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Characterisation of a secreted form of recombinant derived human growth hormone, expressed in *Escherichia coli* cells*

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Abstract: Recombinant DNA derived human growth hormone (rhGH), Genotropin®, has been expressed in *E. coli* cells as a pre-hormone, where the heat stable enterotoxin II signal peptide (STII) was linked to hGH to get secretion of the hormone to the periplasmatic space. The pre-hormone was efficiently cleaved during secretion, by an endogenous signal peptidase generating the correct N-terminal (Phe) end as shown by protein sequence analysis. The purity of rhGH was studied by SDS-PAGE, in combination with laser densitometry and HI-HPLC. These techniques showed that the level of modified rhGH forms, e.g. aggregated and proteolytically cleaved (16 and 6 kDa) in the preparation was in the 0.5–1% range.

Furthermore, evidence that the correct disulphide bonds (Cys_{53}–Cys_{165}; Cys_{182}–Cys_{189}) were formed in rhGH during secretion has been shown by a combination of tryptic fingerprint and amino acid analysis. CD-spectroscopic analysis suggested an identical secondary structure to that of pituitary derived human growth hormone (pit-hGH). Isoelectric focusing revealed an isoelectric point (pI) for rhGH of 5.0 similar to pit-hGH and in excellent agreement with the theoretical value 5.1, based on the primary sequence. Finally, an apparent molecular weight of 22,000 was obtained for rhGH, by SDS-PAGE. All these physico-chemical studies suggest that rhGH is structurally identical to pit-hGH, somatotropin.

Keywords: *Human growth hormone, recombinant DNA, secretion,* E. coli.

Introduction

A major task in modern biotechnology has been to establish the safety and efficacy of recombinant DNA-derived protein pharmaceuticals. One obvious advantage of recombinant DNA produced products is the possibility of well controlled production

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Abbreviations: SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; kDa, kilodalton; ELISA, enzyme-linked immunosorbent assay; DTE, dithioerythritol; RP-HPLC, reverse phase high-performance liquid chromatography; HI-HPLC, hydrophobic interaction high-performance liquid chromatography; CD, circular dichroism; pit-hGH, pituitary derived human growth hormone; STII-rhGH, heat stable enterotoxin II signal peptide (23 amino acid residues) linked to rhGH (191 amino acid residues).

systems. However, by heterologous expression in *E. coli* or yeast for example, several questions have been raised concerning: (i) the possibility of wrongly folded proteins; (ii) the presence of secreted precursor forms of signal peptide-containing proteins; and (iii) proteins having post-translational modifications caused by deamidation, proteolytic cleavage etc.

Human growth hormone (met-hGH) has been synthesised in *E. coli*, as a cytoplasmic protein with an extra methionine residue in the N-terminal end [1]. However, recent advances in the understanding of the underlying mechanisms of protein secretion in *E. coli*, has made it possible to make a secreted form of the hormone. This paper describes the synthesis and secretion of authentic human growth hormone (rhGH), Genotropin®, in *E. coli*. The secretion, which mimics the natural process of the somatotropic cells in the pituitary gland, was achieved by linking the gene for the heat-stable enterotoxin II (STII) signal peptide (23 amino acids) [2], to the hGH gene (191 amino acids). By this construction rhGH is secreted to the periplasmic space [3], from where it could be easily purified to homogeneity. The analytical techniques which have been used to characterise the rhGH preparation with respect to structure and purity are presented here.

Materials and Methods

Protein sequence analysis was carried out on an Applied Biosystems Inc. gas phase protein sequencer 470A (Applied Biosystems, Foster City, CA, USA).

SDS-PAGE was carried out essentially as described by Laemmli [4]. Quantitation of silver-stained SDS-PAGE gels was done by laser densitometry (633 nm) using an LKB UltroScan XL laser densitometer (LKB, Bromma, Sweden) [5]. The hydrophilicity plot of rhGH based on the primary sequence was obtained using a computer program "DNA Inspector II" (Textco, New Hampshire, USA).

HI-HPLC was carried out on a Hewlett-Packard HP 1090 chromatographic system (Waldbronn, FRG) using a TSK-Phenyl 5PW column (75 × 7.5 mm; Tosoh, Japan [6]).

