*Journal of Chromatography*, 361 (1986) 209–216
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 588

# HYDROPHOBIC ADSORBANTS FOR THE ISOLATION AND PURIFICATION OF BIOSYNTHETIC HUMAN GROWTH HORMONE FROM CRUDE FERMENTATION MIXTURES*

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(First received September 2nd, 1985; revised manuscript received February 4th, 1986)

## SUMMARY

Hydrophobic interaction chromatography was used as the initial step for the concentration and partial purification of biosynthetic human gowth hormone (hGH). This molecule was extracted from the culture medium of a transformed monkey kidney cell line by batch adsorption either to Octyl-Sepharose or Phenyl-Sepharose. The adsorbate was decanted and packed into a column for elution. This initial work-up procedure rendered a concentrated hGH solution that was further purified by ion-exchange and gel filtration chromatography. The hGH obtained was homogeneous, based on polyacrylamide gel electrophoresis, N-terminal sequence and fingerprint analysis.

## INTRODUCTION

Many potential therapeutic proteins of human and other eukaryotic origin have been produced on a large scale in bacteria using recombinant DNA technology. However, some biosynthetic products differ from the natural proteins by an extra methionine at their N-termini[1–3]. New systems that can produce the authentic polypeptides directly by processing from a precursor have been developed[4,5]. These systems, mainly based on the secretion of proteins to the culture medium, often result in large volumes of diluted solutions that may require an initial concentration and elimination of insoluble particles. An attractive initial work-up procedure is the batch-wise adsorption to recover the product on a solid phase[6–8].

We present here the application of this methodology to the purification of a 191 amino acid protein, human growth hormone (hGH)[9], from the culture medium of an established monkey kidney cell line transformed with a plasmid containing the cDNA sequence of the hGH precursor[5].

* Presented at the *6th International Symposium on Bioaffinity Chromatography and Related Techniques, Prague, September 1–6, 1985.*

0021-9673/86/$03.50 

## MATERIALS AND METHODS

Biosynthetic hGH was produced by a recombinant-plasmid transformed monkey kidney cell line as described[5]. Pituitary-derived hGH was obtained from Association France-Hypophyse, Hôpital des Enfants Malades, Paris, France. Octyl-Sepharose CL-4B, Phenyl-Sepharose CL-4B and DEAE-Sepharose were purchased from Pharmacia Fine Chemicals. Ultrogel AcA-44 was purchased from Reactifs IBF, Villeneuve-La-Garenne, France. Dithiothreitol was obtained from Calbiochem. Trypsin-TPCK was obtained from Worthington. All water used was from a Millipore Super Q polishing system. HPLC-grade acetonitrile was obtained from Fisons, Loughborough, U.K., and trifluoracetic acid from Merck. All sequencer reagents and solvents were purchased from Applied Biosystems, CA, U.S.A. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence and absence of reducing agents was performed by the methods of Laemmli[10]; proteins were visualized by using a silver-staining technique (Bio-Rad). Tryptic digestion was performed in 1% ammonium bicarbonate (pH 9.0) at room temperature for 16 h. Trypsin was added to the sample in a ratio of 1:66 (w/w). The resulting peptides were separated on a Perkin-Elmer Series 5 system equipped with a LiChrosorb RP-18, 5 $\mu$m column (25 cm $\times$ 4.6 mm I.D.); the elution conditions were essentially as described by Kohr *et al.*[11].

The $NH_2$-terminal sequence was determined with a gas-phase protein sequencer (Model 470A, Applied Biosystems), using a program as described by Strickler *et al.*[12] with minor modifications. Phenylthiohydantoin-amino acid derivatives were identified on a Hypersil, 5 $\mu$m, 20 cm $\times$ 2.1 mm I.D. column (Hewlett-Packard). The protein levels were determined with a Bio-Rad kit using bovine serum albumin as standard. Concentrations of hGH were determined by a commercially available radioimmunoassay (Institut Pasteur Production).

