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(54) Title: A METHOD OF PURIFYING HUMAN GROWTH HORMONE AND PURIFIED GROWTH HORMONE THEREOF

(57) Abstract: The present invention relates to a method of purifying human growth hormone from a mixture containing at least one related impurity; and a purified growth hormone with purity of at least 85%.

A METHOD OF PURIFYING HUMAN GROWTH HORMONE AND PURIFIED GROWTH HORMONE THEREOF

FIELD OF THE INVENTION

The invention provides for a method for purifying a desired heterologous polypeptide resulting from methylotrophic yeast fermentation broth. Specifically, the present invention relates to a process for the purification of a human growth hormone. More particularly the instant invention employs a chaotrope and a detergent to purify the desired protein from pigment related contaminants generated during the methanol induction phase of the fermentation. The invention relates to a simplified and optimized purification process of a polypeptide particularly Human growth hormone from a composition comprising the said polypeptide and at least one related pigment impurity by chromatographic procedures enabling high yields, selectivity and purity of the desired end product.

BACKGROUND OF THE INVENTION

A number of proteins are expressed using yeast expression systems in view of various advantages attributed to employing them. They are ideally suited for large-scale production of recombinant eukaryotic proteins as being easily adaptable to fermentation, easier and less expensive to work with and well characterized known to perform large post-translational modifications. A large number of proteins are produced using *Pichia pastoris* as the expression host.

The target recombinant proteins produced during fermentation of methylotrophic yeast contain many impurities such as proteinous material other than target end product, non-proteinous materials like host-cell DNA, other biomolecules which are expressed extracellularly, pigment components, degraded proteins produced by proteolysis, pyrogenic components and other unutilized fermentation media ingredients which are soluble in cell free liquid and such other impurities.

However, one of the most undesirable attribute of employing yeasts such as Pichia pastoris for expression are the unwanted pigments produced by them. Most of these pigment impurities are produced normally /naturally during the methanol induction phase irrespective of the target recombinant product produced. The pigmentation of the

cell-free supernatant after removal of biomass, is intense, for example brownish, greenish brown, light green and such other pigmentations and combinations thereof. Some of these react with the target proteins, subsequently altering the nature of the end product. During the downstream purification process, the pigments bind to the target molecules reducing the loading capacity and the effective life span of the capturing matrix. This has a considerable impact on the economy of their capture through chromatography leading to reduced yields and lesser purity.

A purified protein is not easily separated from pharmaceutically unacceptable contaminants such as green fluorescent pigments secreted by P. pastoris. The chromatographic behavior of the pigments reflects on their polarity and adsorbality as influenced by their molecular structure. Extensive work has been done on characterizing the coloring pigments in pichia supernatants. It is understood that the yellow-green colour to pichia fermentation supernatant is because of AOX crystalloids which are though made inside the cell, give color to supernatant because of its constant leakage in the outside environment. The level of these pigments is increased with increased methanol concentration (L. M. Damasceno et. al., Protein Expression & purification 37 (2004) 18-26).

Aforesaid problems are more intensely characterized during expression of certain specific proteins such as Human growth hormone. Human growth hormone has very high density of non polar amino acids on surface. This makes it a highly hydrophobic protein. Because of this nature, growth hormone interacts with other hydrophobic protein surfaces. Additionally, Pichia expresses huge amount of AOX crystalloids, resulting in a strong possibility that the growth hormone interacts with these crystalloids by multiple interactions thereby changing hydrophobic and charge properties of growth hormone.

Accordingly there is a perceived need to address the technical problem of removal of impurities from the fermentation broth containing the targeted end products and more particularly the problem of removing pigment impurities. Desirable is a purification method that can be conducted with as few chromatographic steps as possible in order to keep technical complexity and costs on a low level and to avoid high losses of protein. First there is an obvious need to purify the related pigment impurities and further also

obtain higher yields and purity of the target protein. Methods whereby all contaminating proteins or impurities are at least partially, preferably completely, removed in a single purification step are preferred over the prior art methods.

As per Pickford et al.,(Methods in Molecular Biology, Vol 278: Protein NMR Techniques) cation exchange is the most convenient first step procedure that serves not only to partially purify the recombinant protein from Pichia supernatant but also removes the contaminating AOX crystalloid pigment. The proteins which are acid labile, or insoluble at acidic pH or is highly acidic, alternative techniques should be worked out. Cation exchange has been extensively used for capture of insulin precursor from Pichia supernatant (WO/2007/043059). US Patent 7351801 discloses a method to capture recombinant human albumin by using Cation exchanger. The insulin analogues like Glargine insulin, Insulin Aspart and Lispro made in Pichia pastoris are also conveniently captured by Cation exchange (Partha et. al. unpublished data). In all of these methods, protein of interest bind to the Cation exchanger and the contaminating pigments flows through.