Circular dichroism spectra were recorded on a JASCO J-41A spectropolarimeter (Japan Spectroscopic Co., Tokyo). The analysis was carried out in 1 mM sodium phosphate, 0.15 M NaCl, pH 6.8, at a protein concentration of 1.5 mg ml^{-1}. The spectra were recorded at room temperature.

Tryptic fingerprint analysis was carried out essentially as follows: rhGH was dissolved in 0.1% bicarbonate buffer (0.7 mg ml^{-1}) and incubated with TPCK-treated trypsin (5 μg ml^{-1}) for 2 h at 37°C. Tryptic fragments were subsequently separated by RP-HPLC on a Vydac 218 TP C_{18}-column, using an acetonitrile (5–60%) gradient in 0.1 M phosphate buffer (pH = 2.0) at a flow rate of 1 ml min^{-1}.

Amino acid determination was carried out on an automated amino acid analyser (LKB 4150 Alpha Plus, LKB-products AB, Sweden), essentially according to the procedure described by the manufacturer. The isoelectric point (pI) of rhGH was determined by isoelectric focusing in LKB Agarose Z using Pharmalyte, pH 4–6.5. The determination of the pH-gradient obtained after isoelectric focusing was achieved by a pH surface electrode (Ingold 430-3-M8). A plot of pH versus distance from cathode was constructed. The linear part (regression coefficient = 0.997) was chosen for the pI determination.

Recombinant derived human growth hormone, rhGH, Genotropin®, used in this study was obtained from KabiVitrum Peptide Hormones AB, Stockholm, Sweden. All reagents used were of highest available purity.

Results

Signal peptide processing

The effective cleavage of the STII signal peptide is carried out by an endogeneous *E. coli* signal peptidase, creating the correct N-terminal end (Phe) of human growth hormone (Fig. 1). Automated Edman degradation analysis of 15 steps (cf. Table 1) completely matched the known sequence of human growth hormone [7]. No alanine or tyrosine residues belonging to the STII signal peptide were detected in the first degradation cycle, showing that the cleavage is highly specific. The detection level for underlying secondary sequences is usually in the 2–5% range.

Purity of rhGH

Figure 2 shows the purity of rhGH following each individual purification step as deduced by SDS-PAGE and silver staining. The 22 kDa molecular weight form of rhGH, was identified in the periplasmic extract (lane 1) by Western blot analysis (not shown). This form was present together with a few other [25–30] *E. coli* polypeptides. Also present in the periplasmic extract was a proteolytically cleaved form of rhGH with an apparent molecular weight of 24 kDa, which upon reduction was split into a 16 and 6 kDa form. The 24 kDa form has been fully characterised and shown to have been cleaved in the polypeptide chain after Tyr_{142}. The 24 kDa form, which is fully biologically active in the hypophysectomised rat weight-gain assay, was removed during purification down to

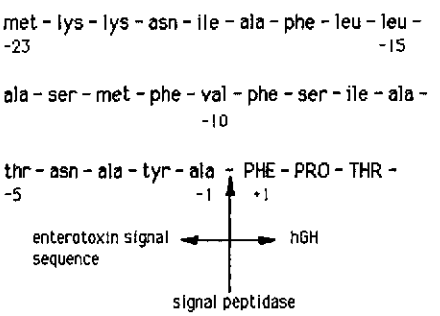

Figure 1
Escherichia coli signal peptidase cleavage of rhGH prehormone (STII-rhGH).

Table 1
Sequence determination of rhGH, Genotropin®

Step	Amino acid identified	Amount (nmol)
1	Phe	31.5
2	Pro	17.5
3	Thr	10.6
4	Ile	16.3
5	Pro	10.4
6	Leu	12.9
7	Ser	4.4
8	Arg	7.5
9	Leu	6.9
10	Phe	7.1
11	Asp	4.5
12	Asn	5.6
13	Ala	5.8
14	Met	9.3
15	Leu	7.4