## RESULTS

Biosynthetic systems based on the secretion of proteins to the culture medium generally result in large volumes of diluted solutions of the product of interest. A convenient way to concentrate and prepurify these products is to adsorb them to appropriate supports in batch-wise operations. Several systems were studied in our laboratory for the purification of hGH from the cell-free culture medium of an engineered monkey kidney cell line. Two of them, based on hydrophobic gels, are presented in this report. The batch adsorption of hGH to Phenyl-Sepharose and Octyl-Sepharose was studied in culture medium without any preconditioning. One volume of hydrophobic gel was added to forty volumes of culture medium. The suspension was continuously stirred at room temperature and aliquots were taken at various times, centrifuged and the supernatant was assayed for hGH immunoreactivity (irhGH). The results are shown in Fig. 1.

Once the hGH was adsorbed on the beads, the stirrer was turned off and the beads were allowed to settle. The supernatant was decanted, the gel was transferred to a column and thoroughly washed with ammonium acetate (0.2 *M*, pH 8.3). When no more proteins were eluted, as indicated by a drop of the detector signal to the baseline level, the elution was done for the Octyl-Sepharose (as indicated in Fig. 2) and for the Phenyl-Sepharose (as indicated in Fig. 3).

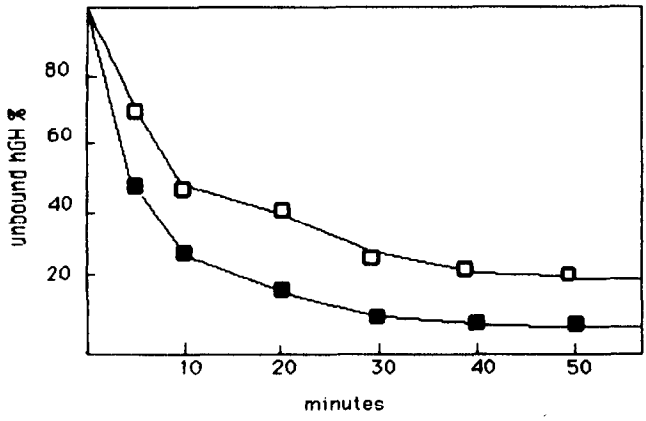


Fig. 1. Batch adsorption of hGH to Octyl-Sepharose and Phenyl-Sepharose. One volume of Octyl-Sepharose (■) or Phenyl-Sepharose (□) was added to 40 volumes of crude transformed monkey kidney cell culture medium. The suspensions were maintained at 20°C and aliquots were taken at various times. The aliquots were centrifuged and the supernatants were assayed for hGH immunoreactivity. After 1 h the adsorptions were considered complete.

The initial adsorption and elution steps from either Octyl-Sepharose or Phenyl-Sepharose resulted both in an approximately 100-fold volume reduction, however the yields and the resulting specific activities were different. The recovery of ir-hGH in the main peak, eluted from the Octyl-Sepharose with 30% acetonitrile, was of 80% with a 17-fold purification. The material eluted with 10% acetonitrile was further purified and characterized as a proteolytically nicked form of the hGH molecule (results not shown). The recovery of ir-hGH in the peak eluted from the Phenyl-Sepharose with water was of 61% with an 8-fold purification. This relatively low

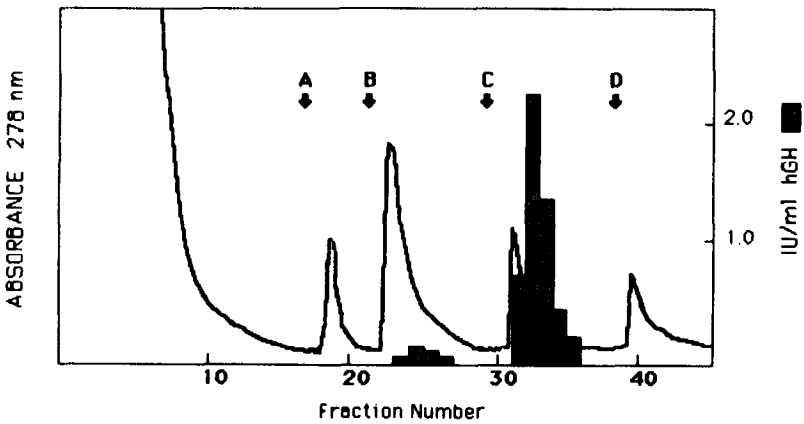


Fig. 2. hGH elution from Octyl-Sepharose gel. Once the maximum hGH adsorption was reached, the culture medium was decanted and the gel was transferred to a glass column (10.0 × 5.0 cm I.D.) and washed with 200 m*M* ammonium acetate buffer (pH 8.3) at a flow-rate of 300 ml/h. The elution was performed by changing the buffer to 10 m*M* ammonium acetate, pH 8.3 (A); 10% acetonitrile in buffer A (B); 30% acetonitrile in buffer A (C); and 50% acetonitrile in buffer A (D). Fractions 33–37 were pooled and used for further purification.

