Expression, secretion and folding of human growth hormone in *Escherichia coli*

Purification and characterization

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An efficient secretion vector containing a gene coding for an *E. coli* signal peptide fused to human growth hormone (hGH) was cloned into *E. coli*. The recombinant fusion protein was expressed and correctly processed hGH was secreted into the periplasmic space at a yield of 10–15 μg hGH/A_{600}. Purification of hGH from the periplasmic fraction by anion exchange and size exclusion gave hGH of greater than 90% purity. Characterization by SDS-PAGE, amino terminal analysis, trypsin mapping, and circular dichroism demonstrated that the fusion protein was correctly processed to authentic hGH and that the *E. coli* periplasm provided an appropriate environment for proper folding of hGH and disulfide bond formation.

(Human) Growth hormone Peptide mapping Amino terminal analysis Recombinant protein Protein purification HPLC

1. INTRODUCTION

Escherichia coli has been used to achieve high-level expression of many recombinant proteins. In most cases, the protein is synthesized and remains in the cytoplasm from which it must be isolated and purified. However, there are certain disadvantages to the cytoplasmic production of proteins; high-level expression often leads to insoluble protein aggregates which may be difficult to solubilize; a refolding step is frequently required to obtain the native conformation and to form correct disulfide bonds; the protein of interest often contains an N-terminal methionine.

Inouye and his co-workers [1,2] have developed a secretion cloning vector by inserting the *E. coli ompA* signal peptide into the high-level expression vector pIN-III [1]. This secretion cloning vector has been used to efficiently express *E. coli* β-lactamase [1] and staphylococcal nuclease A [2]. The two proteins were processed correctly and secreted into the *E. coli* periplasm.

We report here the high-level secretion of human growth hormone (hGH) into the *E. coli* periplasm using a secretion vector with the *ompA* signal peptide gene. The secreted hGH has been purified from the periplasmic fraction and analyzed by a number of assays. By peptide mapping, we show that the secreted hGH has the correct primary structure and the correct disulfide bonds. Sequence analysis has revealed the correct amino-terminal sequence demonstrating correct processing of the precursor and the secondary structure of the secreted hGH was found to be indistinguishable from authentic hGH by circular dichroism.

2. EXPERIMENTAL

2.1. Bacteria strain and media

The host strain used for all the recombinant plasmids was *E. coli* K-12 RV308 [*su*$^-$, Δ*lac*X74, *gal* ISII::OP308, *rps*L]. Cells were grown on TY broth (Difco) with ampicillin (50 μg/ml).

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2.2. *Large-scale preparation of periplasmic fraction for hGH isolation*

The periplasmic fraction used as the starting material for the hGH purification was prepared from *E. coli* K-12 RV308 harboring the pOmpA-hGH2 vector. Cells were grown in TY medium at 37°C to a cell density of approx. $A_{600} = 1.6$ and the periplasmic fraction was prepared by an osmotic shock procedure as described [3].

2.3. *Peptide mapping*

Protein samples (1–2 mg/ml) were digested with trypsin (TPCK-trypsin, Cooper Biomedical) at 37°C at an enzyme:substrate weight ratio of 1:25 for 6 h. The resulting tryptic peptides were separated by reversed-phase HPLC by the method described in the legend to fig.2.

2.4. *Standards*

The hGH used as a standard for SDS-PAGE and other studies was biosynthetic hGH produced by recombinant DNA techniques at Eli Lilly. The hGH standard has been extensively characterized and is indistinguishable from pituitary hGH.

2.5. *Amino-terminal sequence analysis*

Automated sequence analysis was performed on a Beckman 890D sequencer. The phenylthiohydantoin derivatives were separated and identified by reversed-phase HPLC (Ultrasphere-ODS, 0.46× 25 cm, Beckman) using published methods [4].

2.6. *Protein and growth hormone assays*

Total protein was measured using the BCA protein assay following the manufacturers recommended protocol [5].

hGH was quantitated using a Mono Q HR 5/5 column (Pharmacia) and appropriate concentrations of standard hGH to construct a standard curve.