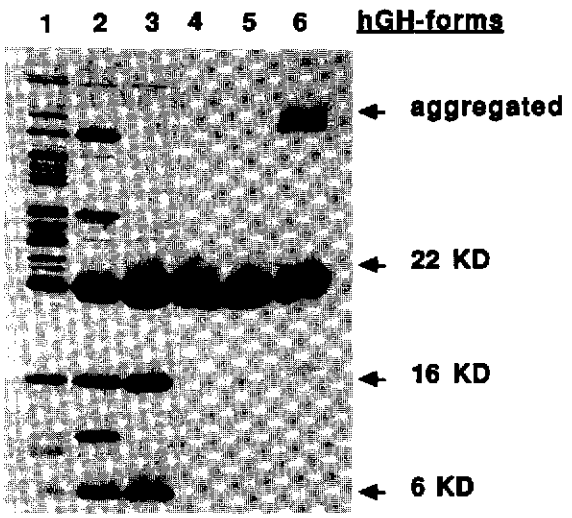

Figure 2
SDS-PAGE of fractions from the purification process. Ten micrograms of protein was loaded on each lane. The sample in lane 6 has been lyophilised prior to electrophoresis. Molecular weight assignments were made relative to molecular weight markers (cf. Materials and Methods).

the 0.1–0.5% level. Furthermore, an aggregated form (molecular weight 44 kDa) appeared after freeze-drying of rhGH (lane 6). The content of this form was dependent on the formulation buffer as well as the freeze-drying parameters chosen. The amount of the aggregated form was usually in the 1–2% range. No aggregated form was found earlier in the purification process. Lanes 2–6 show the removal of contaminating polypeptides during the purification process. The level of *E. coli* proteins after the final purification step was extremely low, usually in the 1 ppm (0.0001%) range and thus could not be quantitated by SDS-PAGE and silver-staining. Accurate quantitation was achieved by an ELISA-method (unpublished).

To more accurately quantitate various molecular weight forms of rhGH present in the final preparation, a laser densitometric method in combination with SDS-PAGE was developed. Figure 3 shows a laser densitometric determination of aggregated rhGH as well as the cleaved (16 and 6 kDa) form of the hormone. The levels of these forms were found to be 2.2 and 0.5%, respectively.

Physico-chemical properties

Hydrophobic surface. Every protein has a unique distribution ("finger print") of hydrophobic and hydrophilic amino acid residues. Figure 4 shows a hydrophilicity plot of STII-rhGH, based on its primary sequence. Seven major and three minor hydrophilic regions were identified within rhGH. The location (surface versus interior) of these regions affects the hydrophilicity–hydrophobicity balance on the surface. This property can be exploited chromatographically using HI-HPLC to separate proteins and demonstrate identity.

The rhGH was chromatographed on a TSK-Phenyl column (Fig. 5). The resolution of the method was found to be very high since methionyl-human growth hormone, Somatonorm®, (t_R = 17 min) could be separated from rhGH, Genotropin®, (t_R = 14 min), differing only in one extra methionine residue in the N-terminal end (Fig. 5A).

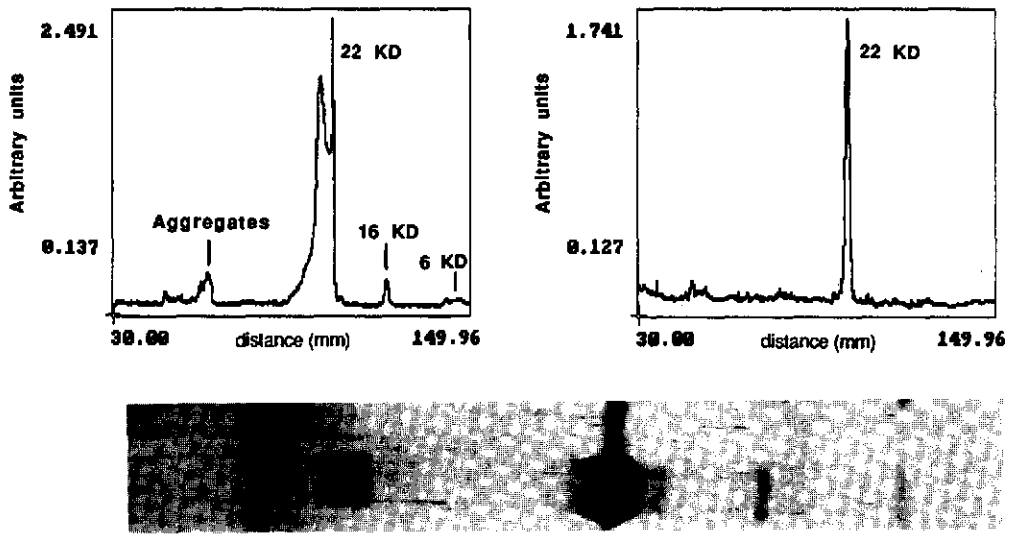

Figure 3
Laser densitometric determination of cleaved (16 and 6 kDa) and aggregated forms of rhGH. 10 and 0.25 µg of rhGH was electrophoresed in each lane. Each lane was densitometrically scanned (633 nm) after staining. The total integrated peak area was determined and used for the estimation of the relative content of each component.