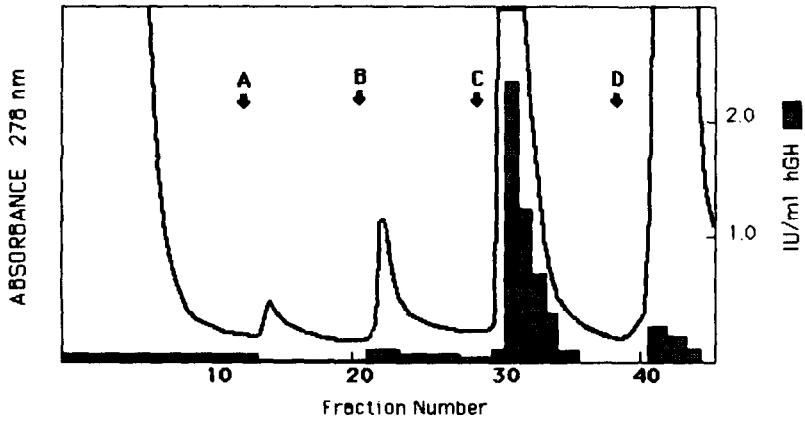


Fig. 3. hGH elution from Penyl-Sepharose gel. The column was prepared as described in the legend to Fig. 2 and then step-eluted with 100 m*M* ammonium acetate, pH 8.3 (A); 50 m*M* ammonium acetate, pH 8.3 (B); water (C) and 20% acetonitrile in water (D). Fractions 30–35 were pooled and used for further purification.

yield could be attributed to a leak of hGH from the column during the packing and washing of the gel.

The material eluting later, with 30% acetonitrile, was also identified as the nicked form of the hGH (results not shown). Following the last acetonitrile elution, both gels were thoroughly washed with water and re-equilibrated in ammonium acetate (0.2 *M*, pH 8.3).

The fractions containing ir-hGH were pooled and loaded directly onto a DEAE-Sepharose fast-flow column equilibrated in ammonium acetate (pH 8.0, 50 m*M* sodium chloride). The elution was performed with a linear gradient of sodium chloride up to 300 m*M* in 60 min, at a flow-rate of 1 ml/cm$^2$. Final purification was obtained by gel filtration chromatography on Ultrogel AcA-44 equilibrated in ammonium bicarbonate. The purification protocols, summarized in Table I, were monitored for hGH by radioimmunoassay (RIA); the purified products were tested for biological activities in the body-weight-gain assay on hypohysectomized rats, as described[13]. The recombinant hGH obtained from both purification protocols showed similar growth-promoting activities as pituitary-derived hGH, *i.e.* 2.4 I.U./mg[14].

TABLE I

PURIFICATION OF BIOSYNTHETIC hGH FROM THE CULTURE MEDIUM OF TRANSFORMED MONKEY KIDNEY CELLS

| *Fraction* | *Octyl-Sepharose* | | *Phenyl-Sepharose* | |
|---|---|---|---|---|
| | *Specific activity (mg hGH/mg protein)* | *Yield (%)* | *Specific activity (mg hGH/mg protein)* | *Yield (%)* |
| Culture medium | 0.03 | 100 | 0.03 | 100 |
| Hydroph. chrom. | 0.50 | 80 | 0.24 | 62 |
| DEAE-Sepharose | 0.85 | 54 | 0.75 | 35 |
| Ultrogel AcA44 | 1.00 | 48 | 1.00 | 28 |

