

A NOVEL PROCESS FOR PURIFICATION OF HUMAN GROWTH HORMONE

Technical field

The invention relates to the purification process for human growth hormone (hGH) in commercial scale by using hydrophobic interactive chromatography, which effectively separates clipped hormone moieties formed during the production of growth hormone by DNA recombinant techniques.

Background of the invention

Human Growth Hormone (hGH) is a pituitary derived protein with a number of important biological functions, including protein synthesis, cell proliferation and metabolism. hGH is a 191 amino acid residue polypeptide of approximately 22 kDa. Recombinant human growth hormone (hGH) has been expressed in E.coli both as intracellular as well as secretory protein. A series of chromatographic and/or non-chromatographic methods are then used to obtain the pure protein. It is reported that the region between 140-150 amino acid residues in human growth hormone is sensitive to a number of proteases. This leads to a proteolytically cleaved form of protein whose physical properties are indistinguishable from that of intact molecule. This variant form of hGH can arise during the purification process and its removal poses a major challenge in the production of therapeutic grade growth hormone protein. To enable the use of hGH for therapeutic purpose, it is necessary to remove the clipped molecules.

Prior art describes techniques, which are different from the present invention in many aspects such as process parameters, equipments used, priority of separation of target moiety, use of number of phases, use of solvents, type of impurity separated. A few prior art documents disclose techniques, which are more useful as analytical techniques rather than as industrial processes of separation.

US 4332717 disclose the use of hydrophobic interaction chromatography for purifying human growth hormone. This patent purifies hGH extracted from the human pituitary glands and is not related to recombinant hGH or its purification. Hence, the impurities arising in this process are different from the one present in the recombinant hormone. Besides use of different media and different columns, the process uses different pressure conditions, different temperature conditions and different binding and elution buffer. The elution gradients are also different. It also describes the use of blue

sepharose or agarose in separation and does not teach use of organic and aqueous mixture for elution, which is one of the essential features of the present invention.

Following references relate to purification of growth hormone obtained by recombinant DNA techniques. In the literature, there are known a few patents like
5 US4861868, which describes the method for producing a recombinant porcine Growth Hormone; US4705848, US4694073, US4731440, US5064943, US4975529, US5023323, US5109117 and US6410694 deal with solubilization and naturation methods as an essential feature, but these are outside the scope of the present invention.

US patent 6022858 is related to the formulation of hGH wherein the hGH is
10 pre- treated with Zn and optionally with lysine or calcium ions, after which benzyl alcohol is added to it and the pH adjusted to 2-9.

US patent 5734024 teaches a method for determination of biological activity of recombinant hGH and is good for analytical purposes.

US patent 5182369 teaches a method where the operations are carried out at a
15 pH less than 6.5 and two-step precipitation is the essential feature. However, process conditions that employ acidic pH may lead to aggregation or acid hydrolysis of proteins and are a disadvantage.

US patent 6451347 describes a purification method for hGH, wherein complexation with metal ions such as Zn ions is carried out which is not a feature of the
20 present invention. This patent describes the variants arising from the degradation of hGH and not the clipped moieties.

US patent 6451987 emphasizes the use of cation exchanger for purification of peptides including hGH, but does not mention the separation of the clipped hormone moiety from the intact hGH molecule

25 US patent 6437101 teaches a technique wherein aqueous biphasic extraction without the use of chaotropic agents is employed. The process is cumbersome and hence not easy to perform.

Patents referred in the document by patent numbers are to be construed to have been included by reference so far as the text of the said patents is concerned.

Even today the real problem in such purification is separation of clipped molecules resulting from mega target molecule. These clipped moieties remain bundled together due to certain other linkages present in the molecule.

Non-patent prior art documents comprises of following three published papers
5 on use of hydrophobic interaction chromatography for separation of hGH variants, including clipped variant.

Pavlu and Gellerfors, Bioseparation (1993), 3: 257-265 disclose hydrophobic interaction chromatography of a recombinant human growth hormone, Genotropin.

Gellerfors et al., Acta Pediatr Scand (Suppl) (1990), 370: 93-100 teaches
10 separation and identification of growth hormone variants by high performance liquid chromatography techniques.

Wu et al., J. Chromatogr. (1990), 500: 595-606 disclose Application of high-performance hydrophobic interaction chromatography to the characterization of DNA derived human growth hormone.