3. RESULTS

3.1. *Expression and secretion of hGH*

A secretion vector, pOmpA-hGH2, containing the gene for hGH fused to a gene coding for the signal peptide of the *E. coli ompA* protein, was constructed from the plasmid pIN-III-ompA3 [1] as described [3]. *E. coli* cells harboring this secretion vector produced mature, correctly processed hGH (fig.1, lane 2) while cells harboring a second plasmid pOmpA-hGH1, with the hGH gene inserted in an incorrect reading frame, produced no hGH (fig.1, lane 3). The amount of hGH present in total cell lysates of *E. coli* cells harboring the pOmpA-hGH2 plasmid was estimated by densitometric scanning of an SDS-polyacrylamide gel to be approx. 6% of the total cellular protein.

3.2. *Purification of secreted hGH*

hGH was purified from the periplasmic fraction

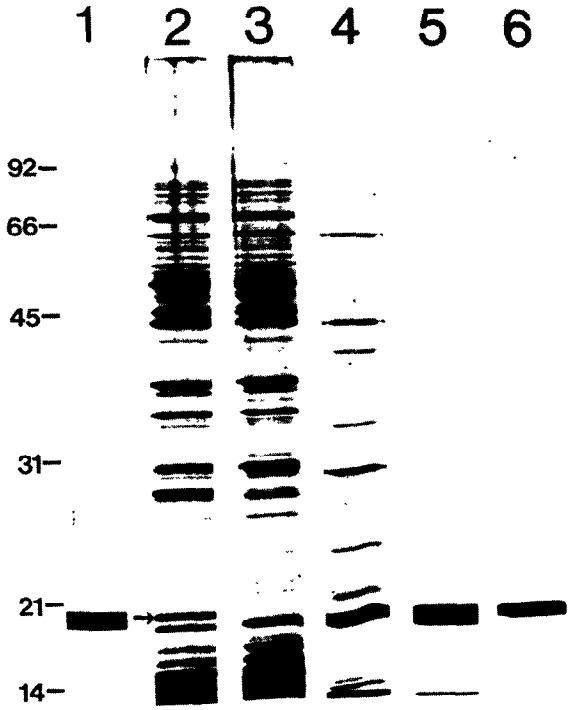


Fig.1. Analysis of hGH purification by SDS-PAGE. The purity of hGH after each purification step was assessed on a non-reducing SDS-polyacrylamide gel. The gel was 12% acrylamide and was run at 20 W constant power. Following electrophoresis the gel was stained with Coomassie blue R-250. Lanes: 1, hGH standard; 2, pOmpA-hGH2 total cell lysate; 3, pOmpA-hGH1 total cell lysate; 4, pOmpA-hGH2 periplasmic fraction (13 μg); 5, Q Sepharose Fast Flow pool (60 μg); 6, Sephacryl S-200 pool (25 μg). The total cell lysates were prepared by centrifuging an aliquot of the cell culture and adding SDS sample buffer to the cell pellet. An amount equivalent to approx. 0.05 A_{600} was loaded into each of lanes 3 and 4. Molecular mass markers are indicated and the arrow points to hGH in the total cell lysate.

prepared from *E. coli* cells harboring the pOmpA-hGH2 plasmid. The purification process, summarized in table 1, consists of two purification steps and one solvent-exchange step prior to lyophilization.

The periplasmic fraction (65 ml) was applied to a column (3.2 × 42 cm) of Q Sepharose Fast Flow (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 8. The bound proteins were eluted with a linear gradient of NaCl (0–0.3 M, 25 h) in the same buffer at a flow rate of 20 cm/h. Fractions containing hGH were localized by assaying on a Mono Q HR 5/5 (Pharmacia) column and those fractions were pooled for the next step. The starting material in the periplasmic fraction is estimated to be about 30% pure; after this initial step the purity has been increased to approx. 91%.

The pooled fractions from the preceding column were applied to a column of Sephacryl S-200 (Pharmacia). A 50 ml sample was applied to a column measuring 4.4 cm × 83 cm. The column was equilibrated and eluted with 50 mM Tris-HCl, pH 8, at a flow rate of 3.9 cm/h.