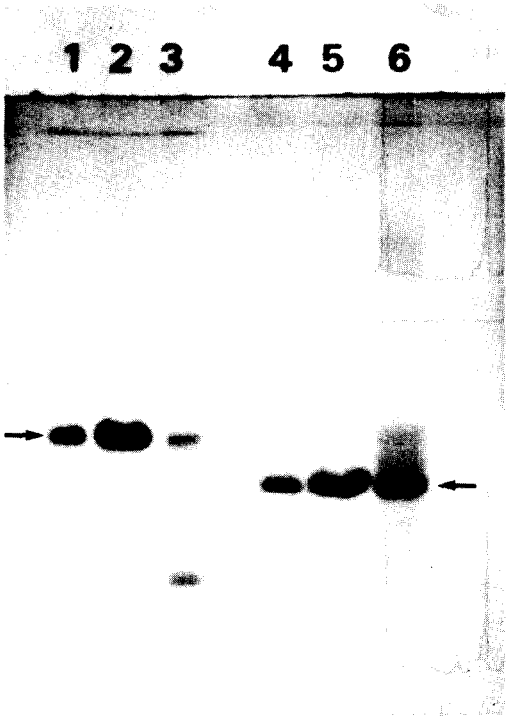


Fig. 4. Sodium dodecyl sulphate gel electrophoresis of biosynthetic hGH (lanes 1 and 4: 1 μg, lanes 2 and 5: 5 μg) and pituitary hGH (lanes 3 and 6: 2 μg). Samples in lines 1–3 were reduced with dithiothreitol before electrophoresis. Samples in lanes 4–6 were not reduced before electrophoresis. Proteins were visualized by silver staining. The arrows indicate the position of reduced and non-reduced hGH.

TABLE II

AMINO-TERMINAL SEQUENCE ANALYSIS OF BIOSYNTHETIC hGH

| *Cycle No.* | *Phenylthiohydantoin-amino acid* | *Yield (pmol)* | *Carry-over (pmol)* |
|---|---|---|---|
| 1 | Phe | 320 | – |
| 2 | Pro | 256 | 19 |
| 3 | Thr | 58 | 23 |
| 4 | Ile | 386 | 6 |
| 5 | Pro | 165 | 18 |
| 6 | Leu | 145 | 22 |
| 7 | Ser | 23 | 10 |
| 8 | Arg | 51 | 4 |
| 9 | Leu | 110 | 19 |
| 10 | Phe | 155 | 9 |

Initial yield: 32%
Average repetitive yield: 90%

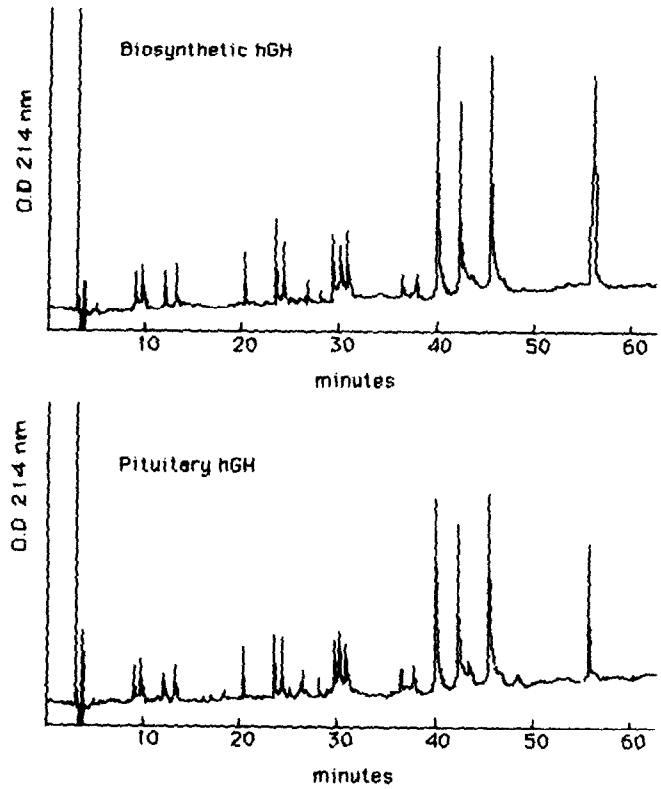


*Fig. 5.* Tryptic peptide map of pituitary-derived and biosynthetic *hGH*. The peptides (from 200 $\mu$g of protein digestion) were separated on a LiChrosorb RP-18 column (25 × 4.6 mm I.D.) with a linear gradient of acetonitrile from 1 to 55% in water–trifluoroacetic acid (99.9:0.1) at a flow-rate of 1.5 ml/min. Elution was monitored at 214 nm.