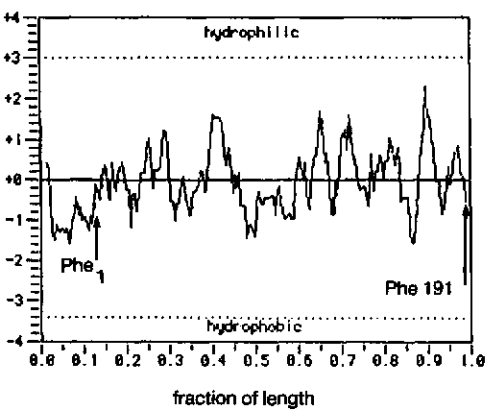

Figure 4
Hydrophilicity plot of STII-rhGH.

Furthermore, rhGH elution time was identical to that for pit-hGH, indicating identical hydrophilic surfaces (Fig. 5B).

Figure 5C shows an expanded HI-HPLC chromatogram of rhGH to clearly identify minor peaks. Four extra peaks beside the main rhGH peak could be identified. These variant forms have been characterised and are present in low amounts (less than 1%) in purified rhGH.

Circular dichroism spectroscopy

The secondary structure of rhGH was studied by CD-spectroscopy. A CD spectrum was recorded between 250 and 320 nm and found to be identical to that for pituitary