In some cases the hGH solution was solvent exchanged on a column of Sephadex G-25 (Pharmacia) which had been equilibrated with water adjusted to pH 8 by the addition of ammonium hydroxide. The same solvent was used to elute the hGH from the column. Following this solvent-exchange step the protein was lyophilized and stored as a dry powder.

Table 1

Purification of hGH from the periplasmic fraction of *E. coli* RV308/pOmpA-hGH2

Step	Protein (mg)	hGH (mg)	Specific activity (%)	Purification (-fold)	Yield (%)
Total cell lysate	ND	ND	6[a]	1	–
Periplasmic fraction	93.4	28.4	30.4	5	100
Q Sepharose Fast Flow	24.6	22.3	90.6	15	78
Sephacryl S-200	ND	20.2	>90[a]	>15	71

Human growth hormone was purified from the periplasmic fraction derived from 1.0 l of culture grown to a cell density of $A_{600} = 1.6$. ND, not determined.
[a]The specific activities of the total cell lysate and of the Sephacryl S-200 pool were estimated by densitometric scanning of an SDS-polyacrylamide gel

The purity of the hGH following each step of the purification process is shown in fig.1. If the starting material is considered to be the total cell lysate (fig.1, lane 2), a remarkable enrichment of hGH is obtained by the osmotic shock procedure and collection of the periplasmic fraction (fig.1, lane 4). The pool from the Q Sepharose Fast Flow column contains, in addition to hGH, a component with an apparent molecular mass of 24 kDa and a low molecular mass component. A few very faint bands were visible on the original gel with apparent molecular masses greater than ~40 kDa. After gel filtration on the Sephacryl S-200 column, only the hGH band and the 24 kDa band are visible on the SDS gel (fig.1, lane 6). The specific activity of hGH after this purification step was estimated to be greater than 90%.

3.3. *Formation of the correct disulfide bonds in the secreted protein*

The purified, secreted hGH was analyzed by a peptide mapping procedure to ascertain whether the correct disulfide bonds had been formed. hGH has two disulfide bonds, one between Cys-53 and Cys-165 and the other between Cys-182 and Cys-189. When native intact hGH is digested with trypsin at pH 8 and the resulting tryptic peptides are separated by reversed-phase HPLC (tryptic mapping), the two disulfide bond-containing peptides can be readily resolved and identified. These two disulfide peptides are labeled 6–16 and 20–21 (fig.2). The tryptic map of secreted hGH (fig.2) clearly shows that these two peptides are present and comparison of this tryptic map with that of standard hGH shows that secreted hGH is indistinguishable from standard hGH.

3.4. *Correct processing of the hGH precursor*

The initial product expressed by *E. coli* cells containing the secretion vector, pOmpA-hGH2, is a higher molecular mass precursor which accumulates in the cytoplasm (not shown). This precursor is hGH with the *ompA* signal peptide intact. The product which accumulates in the periplasm has an apparent molecular mass identical to that of standard hGH as determined by SDS-PAGE (fig.1, lanes 1 and 4). To prove that the hGH in the periplasm had been correctly processed to remove