15 The techniques disclosed in all these references make use of a surfactant Brij (Polyoxyethylene 23 lauryl ether) in mobile phase B and peaks corresponding to clipped molecule appear after the main peak. These papers disclose use of acetonitrile percentage of 0.5% in mobile phase A and 5% in mobile phase B along with 0.075% Brij. Brij 35 was used to improve recovery of the product from 70% to 99%. Brij 35,
20 being a detergent helps in lowering the interaction of the protein with the matrix thereby and improving recovery. Interestingly, the peak of target molecule precedes the peak of variants. These papers essentially describe HPLC techniques and are better used for analytical purposes rather than for industrial production purposes. The main drawback of these methods is that the use of detergents like Brij is not favoured in
25 industrial scale since its removal from the protein containing solution may be difficult.

The present invention makes use of organic solvents in much higher proportions and eliminates the use of Brij. Further it is noticed that the peak corresponding to variant precedes the peak of target molecule.

Prior art mentions resolution between clipped and intact molecules at analytical
30 level loadings of approximately 50-150 µg semi-purified protein per 3.3 ml column

volume. In the present invention resolution is achieved at a loading of 1 mg semi-purified protein per ml column volume.

The limitation of the prior art is the use of a non-ionic surfactant, Brij 35 to enhance the process recovery at an analytical level. This presence of non-ionic surfactant may be undesirable at preparative level, as it is difficult to remove surfactants that remain bound to protein molecules. The claimed process circumvents the need to use surfactant for improving process recovery by using higher amounts of organic solvent in the eluant that can be easily removed by tangential flow filtration or gel filtration chromatography.

10 Objects of the invention

The main object of the invention is to describe a process for purification of human growth hormone that can effectively separate the clipped molecules from the target molecule.

Another object of the invention is to provide a process for purification of human growth hormone that effectively removes the clipped molecules first thereby enabling better control over production of target molecule.

Yet another objective of the present invention is to describe a process for purification of human growth hormone that can be used for multiple purposes such as analytical method for hGH, isolation of the hGH as well as industrial purification of hGH.

Still another objective of the invention to provide a purification process that can be effectively carried out in the pH range of 8.0 to 9.0.

Still yet another objective of the present invention is to develop a process for purification of human growth hormone that does not make use of any detergents or surfactants.

Summary of the Invention

The invention relates to a novel process for purification of hGH obtained by recombinant technique using hydrophobic interactive chromatography. The invention further relates to the use of polymeric hydrophobic beads as solid support and mixture of aqueous buffers and organic solvents as an eluant to separate target hGH molecule

from clipped hormone moieties present as impurities and finally desalting by gel filtration and lyophilizing to obtain purified hGH.

The features of the present invention will become more apparent from the following description of the inventive concept and the description of the preferred
5 embodiments.

Brief description of the accompanying Figures

The following table describes chromatographic separation conditions for semi-purified hGH with respect to each figure.

Figure #	Chromatography	Buffer A	Buffer B	Sample details
1	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate/ 0.4M K_2HPO_4 , pH 9.0	20 mM Sod-Phosphate, pH 9.0 / 50 % Acetonitrile	Sample: 1.0 mg Detection 280 nm
2	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate/ 0.4 M K_2HPO_4 , pH 9.0	20 mM Sod-Phosphate, pH 9.0 / 40 % Acetonitrile	Sample volume: 1.0 ml Detection 280 nm
3	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate/ 0.4 M K_2HPO_4 , pH 9.0	20 mM Sod-Phosphate, pH 9.0 / 30 % Acetonitrile	Sample volume: 1.0 ml Detection 280 nm
4	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate/ 0.4M K_2HPO_4 , pH 8.0	20 mM Sod-Phosphate, pH 8.0 / 50 % Acetonitrile	Sample 1 mg Detection 280 nm
5	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate / 0.4 M K_2HPO_4 , pH 7.0	20 mM Sod-Phosphate, pH 7.0 / 50 % Acetonitrile	Sample 1.0 mg Detection 280 nm
6	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate / 0.4 M K_2HPO_4 , pH 6.0	20 mM Sod-Phosphate, pH 6.0 / 50 % Acetonitrile	Sample 1.0 mg Detection 280 nm
7	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate/ 0.4 M K_2HPO_4 , pH 9.0	20 mM Sod-Phosphate, pH 9.0 / 50 % Methanol	Sample: 1.0 mg Detection 280 nm
8	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate/ 0.4 M K_2HPO_4 , pH 9.0	20 mM Sod-Phosphate, pH 9.0 / 40 % Methanol	Sample: 1.0 mg Detection 280 nm
9	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate/ 0.4 M K_2HPO_4 , pH 9.0	20 mM Sod-Phosphate, pH 9.0 / 30 % Methanol	Sample 1.0 mg Detection 280 nm
10	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K_2HPO_4 , pH 8.0	20 mM Sodium Phosphate, pH 8.0/ 50 % Methanol	Sample: 1 mg Detection: 280 nm
11	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K_2HPO_4 , pH 7.0	20 mM Sodium Phosphate, pH 7.0/ 50 % Methanol	Sample: 1 mg Detection: 280 nm
12	Column : Resource Phenyl (1.0 ml)	20 mM Sodium Phosphate/ 0.4 M	20 mM Sodium Phosphate, pH 6.0/	Sample: 1 mg Detection: 280 nm