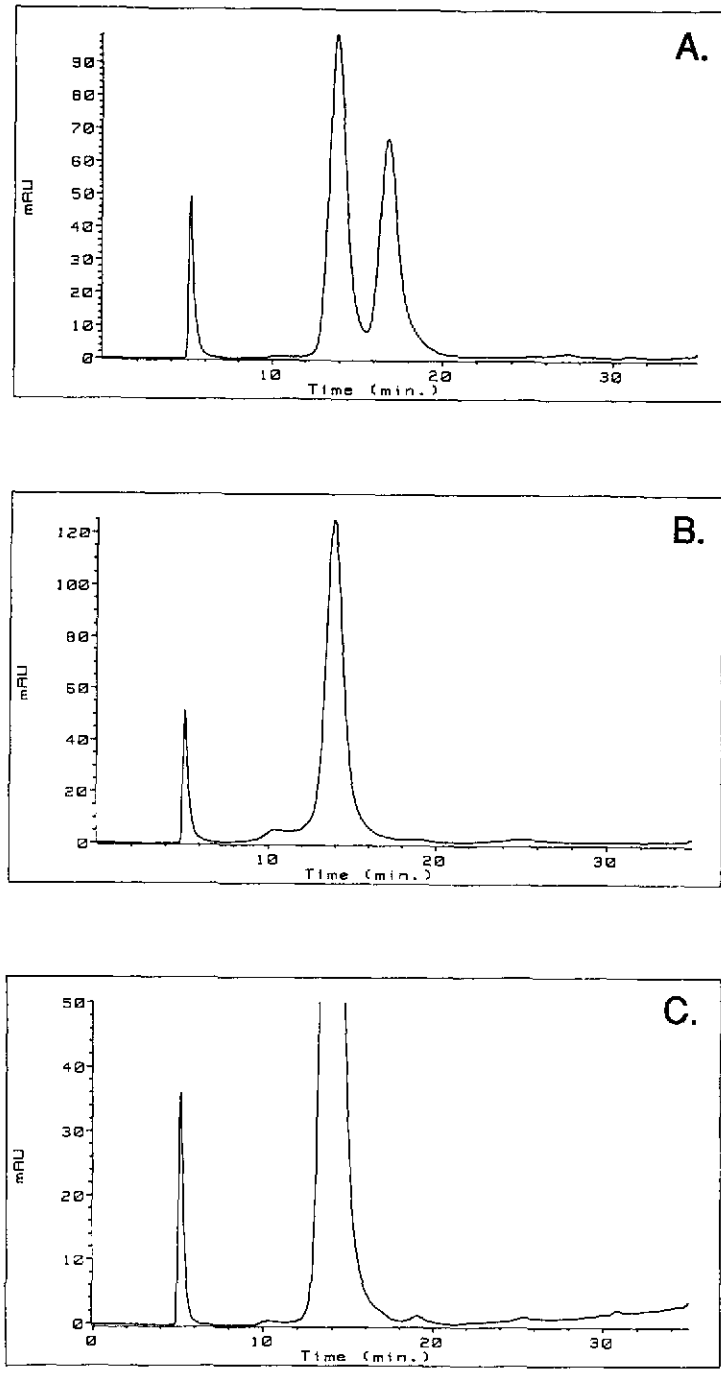

Figure 5
Hydrophobic interaction chromatography (HI-HPLC) of somatotropin preparations. A. rhGH and methionyl-hGH (1:1) B. rhGH plus pituitary-hGH (1:1); C. rhGH (expanded 3.4-fold).

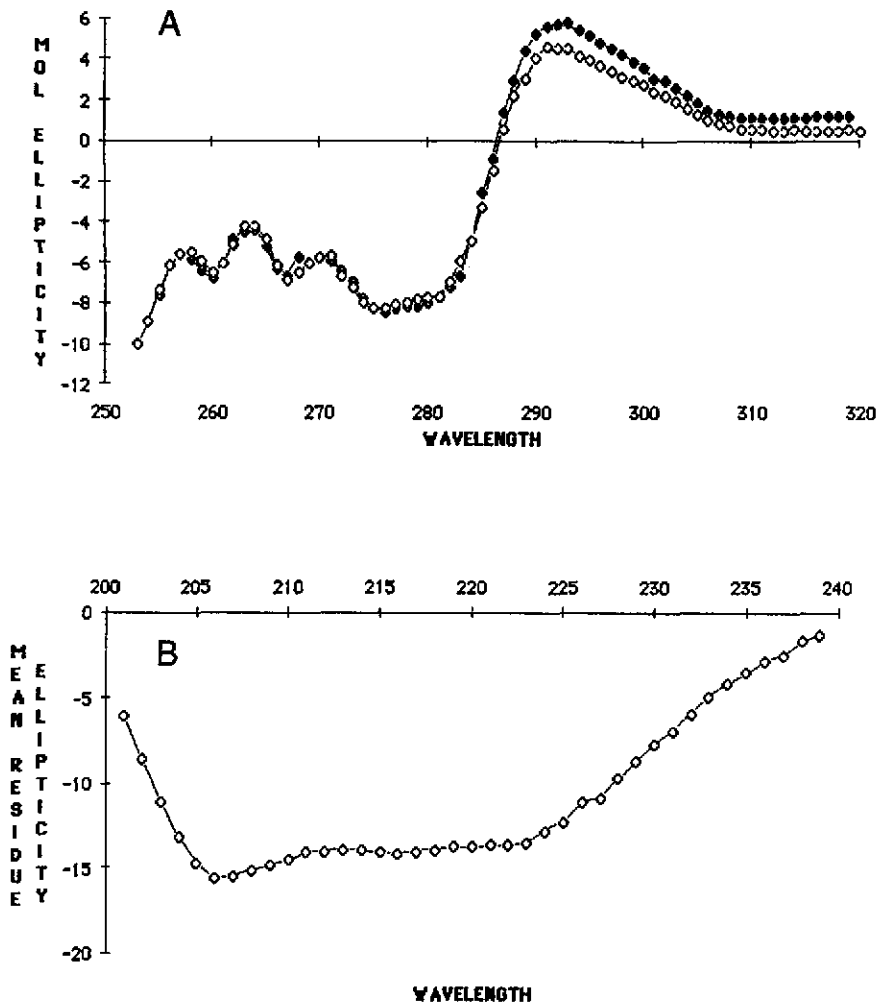

Figure 6
CD spectrum of rhGH. Panel A. rhGH (◇) and pit-hGH (◆); panel B. Far-ultraviolet circular dichroism spectra of rhGH. Molar ellipticity (θ) is expressed as (deg. cm^2 d^{-1} mol).

derived human growth hormone (Fig. 6A). Both preparations exhibited a strong maximum at 292 nm indicative of tryptophan transitions. The CD spectrum in the far-ultraviolet region (Fig. 6B), was identical to that reported for pituitary derived hGH [8] as well as for another recombinant DNA derived hGH preparation [9]. This showed the characteristic high content of α-helix, with a minimum at 206 nm and a shoulder at 223 nm, in agreement with recent X-ray crystallographic data [10].