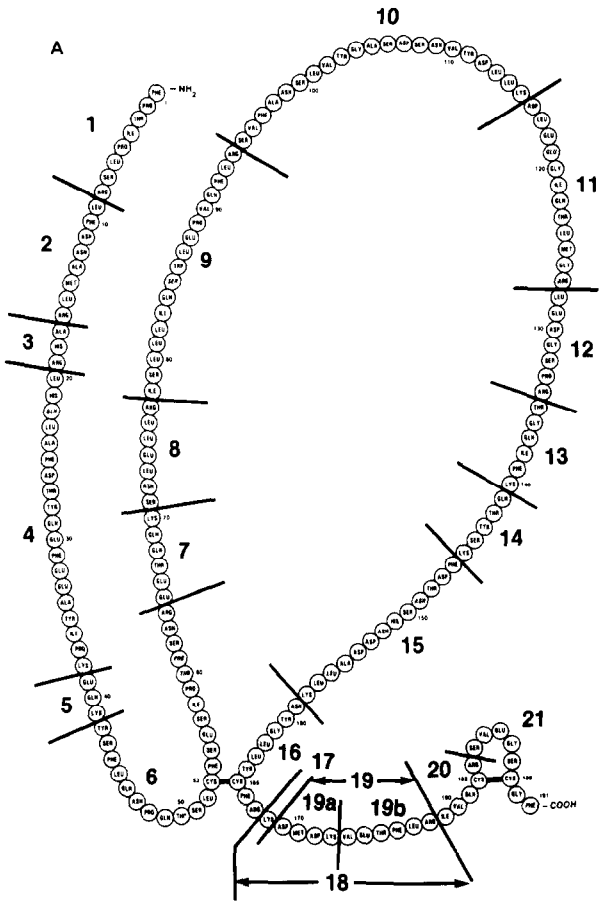
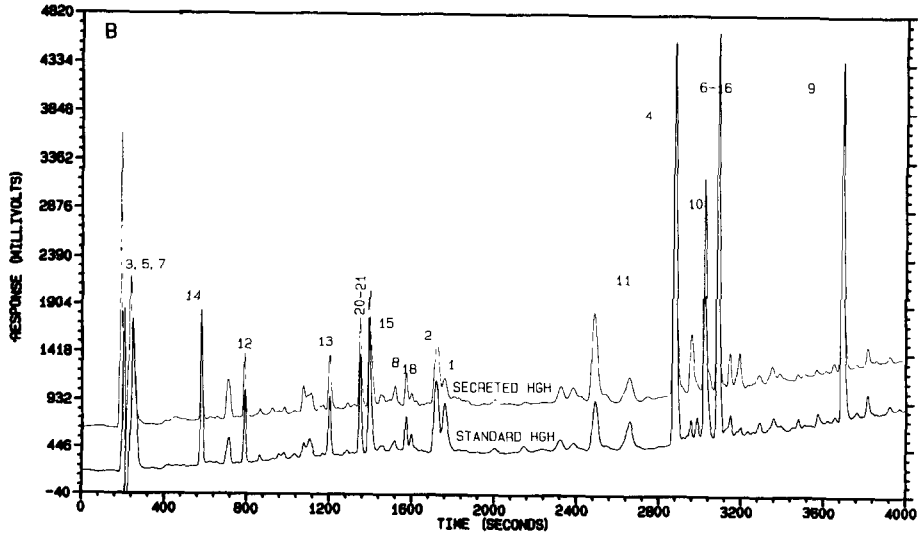


Fig.2. Tryptic maps comparing secreted hGH with hGH standard. Tryptic digests of secreted hGH and standard hGH were prepared as described in section 2. The tryptic peptides were separated by reversed-phase HPLC on an Aquapore' RP-300 column (0.45 × 25 cm, Brownlee Labs) using a gradient generated from two solvents: a, 0.1% trifluoroacetic acid in water; and b, 0.1% trifluoroacetic acid in acetonitrile. The gradient was 0–20% b in 20 min, 20–25% b in 20 min, and 25–50% b in 25 min. The flow rate was 1.0 ml/min. A 100 μl aliquot of each digest was injected onto the column and the elution of the peptides was monitored spectrophotometrically at 220 nm. Panel A shows the hGH primary structure and the tryptic peptides derived from this sequence [7]. Panel B shows the tryptic map of secreted hGH superimposed on the tryptic map of standard hGH. The numbers correspond to the numbered peptides shown in panel A. The identification of each peptide in the tryptic map of hGH is reported in [7].