Based on SDS-PAGE under reducing and non-reducing conditions, the biosynthetic hGH preparation was homogeneous (Fig. 4). The homogeneity of this molecule was further investigated by direct N-terminal sequencing and peptide mapping. The N-terminal sequence (Table II) is identical to the published sequence of the 22-kilodalton pituitary-derived *hGH*[8]. The quantitative recovery of phenylalanine-phenylthiohydantoin on the first Edman degradation cycle showed that the pre-hGH molecule was correctly processed during the secretion procedure. Fingerprint analyses of biosynthetic hGH and pituitary-derived hGH were unable to detect any difference between both molecules (Fig. 5). Each peak was identified and assigned to the expected tryptic peptides by amino acid analysis and sequencing[11–14]. The SDS-PAGE, N-terminal sequence and peptide mapping presented were done on the purified biosynthetic hGH initially adsorbed on Octyl-Sepharose. Identical results were obtained with the purified biosynthetic hGH intially adsorbed on Phenyl-Sepharose.

## DISCUSSION

This report describes a simple procedure for the purification of biosynthetic

hGH from large-scale cell fermentation. The initial step used to concentrate and prepurify the molecule is a batch adsorption step of the cell-free culture medium. We have used two hydrophobic gels: Phenyl-Sepharose and Octyl-Sepharose; better results were obtained with the Octyl-Sepharose gel, however the elution conditions require the utilization of acetonitrile which is not always compatible with the native conformation of some proteins. The first step yield, using Phenyl-Sepharose, could be improved if the adsorption and washing of the gel were done in the presence of 1 *M* ammonium sulphate, however the gain in yield was accompanied by a loss in specific activity of the resulting hGH fraction, which made it very difficult to obtain an homogeneous product without important modifications of the purification protocol.

Following the hydrophobic adsorption–elution step, the hGH-containing fractions were pooled and finally purified by ion-exchange chromatography on DEAE-Sepharose and size-exclusion chromatography on Ultrogel AcA-44.

Based on SDS-PAGE, the purified material is homogeneous. Further characterization by N-terminal sequencing and fingerprint analysis show the identity of the biosynthetic hGH and the pituitary-derived hGH. The quantitative recovery of phenylalanine-phenylthiohydantoin during the first Edman degradation step proved that the pre-hGH was correctly processed by this transformed monkey kidney cell line, during the secretion procedure.

A proteolytically nicked variant of the hGH initially present in this culture medium was almost completely eliminated by a correct adjustment of the elution conditions for the hydrophobic interaction chromatography. These two forms of hGH, very similar in structure, were not resolved by affinity chromatography using several Sepharose-fixed monoclonal anti-hGH antibodies (results not shown). Interestingly, this nicked variant is similar to the nicked variant present in pituitary-derived hGH preparations[15].

Previous studies have shown that it is possible to use different *n*-alkylsilica supports for reversed-phase high-performance liquid chromatographic isolation of hGH[16–19]. However, those systems that proved to be very useful for analytical and semi-preparative work, are technically difficult and expensive to apply in large-scale purification. Furthermore, conformational unfolding of the protein on the bonded-phase surface during chromatography has been described[20–22]. Hydrophobic interaction chromatography, based on a milder adsorption process, significantly minimizes this problem[23–25].

The initial batch adsorption to hydrophobic gels, and step elution, represents a rapid and efficient system applicable to large volumes for the concentration and initial separation of products that do not bind or bind poorly to ion-exchange gels under the conditions used for the fermentation. The technique presented also provides a mild method that does not require any preconditioning of the culture medium and can be used in the presence of suspensions of particulate matter, *i.e.* cell debris. The step-wise elution described simplifies the scaling-up of the procedure.

Finally, with proper handling during washing and re-equilibration, we have seen that the hydrophobic gels can be used repeatedly over periods of months without any change in their adsorption–desorption properties.

ACKNOWLEDGEMENTS

We thank Drs. C. Lebouteiller-Labit and J. Lupker for providing the broths for the purification, Dr. W. Roskam for useful discussions, Drs. M. Magazin and L. Olsen for critical reading of the manuscript, Mr. J. Capdevielle for technical assistance and Mrs. E. Cruse for typing the manuscript.

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