Disulphide bonds

Two disulphide bonds viz., Cys_{53}–Cys_{165} and Cys_{182}–Cys_{189}, have been identified in pituitary human growth hormone [7]. The correct disulphide bond pairing in rhGH has been studied by a combination of tryptic fingerprint and amino acid analysis. Figure 7 shows the hGH amino acid sequence with tryptic cleavage sites indicated. Cleavage of

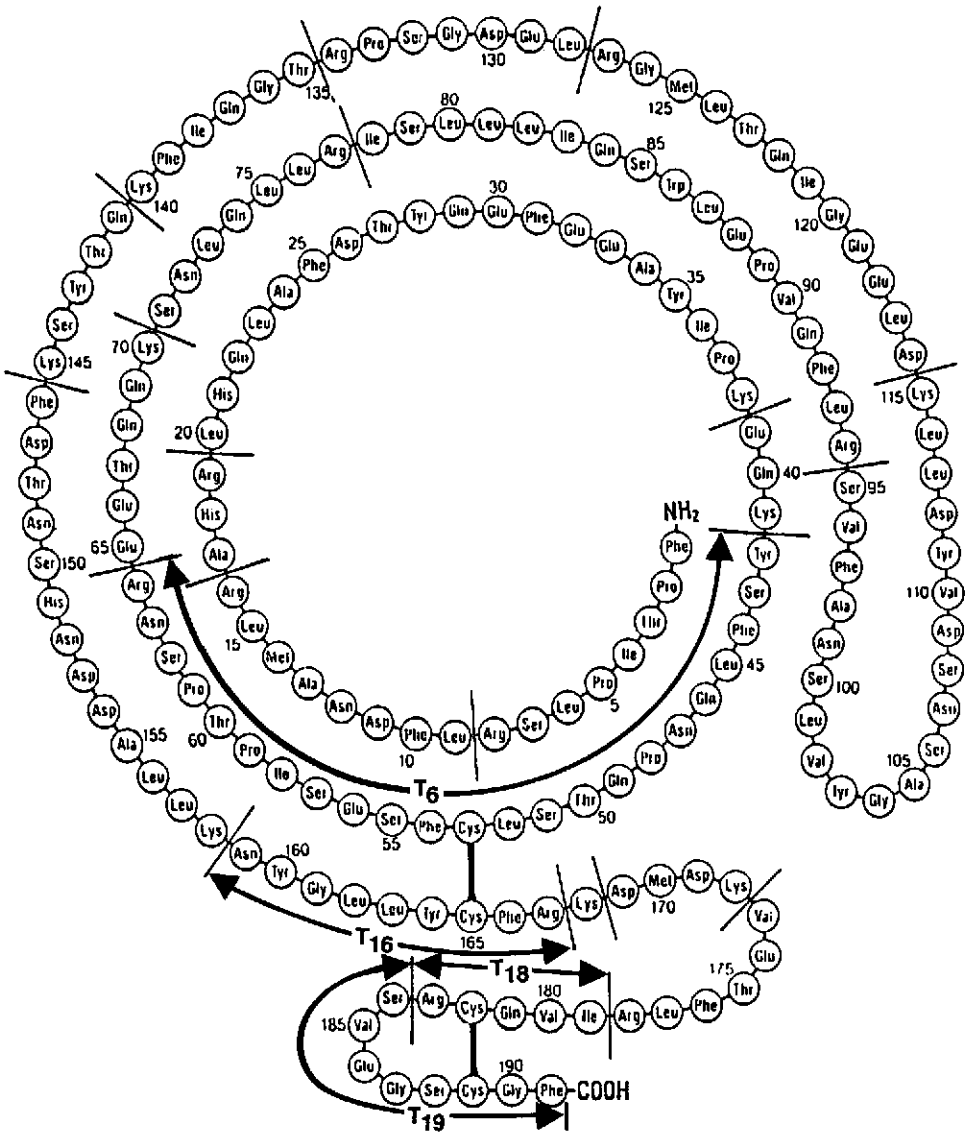

Figure 7
Primary sequence, disulphide bonds and tryptic cleavage sites in human growth hormone.