	Amersham	K ₂ HPO ₄ , pH 6.0	50 % Methanol	
13	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 9.0	20 mM Sodium Phosphate, pH 9.0/ 50 % isopropanol	Sample: 1 mg Detection: 280 nm
14	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 9.0	20 mM Sodium Phosphate, pH 9.0/ 40 % isopropanol	Sample: 1 mg Detection: 280 nm
15	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 9.0	20 mM Sodium Phosphate, pH 9.0/ 30 % isopropanol	Sample: 1 mg Detection: 280 nm
16	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 8.0	20 mM Sodium Phosphate, pH 8.0/ 50 % isopropanol	Sample: 1 mg Detection: 280 nm
17	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 7.0	20 mM Sodium Phosphate, pH 7.0/ 50 % isopropanol	Sample: 1 mg Detection: 280 nm
18	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 6.0	20 mM Sodium Phosphate, pH 6.0/ 50 % isopropanol	Sample: 1 mg of ion-exchange purified sample Detection: 280 nm
19	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 9.0	20 mM Sodium Phosphate, pH 9.0/ 25 % Acetonitrile / 25% methanol	Sample: 1 mg Detection: 280 nm
20	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 9.0	20 mM Sodium Phosphate, pH 9.0/ 25 % Acetonitrile / 25% isopropanol	Sample: 1 mg Detection: 280 nm
21	Column : Resource Phenyl (1.0 ml) Amersham	20 mM Sodium Phosphate/ 0.4 M K ₂ HPO ₄ , pH 9.0	20 mM Sodium Phosphate, pH 9.0/ 25% isopropanol / 25 % Methanol	Sample: 1 mg Detection: 280 nm
22	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate, pH 8.0 / 0.4 M K ₂ HPO ₄	20 mM Sod-Phosphate, pH 8.0 / 50 % Acetonitrile	Sample: GH, IEX fractions, Sample volume: 1.0 ml, Detection 280 nm
23	Column: Resource PHE (1.0 ml) Amersham	20 mM Tris/ 0.4 M K ₂ HPO ₄ , pH 9.0	20 mM Tris, pH 9.0 / 50 % Acetonitrile	Sample: 1.0 mg Detection 280 nm
24	Column: Sephacryl S-200 HR (Amersham) 100 ml bed volume	9 mM sodium phosphate, pH 8.0		Detection: 280 nm
25	Column: Resource PHE (1.0 ml) Amersham	20 mM Sod-Phosphate / 0.4 M K ₂ HPO ₄ , pH 8.0	20 M Na-P, pH 8.0	Sample: 1 mg, Detection 280 nm
26	Column: Phenyl Sepharose FF (1.0 ml), Amersham	20 mM Sod-Phosphate / 0.4 M K ₂ HPO ₄ , pH 8.0	20 M Na-P/ 50 % acetonitrile, pH 8.0	Sample: 1 mg, Detection 280 nm