The various other kinds of chromatographic purifications techniques employed are adsorption chromatography, hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC), affinity chromatography, and reverse phase chromatography.

This application is directed to various aspects of an invention which provides an overall solution to what has emerged as a generalized problem. The present invention relates to a simple yet effective purification procedure that enables the efficient removal of the contaminating pigments thereby providing a more efficient, effective, cheaper subsequent downstream purification process resulting in increased recovery of the target protein.

A highly advantageous process which forms the basis of this invention has been identified for enhancing the purity of target end protein from pigment contaminations thereof obtained via recombinant DNA methods using methylotrophic yeast expression systems.

OBJECTS OF THE INVENTION

The main object of the present invention is to obtain a method of purifying human growth hormone from a mixture containing at least one related impurity.

Another object of the present invention is to obtain growth hormone with purity of at least 85%.

STATEMENT OF THE INVENTION

Accordingly, the present invention relates to a method of purifying human growth hormone from a mixture containing at least one related impurity, said impurity being a pigment impurity comprising contacting the mixture with a detergent and a chaotrope for a sufficient contact time under conditions that enable breakage of interaction between the human growth hormone and the pigment; a method of purifying human growth hormone from a mixture containing at least one related impurity, said impurity being a pigment impurity comprising contacting the mixture with a detergent and a chaotrope for a sufficient contact time under conditions that enable breakage of interaction between the human growth hormone and the pigment thereby subjecting mixture to chromatography comprising steps: (a) packing the chromatographic column with a silica based polymer resin equilibrated with a buffer; (b) loading the mixture on the column at a flow rate of at least about 150cm/h cm/hr; and (c) eluting purified product from the column with 0.5M sodium chloride, 0.3M of L-arginine and 5% mannitol in 50 mM Phosphate buffer; and purified growth hormone with purity of at least 85%.

BRIEF DESCRIPTION OF ACCOMPANYING FIGURES

FIG 1: Chromatogram depicting purity of hGH final product being 97.8%. The chromatogram shows the RP-HPLC purity of hGH at the end of the complete process. RP-HPLC method: using C 4 column 250x 4.6mm 300Ao 5u. Flow rate 0.5ml/min 29% n-propanol in Tris 50mM pH 7.5. monitored at 220nm. Column thermostat is maintained at 45°C.

FIG 2: Chromatogram depicting HMW purity of 99.1%. The chromatogram shows the SE-HPLC purity of hGH at the end of the complete process. SE-HPLC method: using

G2000 SWxl column 300x 7.8mm 125A° 5u. Flow rate 0.5ml/min. 63mM phosphate buffer pH 7.0 3% 2-propanol. Monitored at 214nm.

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to a method of purifying human growth hormone from a mixture containing at least one related impurity, said impurity being a pigment impurity comprising contacting the mixture with a detergent and a chaotrope for a sufficient contact time under conditions that enable breakage of interaction between the human growth hormone and the pigment.

In another embodiment of the present invention, the breakage of interaction is afforded by incubation of the mixture with at least 2% of the detergent and at least 2M of the chaotrope.

In yet another embodiment of the present invention, the detergent is selected from a group comprising Triton and Tween.

In still another embodiment of the present invention, the chaotrope is selected from a group comprising guanidine hydrochloride, guanidinium hydrochloride, sodium thiocyanate and urea.

In still another embodiment of the present invention, pH is maintained at 2.

In still another embodiment of the present invention, the mixture is incubated for at least 2 hours.

In still another embodiment of the present invention, the mixture is subsequently subjected to one or more chromatography steps.

The present invention relates to a method of purifying human growth hormone from a mixture containing at least one related impurity, said impurity being a pigment impurity comprising contacting the mixture with a detergent and a chaotrope for a sufficient contact time under conditions that enable breakage of interaction between the human growth hormone and the pigment thereby subjecting mixture to chromatography comprising steps:

a) packing the chromatographic column with a silica based polymer resin equilibrated with a buffer;

b) loading the mixture on the column at a flow rate of at least about 150cm/h cm/hr; and

c) eluting purified product from the column with 0.5M sodium chloride, 0.3M of L-arginine and 5% mannitol in 50 mM Phosphate buffer.

In still another embodiment of the present invention, the yield is at least 90%.

In still another embodiment of the present invention, the human growth hormone obtained has a purity of at least 80%.

In still another embodiment of the present invention, the human growth hormone obtained has a purity of at least 85%.

In still another embodiment of the present invention, the human growth hormone obtained has a purity of at least 90%.

In still another embodiment of the present invention, the human growth hormone obtained has a purity of at least 95%.