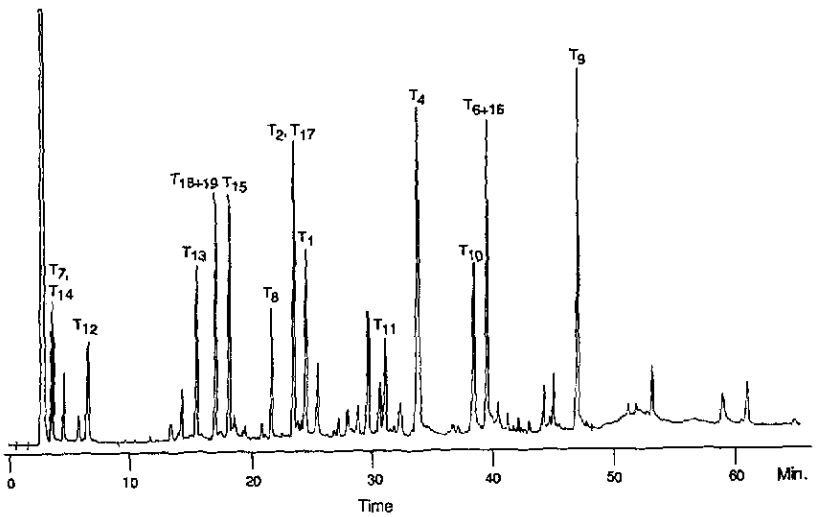

Figure 8
Tryptic fingerprint of rhGH. rhGH was cleaved with TPCK-treated trypsin. Tryptic fragments literated were subsequently separated by RP-HPLC on a Vydac C_{18}-column. Tryptic fragment assignments are based on amino acid composition determinations. Tryptic fragment T_{18+19} and T_{6+16} are linked by disulphide bonds.

Table 2
Amino acid composition for reduced tryptic S-S fragments

Amino acid	Sample T_{18} (179–183)	Sample T_{19} (184–191)	Sample T_6 (42–64)	Sample T_{16} (159–167)
Asx			2.0 (2)	1.1 (1)
Thr			1.9 (2)	0.1
Ser		2.0 (2)	5.0 (5)	0.4
Glx	1.1 (1)	1.0 (1)	2.8 (3)	0.1
Pro			3.0 (3)	
Gly		2.0 (2)	0.4	1.1 (1)
Ala			0.1	
Cys	1.1 (1)	0.9 (1)	0.9 (1)	0.9 (1)
Val	0.8 (1)	1.0 (1)	0.1	0.1
Met				
Ile	0.7 (1)		0.9 (1)	
Leu			1.9 (2)	1.8 (2)
Tyr			0.9 (1)	1.4 (2)
Phe		0.9 (1)	1.8 (2)	0.8 (1)
His				
Lys			0.2	0.1
Trp				
Arg	1.1 (1)		0.9 (1)	0.9 (1)

Values are expressed as nmol peptide.
Values in parentheses are the theoretical values (Ref. 6).
Tryptophan was not determined.
Asparagine and glutamine are expressed as aspartic acid and glutamic acid, respectively.

rhGH in the non-reduced state (intact disulphide bonds) with trypsin generated 19 tryptic fragments. All of them, except T_3 and T_5 which were too small and eluted in the solvent front, were separated by RP-HPLC and identified by amino acid analysis (Fig. 8). Four of these fragments, e.g. T_6–T_{16} and T_{18}–T_{19}, were linked by disulphide bonds to each other and could be isolated. After disulphide bond cleavage (DTE), each individual tryptic fragment (T_6, T_{16}, T_{18} and T_{19}) was again separated by RP-HPLC (not shown), and identified by amino acid analysis (Table 2), to establish the correct disulphide pairing of rhGH.