27	Column; Source 15 Q, Amersham	10 mM NH ₄ Cl / 10 mM Tris, pH 8.0 / 3 % EtOH	20 mM NH ₄ Cl, pH 8.0 / 7.5 % EtOH / 500 mM NaCl	Sample: Cu-IDA purified hGH having clipped molecule Detection : 280 nm Fractions: 1 ml System used: FPLC at 1 ml/min flow rate Analyses: 10 µl of each fraction analysed on a SDS-PAGE gel followed by silver staining
28	Column: Superdex 75 HR 10/30, Amersham	20 mM Tris, pH 8.0 / 5 % Glycerol / 150 mM NaCl		Sample: hGH having clipped molecule – denatured in 6 M urea and reduced with 50 mM DTT for 2 hrs at RT Detection : 280 nm Fractions: 1 ml System used: FPLC at 0.5 ml/min flow rate Analyses: 10 µl of each fraction analysed on a SDS-PAGE gel followed by silver staining
29	Column: HiTrap 1 ml columns of Phenyl Sepharose FF (high sub) / Butyl Sepharose FF / Phenyl Sepharose HP / Octyl Sepharose FF	20 mM Sodium phosphate, pH 7.0 / 0.5 M (NH ₄) ₂ SO ₄	20 mM Sodium phosphate, pH 7.0	Sample: hGH having clipped molecule Detection : 280 nm Fractions: 1 ml System used: AKTA Explorer at 1 ml/min flow rate

Detailed description of the invention

In accordance with the object, the present invention discloses a purification technique for hGH. *E. coli* cells containing recombinant human growth hormone (hGH) gene were grown under standard conditions in a 1 L shake flask. After 8-10 hrs of induction, cells were harvested by centrifuging for 15 min at 4 - 8°C. The supernatant was discarded and the cell pellet was suspended in Lysis buffer containing 20 - 50 mM Tris, pH 7.0 - 9.0 and 100 - 500 mM NaCl. Cells were disrupted using ultrasonication for 30 min. Temperature during disruption was maintained at 4 to 8°C by keeping the samples on ice.

The crude lysate was clarified by centrifugation at 16,000 rpm for 1 hr at 4°C. After centrifugation the pellet was discarded and to the clear supernatant, imidazole was added to give a final concentration of 20 - 40 mM. This was loaded onto a 10 ml column of Chelating sepharose beads charged with NiSO₄. The column was equilibrated at a flow rate of 5-20 ml/ min with buffer containing 20 - 50 mM Tris pH 7.0 - 9.0, 100 - 500 mM NaCl, 20 - 40 mM Imidazole. Unbound material washed away using the equilibration buffer and after the absorbance at 280 dropped to baseline, elution of bound proteins was carried out using elution buffer containing 20 - 50 mM Tris pH 7.0 - 9.0, 100 - 500 mM NaCl, 200 - 500 mM Imidazole. Protein concentration in the elution was measured, made to 5 - 10 mg/ml concentration and kept for enzymatic digestion at 4 - 10°C. Digestion was carried out for 15 - 24 hrs and stopped by adding 2 M solution of K₂HPO₄, pH 7-9.0 to a give a final concentration of 0.2 - 0.4M. The digested sample was loaded onto a Phenyl Sepharose FF column (column vol. = 15 ml) equilibrated with buffer containing 20 - 50 mM Tris, 0.2 - 0.4 M K₂HPO₄, pH 7.0 - 9.0 at a flow rate of 5-10 ml /min - Bound protein eluted with water and the peak fraction collected. This was loaded onto Q Sepharose FF column (column vol = 10 ml) equilibrated with 20 - 50 mM Tris pH 7.0 - 9.0. The elution was done using a 15 - 30 column volume linear gradient of 0% A to 30% (v/v) of buffer "A" containing 0.5 - 2M NaCl. The major peak at 280 nm containing human growth hormone was collected and a solution of 2 M K₂HPO₄ was added to achieve a final concentration of 0.2 - 0.6M.

This sample was analyzed varying pH, equilibrating buffers and eluants conditions using Hydrophobic Interaction Chromatography.

According to the invention, there is provided a process for the purification of human growth hormone from its clipped moieties of hGH molecule, by using hydrophobic interaction chromatography technique, the said process comprising steps of:

- a. loading the sample on the column in presence of high inorganic salt concentration,
- b. equilibrating the column loaded with sample with an - aqueous buffer,
- c. eluting the equilibrated column of step(b) with linear gradient of aqueous buffer and organic solvent mixture,

- d. collecting and combining eluted fractions corresponding to hGH peak, concentrating the combined fractions,
- e. desalting and lyophilizing the concentrated fractions of step(d) by filtering on Sephacryl S-200 gel equilibrated with disodium hydrogen phosphate solution of pH 6.0-9.0, and
- f. obtaining purified human growth hormone.

The process utilizes semi purified human growth hormone as a sample for purification.