In still another embodiment of the present invention, the human growth hormone obtained has a purity of 100%.

The present invention relates to a purified growth hormone with purity of at least 85%.

This invention is directed to a process for separating pigment related impurities from an impure mixture containing at least one related impurity and the resultant expressed target protein leading to substantially complete recovery of said end product.

More specifically, the invention relates to a purification of Human growth hormone.

The invention further relates to the use of a mixture of non-ionic detergents and chaotropic salts to separate target human growth molecule from pigment crystalloids to obtain a purified Human growth hormone.

It is an object of the present invention to provide for a subsequent chromatographic medium/solvent system wherein the process is based upon Hydrophobic Interaction

chromatography [HIC], cation-exchange chromatography and anion exchange chromatography.

In a broad aspect, the present invention relates to a chromatographic process for purifying a protein particularly Human growth hormone from a mixture comprising said protein and related pigment impurities, comprising the steps of:

Separating said protein and said related pigment impurities by incubation with a chaotrope and detergent, loading solution of the pre-incubated protein on the column at a desired flow rate, eluting the purified protein product by performing a isocratic elution with an optimized buffer system.

Those skilled in the art will recognize that there are various variables which can be adjusted during the chromatographic procedures of the present invention. Such variables include loading and eluting conditions, such as ionic strength, buffer composition, pH, temperature, addition of a small amount of an organic solvent, etc. However, such variables are routinely adjusted in this field and those skilled in the art can readily establish optimum conditions.

Before explaining at least one embodiment of the invention in detail by way of experimentation, results, and procedures, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings, experimentation and/or results. The invention is capable of other embodiments or of being practiced or carried out in various ways. As such, the language used herein is intended to be given the broadest possible scope and meaning; and the embodiments are meant to be exemplary, not exhaustive. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason.

Reference will now be made in detail to the presently preferred embodiments of the invention which, together with the following example, serve to explain the principles of

the invention. The Examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be construed to, limit its scope in any way.

DEFINITIONS:

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out herein. Thus, for example, reference to "hGH" is a reference to one or more such proteins and includes equivalents thereof known to those of ordinary skill in the art, and so forth.

The term "protein" as used herein, includes a polymer or complex of various polymers of amino acids and does not connote a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, and polypeptide are included within the definition of protein, whether produced using recombinant techniques, chemical or enzymatic synthesis, or naturally occurring. The term also includes peptides, oligopeptides, and polypeptides that have been modified or derivatized, such as by glycosylation, acetylation, phosphorylation, and the like. The term "protein" specifically includes variants, as defined herein. The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. That is, a description directed to a polypeptide applies equally to a description of a peptide and a description of a protein, and vice versa.

As used herein, "growth hormone" or "GH" shall include those polypeptides and proteins that have at least one biological activity of a human growth hormone, as well as GH analogs, GH isoforms, GH mimetics, GH fragments, hybrid GH proteins, fusion proteins, oligomers and multimers, homologues, glycosylation pattern variants, variants, splice variants, and muteins, thereof, regardless of the biological activity of

same, and further regardless of the method of synthesis or manufacture thereof including, but not limited to, recombinant (whether produced from cDNA, genomic DNA, synthetic DNA or other form of nucleic acid), in vitro, in vivo, by microinjection of nucleic acid molecules, synthetic, transgenic, and gene activated methods.

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. It has eight tyrosines and one tryptophan which make it highly hydrophobic protein. The term "hGH polypeptide" or "hGH" encompasses hGH polypeptides comprising one or more amino acid substitutions, additions or deletions. Exemplary substitutions in a wide variety of amino acid positions in naturally-occurring hGH including substitutions that increase agonist activity, increase protease resistance, convert the polypeptide into an antagonist, modulate immunogenicity, modulate receptor binding, etc. are encompassed by the term "hGH polypeptide."

The term "hGH polypeptide" also includes glycosylated hGH, such as but not limited to, polypeptides glycosylated at any amino acid position, N-linked or O-linked glycosylated forms of the polypeptide. Variants containing single nucleotide changes are also considered as biologically active variants of hGH polypeptide. In addition, splice variants are also included. The term "hGH polypeptide" also includes hGH polypeptide heterodimers, homodimers, heteromultimers, or homomultimers of any one or more hGH polypeptides or any other polypeptide, protein, carbohydrate, polymer, small molecule, linker, ligand, or other biologically active molecule of any type, linked by chemical means or expressed as a fusion protein, as well as polypeptide analogues containing, for example, specific deletions or other modifications yet maintain biological activity.