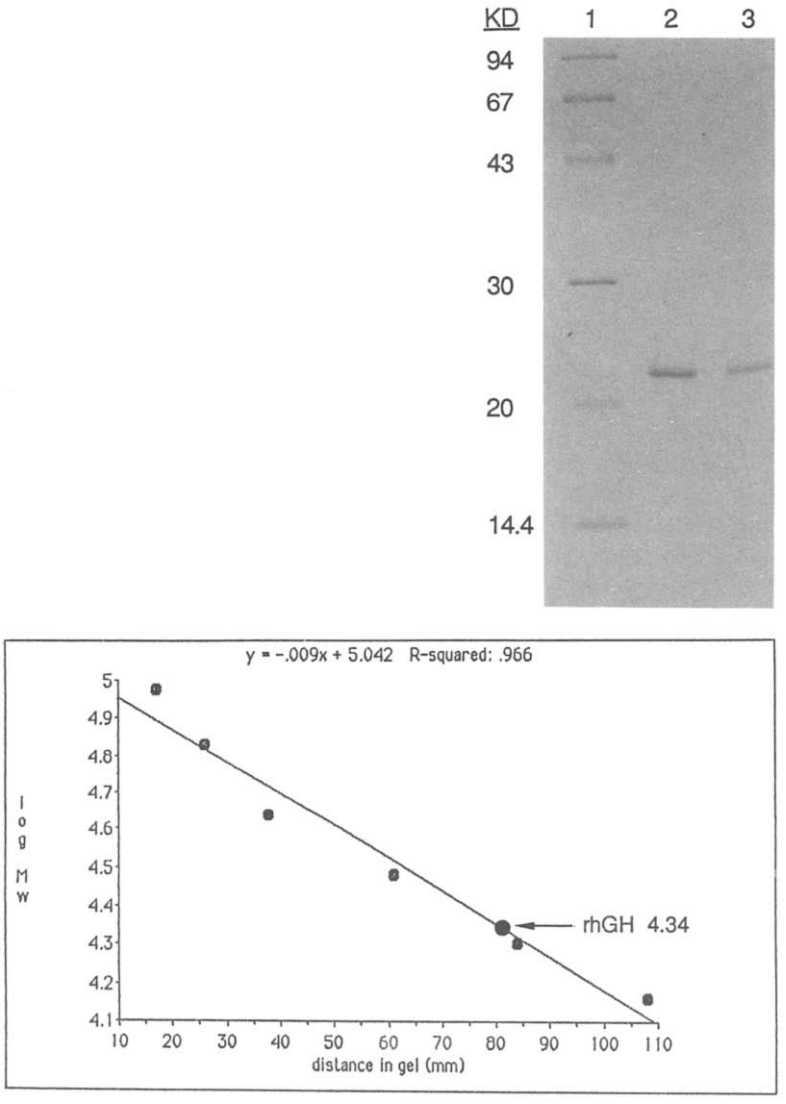

Figure 9
rhGH molecular weight determination by SDS-PAGE. Upper panel. Lane 1, standard proteins with known molecular weight; phosphorylase b (94,000); albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); trypsin inhibitor (20,100); α-lactalbumin (14,400). Lane 2, rhGH; lane 3, pit-hGH (Crescormon®). Lower panel. A plot of $\log_{10}$ molecular weight (MW) versus migration distance.

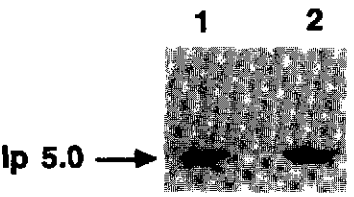

Figure 10
Isoelectric focusing of rhGH. Isoelectric focusing was carried out in the pH interval of 4.0–6.5. pH across the gel was measured by a surface electrode. Bands were subsequently visualised by Coomassie Blue staining. The isoelectric point of rhGH was determined relative to the measured pH gradient. Lane 1, rhGH; lane 2, pit-hGH.

Table 3
Properties of rhGH, Genotropin®

Test	Value
Primary structure	Single polypeptide chain of 191 amino acid residues
N-terminal amino acid	Phe
Disulphide bonds	2 (Cys_{53}–Cys_{165}; Cys_{182}–Cys_{189})
Mw (SDS-PAGE)	22.000 ± 500
Mw (theoretical)	22.096
Molecular form	Monomer
Isoelectric point	5.0
Net charge at pH 7	−5

Molecular weight and isoelectric point of rhGH. The apparent molecular weight of rhGH was determined by SDS-PAGE relative to several standard proteins. A molecular weight of approximately 22,000 (log 4.34) was obtained (Fig. 9), which is in excellent agreement with that for pit-hGH (21,500) and the theoretical value (22,098), based on the primary sequence for hGH [7]. Furthermore, the isoelectric point of rhGH was determined by isoelectric focusing (Fig. 10). A value of 5.0 was found, in good agreement with pit-hGH. A summary of physico-chemical properties obtained for rhGH is given in Table 3.

Conclusion

The results presented in this paper indicate that rhGH, when expressed and secreted from *E. coli* cells, has all the physico-chemical properties of authentic pituitary derived human growth hormone.

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