The column used is hydrophobic resin is a cross-linked polystyrene divinyl benzene polymer resin having attached hydrophobic ligand selected from a group consisting of ether, isopropyl, butyl, octyl and phenyl.

The hydrophobic ligand is preferably phenyl group.

The inorganic salt is selected from a group consisting of ammonium sulfate, disodium hydrogen phosphate, dipotassium hydrogen phosphate or sodium chloride, preferably disodium hydrogen phosphate and most preferably dipotassium hydrogen phosphate.

The inorganic salt concentration used is in the range of 0.2 - 0.6 M, more preferably 0.3 - 0.4 M.

The buffer used for equilibrating the column is a mixture of aqueous disodium hydrogen phosphate and dipotassium hydrogen phosphate. The pH of the equilibrating buffer ranges between 6.0 and 9.0, preferably in the range of 8.0 to 9.0.

The buffer used for eluting protein is a mixture of disodium hydrogen phosphate or tris buffer and an organic solvent.

The pH of eluting buffer preferably ranging between 8.0 and 9.0

The organic solvent used for eluting protein is selected from the group consisting of C₁ to C₄ alcohol, acetonitrile and mixtures thereof.

The organic solvent in the eluting mixture ranges between 40-70% v/v, more preferably 40% - 50% v/v.

The temperature for chromatography separations is preferably in the range of 20-30°C, more preferably 22-24°C.

The growth hormone peak eluted from the above hydrophobic interaction chromatography step was concentrated and further desalted on a Sephacryl S-200 gel filtration column equilibrated with 2 - 10 mM disodium hydrogen phosphate of pH 7.0 - 9.0. Desalted fraction collected and lyophilized to obtain pure hGH.

It is known that 0.5 to 5% acetonitrile cannot separate the clipped moieties from the recombinant hGH. (Gellerfors P et al. Acta Pediatr Scand (Suppl) (1990) 370, 93-100, Separation and identification of growth hormone variants by high performance liquid chromatography techniques In the present invention, it was found that use of acetonitrile in the range of 50%±10% effectively separates the unwanted clipped molecules first and subsequently the target molecule can be effectively separated with the purity of > 99.5 %. pH also plays an important role in the efficiency of separation. When the experiments were performed, it was observed that when the pH is acidic there was no effective separation whereas when the pH was increased above neutrality the separation became more and more effective. It was observed that pH range of 8 to 9 yields optimum purification and hence the desirable quality product (Figure 1, Figure 2). After conducting series of experiments it is concluded that pH does play an important role in achieving resolution between the clipped and intact hGH molecules using Resource Phenyl chromatography. Separation is most efficient at pH 8 - 9. Decreasing the pH below 8.0 reduces resolution (Figure 3) so much that at pH 6.0 both molecules elute as a single peak. In all the chromatograms the peak on the left corresponds to clipped hGH and on the right is of intact hGH.

Applicant tried various other techniques to separate clipped molecules from intact hGH were tried as mentioned herein below:

1. Ion-exchange with both polymer as well as sepharose beads- using aqueous buffer as well as mixture of aqueous-organic solvents and detergents
2. Hydrophobic interaction chromatography using sepharose beads of different hydrophobicities
3. Gel filtration chromatography uses native as well as reduced-denatured conditions

A brief account of the details of conditions used for purification and the outcome features below.

Experimental details

- Ion-exchange chromatography:** Ion exchange chromatography using Source 15 Q beads were tried to separate the intact from clipped hGH molecules using sodium chloride gradient for elution. (Figure 27)

Observation: No separation of Clipped molecules from intact hGH is observed in the above chromatographic process in any of the fractions analysed by SDS-PAGE gel.

Gel filtration chromatography-

Column: Superdex 75 HR 10/30, Amersham

5 Buffer: 20 mM Tris, pH 8.0 / 5 % Glycerol / 150 mM NaCl

Sample: hGH having clipped molecule –denatured in 6 M urea and reduced with 50 mM DTT for 2 hrs at RT

Detection: 280 nm

Fractions: 1 ml

10 System used: FPLC at 0.5 ml /min flow rate

Analyses: 10 ul of each fraction analysed on a SDS-PAGE gel followed by silver staining (Figure 28)

Observation: No separation of Clipped molecules from intact hGH is observed in the above chromatographic process in any of the fractions analysed by SDS-PAGE gel.