The term "substantially purified" refers to hGH polypeptide that may be substantially or essentially free of components that normally accompany or interact with the protein as found in its naturally occurring environment, i.e. a native cell, or host cell in the case of recombinantly produced hGH polypeptide. hGH that may be substantially free of cellular material includes preparations of protein having less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, less than

about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% (by dry weight) of contaminating ingredient.

When the hGH polypeptide or variant thereof is recombinantly produced by the host cells, in this case methylotrophic yeast cells, the protein may be present at about 30%, about 25%, about 20%, about 15%, about 10%, about 5%, about 4%, about 3%, about 2%, or about 1% or less of the dry weight of the cells. When the hGH polypeptide or variant thereof is recombinantly produced by the host cells, the protein may be present in the culture medium at about 5 g/L, about 4 g/L, about 3 g/L, about 2 g/L, about 1 g/L, about 750 mg/L, about 500 mg/L, about 250 mg/L, about 100 mg/L, about 50 mg/L, about 10 mg/L, or about 1 mg/L or less of the dry weight of the cells. Thus, "substantially purified" hGH polypeptide as produced by the methods of the present invention may have a purity level of at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, specifically, a purity level of at least about 75%, 80%, 85%, and more specifically, a purity level of at least about 90%, a purity level of at least about 95%, a purity level of at least about 99% or greater

An "impurity" is a material that is different from the desired polypeptide product or protein of interest. The impurity includes, but is not limited to pigment contaminants with specific reference to AOX crystalloid pigments. The pigmentation of the cell-free supernatant after removal of biomass, is intense, for example brownish, greenish brown, light green and such other pigmentations and combinations thereof.

"Fermenting Organism" refers to any microorganism suitable for use in a desired fermentation process. Examples of fermentating organisms include fungal organisms such as yeasts. More specifically the methylotrophic yeast. Examples of fermenting organisms in context of the present invention are Pichia pastoris, Pichia sp., Saccharomyces sp., Saccharomyces cerevisiae, Kluyveromyces sp., or Hansenula polymorpha.

The term "recombinant", as used herein describes a protein or polypeptide means a polypeptide produced by expression of a recombinant gene in a host organism. The term "recombinant", as used herein in reference to cells, means cells that can be or have

been used as recipients for recombinant vectors or other transfer DNA, and include progeny of the original cell which has been transfected.

The term "chromatography" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term "High Performance liquid chromatography", as used herein, refers to that chromatographic procedure in which the particles (stationary phase) used in the column packing are small (between 3 and 50 microns) and regular with little variation from the selected size. Such chromatography typically employs relatively high (around 500-3500 psi) inlet pressures.

The term "ion-exchange" and "ion-exchange chromatography" refers to the chromatographic process in which a solute of interest (such as a protein) in a mixture interacts with a charged compound linked (such as by covalent attachment) to a solid phase ion exchange material such that the solute of interest interacts non-specifically with the charged compound more or less than solute impurities or contaminants in the mixture. The contaminating solutes in the mixture elute from a column of the ion exchange material faster or slower than the solute of interest or are bound to or excluded from the resin relative to the solute of interest. "Ion-exchange chromatography" specifically includes cation exchange, anion exchange, and mixed mode chromatography. The Cation-exchange chromatography step may follow the RP-HPLC step, or vice versa. Preferably, the cation exchange chromatography step is followed by other chromatographic steps.

"Cation exchange chromatography" is a process in which positively charged ions bind to a negatively charged resin.

"Ion exchange chromatography resin" or "ion exchange resin" refers to a solid support to which are covalently bound ligands that bear a positive or negative charge, and which thus has free counterions available for exchange with ions in a solution with which the ion exchange resin is contacted.

A chaotropic agent is a compound, including, without limitation, guanidine hydrochloride (guanidinium hydrochloride, GdmHCl), sodium thiocyanate, urea and/or a detergent which disrupts the noncovalent intermolecular bonding of the protein, permitting the breakage of interaction of the pigment contaminants with the amino acids of the target protein. The use of the chaotropic agent ranges in concentration from about 2 M to about 8 M. Suitable incubation conditions, such as temperature and length of incubation may be readily determined for each agent to be used.

Buffering agents are advantageously used in chromatographic purifications to maintain a desired pH value or pH range. Inorganic buffer systems (phosphate, carbonate, among others) and organic buffer systems (citrate, Tris, MOPS, MES, HEPES, among others) are well known to the art.

