution of sinapinic acid (SA) and spotted on top of a pre-formed layer of SA matrix. Mass spectra were obtained on a Microflex LT mass spectrometer (Bruker Daltonics; Billerica, MA) operated in linear, positive mode at a laser frequency of 60 Hz (100 shots total). The spectra were calibrated using the

Table 1
Primers used in this study.

Vector	Primer pair	Restriction site
pET22b-pelB hGH	F = 5′-GATGGCCATGGGCTTCCCAACCATTCCCTTATC-3′	NcoI
	R = 5′-GGTGCTCGAGGCTGCCGCGCGGCACCAGGAAGCCACAGCTGCCCTC-3′	XhoI
pET22b-ompA hGH	F = 5′-CATACATATGAAAAAAACCGCGATTGCGATTGCGGTGGCGTTAGCGGGCT TTGCGACCGTGGCGCAGGCGTTCCCAACCATTCCCTTATC-3′	NdeI
	R = 5′-GGTGCTCGAGGCTGCCGCGCGGCACCAGGAAGCCACAGCTGCCCTC-3′	XhoI