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Hydrophobic interaction Chromatography on Sepharose beads- (Figure 29)

Column: HiTrap 1 ml columns of Phenyl Sepharose FF (high sub) / Butyl Sepharose FF / Phenyl Sepharose HP / Octyl Sepharose FF

Buffer A: 20 mM Sodium phosphate, pH 7.0 / 0.5 M $(\text{NH}_4)_2\text{SO}_4$

20 Buffer B: 20 mM Sodium phosphate, pH 7.0

Sample: hGH having clipped molecule

Detection: 280 nm

Fractions: 1 ml

System used: AKTA Explorer at 1 ml /min flow rate

25 **Observation:** No separation of Clipped molecules from intact hGH is observed in the above chromatographic process

The following examples are illustrative of the invention but not to be construed to limit the scope of the present invention. The present invention has been described in terms of its specific embodiments and certain modifications and equivalents will be apparent to those skilled in the art and are intended to be included within the scope of present invention.

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EXAMPLES

Example 1

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 50 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 1**). Highly pure form of human growth hormone was obtained. (Purity : > 99.5%, Yield: 85%)

10 Example 2

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 40 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 2**). Highly pure form of human growth hormone was obtained. (Purity : > 99.5%, Yield: 85%)

Example 3

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 30 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule did not resolve from intact molecule (**Figure 3**).

25 Example 4

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 8.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 8.0 / 50 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 4**). Highly pure form of human growth hormone was obtained. (Purity : > 99.5%, Yield: 85%)

Example 5

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 7.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 7.0 / 50 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE (Figure 5). (Purity : >80%, Yield: 35%)

Example 6

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 6.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 6.0 / 50 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule did not resolve from intact molecule (Figure 6).

Example 7

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 50 % Methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 7). Highly pure form of human growth hormone was obtained. (Purity : > 99.5%, Yield: 85%)

Example 8

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 40 % Methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 8). Highly pure form of human growth hormone was obtained. (Purity : > 99.5%, Yield: 85%)

Example 9

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 30 % Methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule did not resolve from intact molecule (**Figure 9**). (Purity : >95 %, Yield: 45%)

Example 10

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 8.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 8.0 / 50 % methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 10**). Highly pure form of human growth hormone was obtained. (Purity >98%; Yield 80%)

Example 11

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 7.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 7.0 / 50 % methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 11**). (No purity achieved)

Example 12

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 6.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 6.0 / 50 % methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 12**). (No purity achieved)

Example 13

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x

6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 50 % isopropanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 13). Highly pure form of human growth hormone was obtained (Purity > 99%; Yield 85%).

Example 14

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 40 % isopropanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 14). Highly pure form of human growth hormone was obtained (Purity >98%; Yield 80%).

Example 15

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 30 % isopropanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 15) (Purity >95%; Yield 60%).

Example 16

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 8.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 8.0 / 50 % isopropanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 16) (Purity not achieved).

Example 17

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x

6.4 mm) equilibrated with 20 mM Na₂HPO₄/ 0.4 M K₂HPO₄, pH 7.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na₂HPO₄, pH 7.0 / 50 % isopropanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 17**) (no purity achieved).

Example 18

To the ion-exchange purified fraction of hGH, K₂HPO₄ is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na₂HPO₄/ 0.4 M K₂HPO₄, pH 6.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na₂HPO₄, pH 6.0 / 50 % isopropanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 18**) (no purity achieved).

Example 19

To the ion-exchange purified fraction of hGH, K₂HPO₄ is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na₂HPO₄/ 0.4 M K₂HPO₄, pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na₂HPO₄, pH 9.0 / 25 % acetonitrile / 25% methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 19**). Highly pure form of human growth hormone was obtained (Purity >99%; Yield 80%).

Example 20

To the ion-exchange purified fraction of hGH, K₂HPO₄ is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na₂HPO₄/ 0.4 M K₂HPO₄, pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na₂HPO₄, pH 9.0 / 25 % acetonitrile / 25% isopropanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (**Figure 20**). Highly pure form of human growth hormone was obtained (Purity >99%; Yield 85%).

Example 21

To the ion-exchange purified fraction of hGH, K₂HPO₄ is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x

6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 25 % isopropanol / 25% methanol. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 21). Highly pure form of human growth hormone was obtained (Purity >99%; Yield 85%).