Examples of suitable binding buffers include those that modify surface charge of an analyte and/or binding moieties, such as pH buffer solutions. pH buffer solutions preferably are strong buffers, sufficient to maintain the pH of a solution in the acidic range, i.e., at a pH less than 7, preferably less than 6.8, 6.5, 6.0, 5.5, 5.0, 4.0 or 3.0; or in the basic range at a pH greater than 7, preferably greater than 7.5, 8.0, 8.3, 8.5, 9.0, 9.3, 10.0 or 11.0. The pH conditions suitable for purifying a target protein group from a sample comprising the target protein group and contaminating proteins range from about 3.5 to about 11, from about 4.0 to about 10.0, from about 4.5 to about 9.5, from about 5.0 to about 9.0, from about 5.5 to about 8.5, from about 6.0 to about 8.0, or from about 6.5 to about 7.5. Typically, binding buffers have a pH range of about 6.5 to about 7.5. In an alternative embodiment of the present invention, binding buffers have a pH range of about 6.5 to about 8.5.

The cation exchange chromatography step may be performed using any suitable cation exchange matrix. Useful cation exchange matrices include, but are not limited to, fibrous, porous, non-porous, microgranular, beaded, or cross-linked cation exchange matrix materials. Such cation exchange matrix materials include, but are not limited to, cellulose, agarose, dextran, polyacrylate, polyvinyl, polystyrene, silica, polyether etc. The cation exchange matrix may be any suitable cation exchanger including strong and weak cation exchangers. Strong cation exchangers may remain ionized over a wide pH range and thus, may be capable of binding hGH over a wide pH range. Weak cation

exchangers, however, may lose ionization as a function of pH. For example, a weak cation exchanger may lose charge when the pH drops below about pH 4 or pH 5. Suitable strong cation exchangers include, but are not limited to, charged functional groups such as sulfopropyl (SP), methyl sulfonate (S), or sulfoethyl (SE). The cation exchange matrix may be a strong cation exchanger, having an hGH binding pH range of about 2.5 to about 6.0. Alternatively, the strong cation exchanger may have a hGH binding pH range of about pH 2.5 to about pH 5.5. The cation exchange matrix may be a strong cation exchanger having an hGH binding pH of about 3.0. Alternatively, the cation exchange matrix may be a strong cation exchanger, having an hGH binding pH range of about 6.0 to about 8.0. The cation exchange matrix may be a strong cation exchanger having an hGH binding pH range of about 8.0 to about 12.5. Alternatively, the strong cation exchanger may have hGH binding pH range of about pH 8.0 to about pH 12.0.

"Non-ionic detergents" are considered to be "mild" detergents because they are less likely than ionic detergents to denature proteins. By not separating protein-protein bonds, non-ionic detergents allow the protein to retain its native structure and functionality, although detergents with shorter hydrophobic chain lengths are more likely to cause protein deactivation. poly(oxyethylene) ethers and related detergents have a neutral, polar head and hydrophobic tails that are oxyethylene polymers (e.g. TWEEN®) or ethyleneglycoether polymers (e.g. TRITON®). The tert-octylphenol poly (ethyleneglycoether) series of detergents, which Includes TRITON X-100, have an aromatic head that interferes with downstream UV analysis techniques (Detergents and solubilization reagents, Biofiles V 3 N0 3, Sigma Aldrich).

Human growth hormone has very high density of non polar amino acids on surface. This makes it highly hydrophobic protein. Because of this growth hormone interacts with other hydrophobic protein surfaces. As along with growth hormone, pichia expresses huge amount of AOX crystalloids, there is strong possibility that growth hormone interacts with these crystalloids by multiple interaction and thus changes hydrophobic and charge properties of growth hormone. This can be the probable reason for it to not interact with the hydrophobic as well as cation and anion exchange resins. The possible interactions between growth hormone and crystalloids are ionic as well as hydrophobic. So to exploit the property of growth hormone during purification, there is

a need to break both types of interactions. The use of urea as one of the preferred reagent in the invention is explained by its use in breaking varied interactions in refractile bodies formed during *E. Coli* expressions. The mechanism which underlines the role of chaotrope shows by molecular dynamics simulations that a 7 M aqueous urea solution unfolds a chain of purely hydrophobic groups which otherwise adopts a compact structure in pure water. The unfolding process arises due to a weakening of hydrophobic interactions between the polymer groups. It is also understood that the attraction between two model hydrophobic plates, and graphene sheets, is reduced when urea is added to the solution. The action of urea is found to be direct, through its preferential binding to the polymer or plates. It is, therefore, acting like a surfactant capable of forming hydrogen bonds with the solvent. The preferential binding and the consequent weakened hydrophobic interactions are driven by enthalpy and are related to the difference in the strength of the attractive dispersion interactions of urea and water with the polymer chain or plate. It is understood that the indirect mechanism, in which urea acts as a chaotrope, is not a likely cause of urea's action as a denaturant. These findings suggest that, in denaturing proteins, urea (and perhaps other denaturants) forms stronger attractive dispersion interactions with the protein side chains and backbone than does water and, therefore, is able to dissolve the core hydrophobic region.