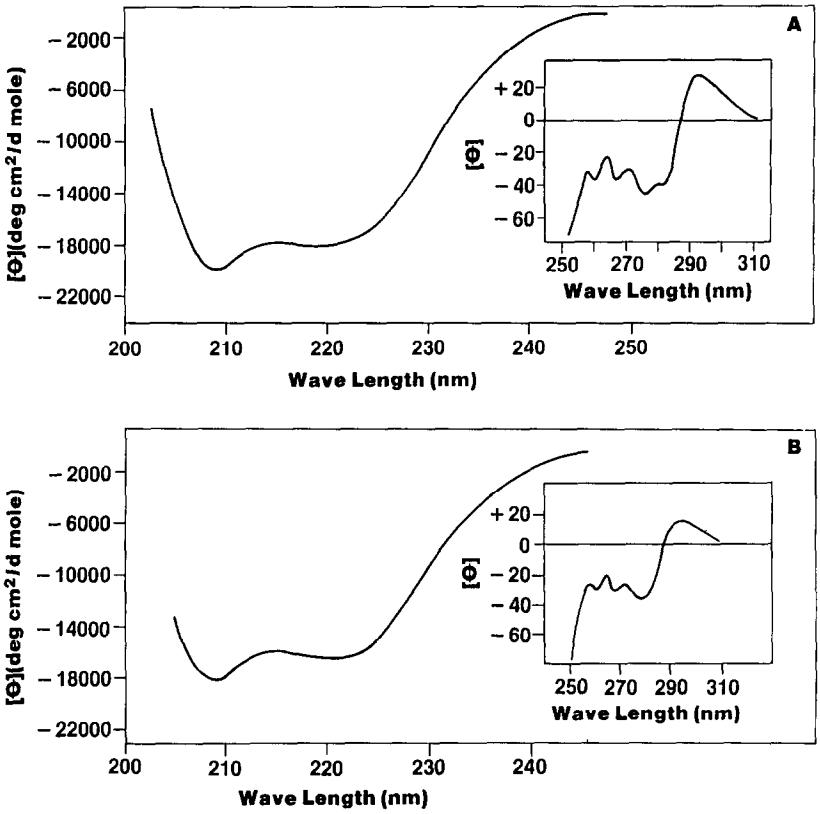


Fig.3. CD spectrum of secreted hGH. The CD spectrum of secreted hGH was scanned from 200 to 350 nm on a Cary 61 spectropolarimeter calibrated with d-10-camphorsulfonic acid. Panel A shows the CD spectrum of standard hGH and Panel B the CD spectrum of secreted hGH.

the signal peptide, the purified, secreted hGH was subjected to automated sequence analysis. The first four cycles of an Edman degradation gave the sequence Phe-Pro-Thr-Ile with Phe being the only amino acid detected at the amino-terminus. This sequence matches exactly the known sequence of hGH [6], thereby demonstrating that the *E. coli* cells correctly processed the precursor, removing the signal sequence, with the secretion of mature, natural sequence hGH into the periplasmic space.

3.5. *Secondary structure of secreted hGH*

To determine whether the conformation of secreted hGH was the same as standard hGH, circular dichroic spectra were obtained (fig.3). Overall the two spectra appeared to be identical each with a minimum at 208 nm and a shoulder at 222 nm, characteristic of a high α-helical content. In the near UV, the two spectra are also quite similar, with a small positive band in 290 nm range and weak negative dichroic bands in the 260–290 nm region. These two spectra are also identical to published CD spectra of pituitary hGH [7]. From these results we conclude that the conformation of the secreted hGH, isolated from the periplasmic space of *E. coli*, is indistinguishable from the conformation of either standard hGH or pituitary hGH.

4. DISCUSSION

The isolation and purification scheme reported here has potential advantages over an isolation from the cytoplasmic fraction from *E. coli*. When high-level expression of a recombinant protein is achieved in *E. coli* without taking advantage of the secretory mechanism of that cell, the protein is often found deposited within the cytoplasm as insoluble granules [8]. The initial isolation of the protein is aided by this phenomenon because the

granules can be easily collected by centrifugation after rupture of the cell. However, the isolated granules are not easily solubilized nor is the protein properly folded, necessitating somewhat rigorous conditions for dissolving the granule and a refolding step to obtain the protein of interest with full biological activity.

As we have shown in this report, high-level expression of hGH can be achieved using the secretion vector pOmpA-hGH2. Instead of being deposited in the cytoplasm as granules, however, the hGH is secreted across the inner membrane into the periplasmic space where it can be easily isolated by rupturing the outer membrane and collecting the supernatant following centrifugation. The hGH in this periplasmic fraction represents 30% of the total protein of the fraction (table 1). The signal peptide has been properly removed, the hGH has been correctly folded with the proper disulfide bonds, and the hGH can be easily purified.

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