Example 22

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a Resource Phenyl column (procured from Amersham Biosciences) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 9.0 / 50 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE according to the method of Laemmli. The gel was run at 25 mA for 45 min and thereafter silver stained to visualize the protein bands (Figure 22). The clipped hGH molecule eluted ahead of the intact GH molecules and is resolved as seen from the gel picture.

Example 23

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Tris/ 0.4 M K_2HPO_4 , pH 9.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Tris, pH 9.0 / 50 % acetonitrile. Fractions of 1 ml were collected and analysed by SDS-PAGE. The clipped hGH molecule eluted ahead of the intact GH molecules (Figure 23). Highly pure form of human growth hormone was obtained (Purity : > 99.5%, Yield: 85%).

Example 24

Resource PHE purified protein fraction was loaded to a 100 ml bed volume of Sephacryl S-200 HR column equilibrated with 9 mM disodium hydrogen phosphate buffer, pH 8.0. Column was eluted with the same buffer at 0.4 ml/min. Protein peak, detected at 280 nm was collected and lyophilised. Pure hGH (>99.5%) was obtained with a yield of >95% (Fig 24).

Example 25

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Resource Phenyl column (30 x 6.4 mm) equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 8.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 , pH 8.0. Fractions of 1 ml were collected and analysed by SDS-PAGE. (Figure 25). No resolution of clipped molecules and intact hGH molecules are obtained.

Example 26

To the ion-exchange purified fraction of hGH, K_2HPO_4 is added to a final concentration of 0.4 M. This was injected onto a 1 ml Phenyl Sepharose FF column equilibrated with 20 mM Na_2HPO_4 / 0.4 M K_2HPO_4 , pH 8.0. Bound proteins were eluted with a 20 ml linear gradient of 20 mM Na_2HPO_4 / 50% acetonitrile, pH 8.0. Fractions of 1 ml were collected and analysed by SDS-PAGE. (Figure 26). No resolution of clipped molecules and intact hGH molecules are obtained.

We claim,

1. A process for the purification of human growth hormone from its clipped moieties of hGH molecule, by using hydrophobic interaction chromatography technique, the said process comprising steps of:
 - 5 a. loading the sample on the column in presence of high concentration of a inorganic salt,
 - b. equilibrating the column of step (a) loaded with sample with an aqueous buffer,
 - c. eluting the equilibrated column of step (b) with linear gradient of an
10 aqueous buffer and organic solvent mixture,
 - d. collecting and combining the eluted fractions corresponding to hGH peak, concentrating the combined fractions,
 - e. desalting and lyophilizing the concentrated fractions of step (d) by
15 filtering on Sephacryl S-200 gel equilibrated with disodium hydrogen phosphate solution of pH ranging between 6.0-9.0, and
 - f. obtaining purified human growth hormone.
2. A process as claimed in claim 1, wherein in step (a) the sample used is semi-purified human growth hormone.
3. A process of claim 1, wherein in step (a) the column used is hydrophobic resin
20 comprising cross-linked polystyrene divinyl benzene polymer resin having attached hydrophobic ligand selected from the group consisting of ether, isopropyl, butyl, octyl and phenyl.
4. A process of claim 3, wherein the hydrophobic ligand is preferably phenyl.
5. A process of claim 1, wherein in step (a) the inorganic salt used is selected from
25 a group consisting of ammonium sulfate, disodium hydrogen phosphate, dipotassium hydrogen phosphate or sodium chloride, preferably disodium hydrogen phosphate, and most preferably dipotassium hydrogen phosphate.
6. A process of claim 1, wherein the salt concentration used is in the range of 0.2 - 0.6 M, more preferably 0.3 - 0.4 M.

7. A process of claim 1, wherein in step (b) the buffer used for equilibrating the column is a mixture of aqueous disodium hydrogen phosphate and dipotassium hydrogen phosphate.
8. A process of claim 7, wherein the pH of the equilibrating buffer ranges between 6.0 and 9.0
9. A process of claim 8, wherein the preferable pH ranges between 8.0 and 9.0.
10. A process of claim 1, wherein in step (c) the buffer used for eluting protein is a mixture of disodium hydrogen phosphate or Tris buffer and an organic solvent.
11. A process of claim 10, wherein the pH of the eluting buffer preferably ranges between 8.0 to 9.0
12. A process of claim 10, wherein the organic solvent used is selected from the group consisting of C₁ to C₄ alcohol, acetonitrile and/or mixtures thereof.
13. A process of claim 10, wherein the organic solvent used ranges between 40 and 70% v/v, preferably 40 to 50% v/v.
14. A process of claim 1, wherein the temperature for chromatography separation is preferably in the range of 20-30°C, more preferably 22-24°C.