The non ionic detergents are largely regarded as the hydrophobic bond breakers. Thus their use in this invention is justified.

Pichia supernatant containing hGH was incubated with chaotrope and detergent and mixed for certain duration. Following different experiments indicate what combinations of detergent and chaotrope are optimum for breaking the interaction between protein and pigments.

One of the most significant aspects of the present invention involves incubating Pichia supernatant containing hGH with an optimal quantity of a chaotrope and detergent for a sufficient contact time to enable the breakage of interaction between hGH and the pigments. Resultant target hGH polypeptide is conveniently purified chromatographically substantially devoid of pigment related impurities.

In one embodiment of the inventive method, the Pichia supernatant containing hGH is incubated with at least 2M solution of the chaotrope and 2% of the detergent for at least about 2 hours at room temperature and diluted with water to get conductivity less than 12mS/cm that allows the breakage of interaction between the hGH and the pigment impurities.

Preferably, the incubation process is carried out at a pH from about pH 1 to about pH 11. Even more preferably, the contacting process is carried out at a pH from about pH 1 to about pH 5. Most preferably, the contacting process is carried out at a pH of about pH 1.5 to about pH 4. The most preferred pH is 2.5-3.5

A method of purifying human growth hormone from a mixture containing at least one related impurity, said impurity being a pigment impurity comprising contacting the mixture with a detergent and a chaotrope for a sufficient contact time under conditions that enable the breakage of the interaction between the human growth hormone and the pigment thereby subjecting mixture to chromatography comprising steps:

a) packing the chromatographic column with agarose based resin equilibrated with a buffer.
b) loading the mixture on the column at a flow rate of at least about 150cm/h cm/hr.
c) eluting the purified product from the column with 0.5M sodium chloride, 0.3M of L-arginine, and 5% mannitol in 50 mM Phosphate buffer pH 8.

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

The inventors tried various other techniques to purify hGH. Those that were tried as mentioned herein below. Ion-exchange with both HIC resins-using aqueous buffer as well as mixture of aqueous-organic solvents was investigated.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to

constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1:

The Pichia pastoris supernatant containing HGH was adjusted to 5% acetonitrile and conductivity of 23mS/cm by Sodium sulfate adjusted to pH 3 using acetic acid. This sample was loaded on to onto a 25 ml Amberlite XAD 7 column (12.5 x 1.6cm) equilibrated with 50 mM Sodium Acetate 150mM Sodium sulfate, 5% acetonitrile pH 3. Bound proteins were eluted with a 50% acetonitrile in Tris 50mM pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. The 47% of loaded protein was found in flow through.

TABLE: 1

	Volume (mL)	Protein (mg)	Yield (%)	Purity (%)
Load	180	125	100	83.3
FT	190	58	47	83.3
Elution	50	Nil	0	

EXAMPLE 2

The Pichia pastoris supernatant containing hGHwas adjusted to conductivity of 23mS/cm by Sodium sulfate, 5% acetonitrile and adjusted to pH 3 using acetic acid. This sample was loaded on to onto a 25 ml Diaion HP 20 SS column pre-swelled in solvent (12 .5 x 1.6cm) equilibrated with 50 mM Sodium Acetate 150mM Sodium sulfate pH 3& 5% acetonitrile. Bound proteins were eluted with a 70% acetonitrile in 50mM acetate buffer pH 3. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. The 93% of loaded protein was found in flow through.

TABLE: 2

	Volume (mL)	Protein (mg)	Yield (%)	Purity (%)
Load	270	180	100	77.14
FT	290	168	93	77.14
Elution	33	---		

EXAMPLE 3

The Pichia pastoris supernatant containing hGHwas adjusted to conductivity of 23mS/cm by Sodium sulfate, 5% acetonitrile and adjusted to pH 3 using acetic acid. This sample was loaded onto a 25 ml Toyopearl Super Butyl 550C column (12.5 x 1.6cm) equilibrated with 50 mM Sodium Acetate 150mM Sodium sulfate pH 3. Bound proteins were eluted with a 40% acetonitrile in Tris buffer 50mM pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method.

TABLE: 3

	Volume (mL)	Protein (mg)	Yield (%)	Purity (%)
Load	200	135	100	83.4
FT	230	118	88	83.4
Elution	50	0		

The 88% of loaded protein was found in flow through.

RESULTS AND DISCUSSION:

Reasons for failure of Polymeric & HIC resins

None of the hydrophobic and reversed phase matrices could retain the protein as well as the pigment material. The probable reasons for this kind of behavior might be saturation of binding surface by contaminating pigment or proteins. Even though matrices used had very good binding capacities and the protein in question being hydrophobic, the protein was not retained by the column. It was presumed to be also

because of the contaminating pigments and host related proteins coating the hydrophobic patches of protein.

EXAMPLE 4

The Pichia pastoris supernatant containing hGH was adjusted to pH 3 by acetic acid and diluted with water to get conductivity not more than 5.5mS/cm.0.5% Tween 20 was added to the load. This sample was loaded onto a 25 ml S hyper D columns (12.5 x 1.6cm) equilibrated with 50 mM acetate buffer 0.5% Tween 20, 30mM of sodium chloride pH3. Bound proteins were eluted with a 0.5M sodium chloride, 0.4M of L-arginine, 2M urea & 5% mannitol in 50 mM Tris buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 71% of loaded protein was found in flow through.

TABLE: 4

	Volume (mL)	Protein (mg)	Yield (%)	Purity (%)
Load	150	107.5	100	79.3
FT	650	77	71	79.3
Elution	25	0		

EXAMPLE 5

The Pichia pastoris supernatant containing hGHwas adjusted to pH 3 by acetic acid and diluted with water to get conductivity not more than 11mS/cm.0.5% Tween 20 was added to the load. This sample was loaded onto a 25 ml SP Sepharose 4FF column (12.5 x 1.6cm) equilibrated with 50 mM acetate buffer 0.5% tween 20, 80mM of sodium chloride pH 3. Bound proteins were eluted with a 0.5M sodium chloride, 0.4M of L-arginine, 2M urea & 5% mannitol in 50 mM Tris buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 96% of loaded protein was found in flow through.

TABLE: 5

	Volume (mL)	Protein (mg)	Yield (%)	Purity (%)
Load	150	105	100	86.4
FT	575	101	96	86.4
Elution	50	0		

EXAMPLE 6

Higher conductivity was assumed as the probable reason for the lesser binding of hGH on SP sepharose. The next experiment was done by lowering the conductivity of broth to 8mS/cm.

The Pichia pastoris supernatant containing hGHwas adjusted to pH 3 by acetic acid and diluted with water to get conductivity not more than 8mS/cm.0.5% Tween 20 was added to the load. This sample was loaded onto a 25 ml SP Sepharose 4FF column (12.5 x 1.6cm) equilibrated with 50 mM acetate buffer 0.5% tween 20, 50mM of sodium chloride pH 3. Bound proteins were eluted with a 0.5M sodium chloride, 0.4M of L-arginine, 2M urea & 5% mannitol in 50 mM Tris buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 80% of loaded protein was found in flow through.

TABLE: 6

	Volume (mL)	Protein (mg)	Yield (%)	Purity (%)
Load	150	106	100	86.4
FT	600	84	80	86.4
Elution	50	13	12	

EXAMPLE 7

Pichia supernatant contains various metals like Ca, Mg, Mn, Cu etc. These metal ions are present in the significant amount in Pichia supernatant containing hGH which can compete with proteins for binding on cation exchange resins because of strong positive charge. To nullify the effect of these metal ions, 2mM EDTA is added.

The Pichia pastoris supernatant containing hGH was adjusted to pH 3 by acetic acid and diluted with water to get conductivity not more than 8mS/cm.0.5% Tween 20 & 2mM EDTA was added to the load. This sample was loaded onto a 25 ml SP Sepharose 4FF column (12.5 x 1.6cm) equilibrated with 50 mM acetate buffer 0.5% tween 20, 50mM of sodium chloride pH 3. Bound proteins were eluted with a 0.5M sodium chloride, 0.4M of L-arginine, 2M urea & 5% mannitol in 50 mM Tris buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 72% of loaded protein was found in flow through.

TABLE: 7

	Volume(ml)	Protein (mg)	Yield (%)	Purity (%)
Load	150	105	100	86.4
FT	550	76.5	72	86.4
Elution	50	15	14	86.4

It was understood from all above experiments that the charge and hydrophobic properties of the protein are masked. The attempts were targeted at creating conditions which does not favor hydrophobic interaction between HGH and pigments so that the protein is not coated and charge properties of the protein can be exploited. As it is known that the hydrophobic interactions are weak at higher pH, attempts were being made to capture protein at higher pH on anion exchange.

EXAMPLE 8

The Pichia pastoris supernatant containing hGHwas buffer exchanged to 10mM Phosphate buffer pH 8 & 2% tween 20 was added to the load. This sample was loaded onto a 25 ml Toyopearl Super Q 650 M columns (12.5 x 1.6cm) equilibrated with 10 mM phosphate 2% tween 20 pH 8. Bound proteins were eluted with a 0.5M sodium chloride in 10 mM Phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 90% of loaded protein was found in flow through.