AMENDED CLAIMS

[received by the International Bureau on 19 September 2005 (19.09.05);
Claims 1-14 replaced by amended claims 1-11
(2 pages)]

1. A process for the purification of human growth hormone from its clipped moieties of hGH molecule, by using hydrophobic interaction chromatography technique, the said process comprising steps of:
 - a. loading the sample on the column in presence of inorganic salt having concentration in the range of 0.2 – 0.5 M,
 - b. equilibrating the column of step (a) loaded with sample with an aqueous buffer having pH in the range of 6.0-9.0,
 - c. eluting the equilibrated column of step (b) with the linear gradient of an aqueous buffer and organic solvent in the range of 40 – 70% v/v,
 - d. collecting and combining the eluted fractions corresponding to hGH peak, concentrating the combined fractions,
 - e. desalting and lyophilizing the concentrated fractions of step (d) by filtering on Sephacryl S-200 gel equilibrated with disodium hydrogen phosphate solution of pH ranging between 6.0-9.0, and
 - f. obtaining purified human growth hormone.
2. A process as claimed in claim 1, wherein in step (a) the sample used is semi-purified human growth hormone.
3. A process of claim 1, wherein in step (a) the column used is hydrophobic resin comprising cross-linked polystyrene divinyl benzene polymer resin having attached hydrophobic ligand selected from the group consisting of ether, isopropyl, butyl, octyl and phenyl.
4. A process of claim 3, wherein the hydrophobic ligand is preferably phenyl.
5. A process of claim 1, wherein in step (a) the inorganic salt used is selected from a group consisting of ammonium sulfate, disodium hydrogen phosphate, dipotassium hydrogen phosphate or sodium chloride, preferably disodium hydrogen phosphate, and most preferably dipotassium hydrogen phosphate.

6. A process of claim 1, wherein in step (b) the aqueous buffer used for equilibrating the column is a mixture of aqueous disodium hydrogen phosphate and dipotassium hydrogen phosphate.
7. A process of claim 1, wherein in step (c) the aqueous buffer is either disodium hydrogen phosphate or Tris buffer.
8. A process of claim 1, wherein in the step (c) the organic solvent used is selected from the group consisting of C₁ to C₄ alcohol, acetonitrile and/or mixtures thereof.
9. A process of claim 1, wherein in step (c) the linear gradient range is preferably 40-50%.
10. A process of claim 1, wherein in step (c) the pH of the aqueous buffer and the organic solvent is preferably in the range of 6.0 - 9.0.
11. A process of claim 1, wherein the temperature for chromatography separation is preferably in the range of 20-30°C, more preferably 22-24°C.

STATEMENT UNDER ARTICLE 19(1)

The present invention relates to a commercial process for purification of recombinant human growth hormone using hydrophobic interactive chromatography, efficient to separate clipped hormone moiety present as impurity using a phenyl column, mixture of aqueous buffers and organic solvent as eluant, followed by desalting, lyophilization to obtain hGH of purity > 99.5%.

Applicant has gone through the written opinion of International Search Report and observes that:

- a) Novelty of claims 1- 14 acknowledged.
- b) Inventive step of claim 13 acknowledged.
- c) Inventive step of claims 1-12, and 14 lacking.
- d) Industrial Applicability of claims 1- 14 acknowledged.

ISR refers to six prior art documents D₁ to D₆. Written Opinion of International Search Report (ISR) reveals that process steps of the present invention could be arrived at from the cited prior art D₁ and D₂ and hence, the process lacks inventive step.

Applicant believes that their process is different from the process described in documents D₁ and D₂ for the following:

- a) Use of a high concentration of organic solvent in the range of 40-70%, preferably 40 – 50%.
- b) Elution does not make use of non-ionic detergent Brij 35.

In addition to the above the process of the present invention involves purification of the recombinant hGH of purity > 99.5% and with a high yield. This unusual result has been obtained by the use of a high concentration of organic solvent in the range of 40 to 70 % which has also been acknowledged in the ISR to have an inventive step and has not been suggested or taught in documents D₁ and D₂. Hence applicant has included this step in the main claim as a result of which all the other claims also fall into the category of having inventive step. No new subject matter has been added.