TABLE: 8

	Volume (mL)	Protein(mg)	Yield (%)	Purity (%)
Load	200	135	100	82.3
FT	1000	122	90	83
Elution	30	13	9.6	80

EXAMPLE 9

The Pichia pastoris supernatant containing hGH was incubated with 5% Triton X 100 and 2M urea at pH 2.5 for 2hours at room temperature and diluted with water to get conductivity less than 12mS/cm. This sample was loaded onto a 24 ml SP Sepharose 4FF column (12 x 1.6cm) equilibrated with Phosphate buffer pH 2.5. Bound proteins were eluted with a 0.5M sodium chloride, 0.3M of L-arginine & 5% mannitol in 50 mM Phosphate buffer pH 8 and 40% acetonitrile in 50mM phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method.

100% protein was bound to the column. 78% of protein was recovered in the elution. As the triton interferes with the purity analysis, estimating purity for these experiments was not possible.

TABLE: 9

	Volume (mL)	Protein(mg)	Yield (%)
Load	300	390	100
FT	1450	0	0
Elution	160	306	78

EXAMPLE 10

The Pichia pastoris supernatant containing hGH was incubated with 3% Triton X 100 and 2M urea at pH 2.5 for 2hours at room temperature and diluted with water to get conductivity less than 12mS/cm. This sample was loaded onto a 30 ml SP Sepharose 4FF column (15 x 1.6cm) equilibrated with Phosphate buffer pH 2.5. Bound proteins were eluted with a 0.5M sodium chloride, 0.3M of L-arginine, & 5% mannitol in 50 mM Phosphate buffer pH 8 and 40% acetonitrile in 50mM phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 100% protein was bound to the column.

79% of protein was recovered in the elution. As the triton interferes with the purity analysis, estimating purity for these experiments was not possible.

TABLE: 10

	Volume (mL)	Protein(mg)	Yield (%)
Load	520	360	100
FT	2500	0	
Elution	150	286	79

EXAMPLE 11

The Pichia pastoris supernatant containing hGH was incubated with 2% Tween 20 and 5M urea at pH 2.5 for 2 hours at room temperature and diluted with water to get conductivity less than 12mS/cm. This sample was loaded onto a 25 ml SP Sepharose 4FF column (15 x 1.6cm) equilibrated with Phosphate buffer pH 2.5. Bound proteins were eluted with a 0.5M sodium chloride, 0.3M of L-arginine, & 5% mannitol in 50 mM Phosphate buffer pH 8 and 40% acetonitrile in 50mM phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method.

34% of loaded protein was found in flow through. 32% of protein was recovered in the elution.

TABLE: 11

	Volume (mL)	Protein(mg)	Yield (%)	Purity (%)
Load	750	345	100	80.8
FT	3600	120	34	80.8
Elution	50	110	32	80.8

EXAMPLE 12

The Pichia pastoris supernatant containing hGH was incubated with 3% Tween 20 and 5M urea at pH 2.5 for 2 hours at room temperature and diluted with water to get conductivity less than 12mS/cm. This sample was loaded onto a 25 ml SP Sepharose 4FF column (15 x 1.6cm) equilibrated with Phosphate buffer pH 2.5. Bound proteins were eluted with a 0.5M sodium chloride, 0.3M of L-arginine, & 5% mannitol in 50 mM Phosphate buffer pH 8 and 40% acetonitrile in 50mM phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method.

No protein was detected in flow through. 70% of protein was recovered in the elution.

TABLE: 12

	Volume (mL)	Protein(mg)	Yield (%)	Purity (%)
Load	750	360	100	89.5
FT	3600			
Elution	140	252	70	89.5

EXAMPLE 13

The Pichia pastoris supernatant containing hGH was incubated with 3% Tween 80 and 5M urea at pH 2.5 for 2 hours at room temperature and diluted with water to get conductivity less than 12mS/cm. This sample was loaded onto a 25 ml SP Sepharose 4FF column (15 x 1.6cm) equilibrated with Phosphate buffer pH 2.5. Bound proteins were eluted with a 0.5M sodium chloride, 0.3M of L-arginine, & 5% mannitol in 50 mM Phosphate buffer pH 8 and 40% acetonitrile in 50mM phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method.

No protein was detected in flow through. 92% of protein was recovered in the elution.

TABLE: 13

	Volume (mL)	Protein(mg)	Yield (%)	Purity (%)
Load	400	285	100	66.5
FT	2500	0		
Elution	180	262	92	64

EXAMPLE 14

The Pichia pastoris supernatant containing hGH was incubated with 2% Triton X 114 and 5M urea at pH 2.5 for 2 hours at room temperature and diluted with water to get

conductivity less than 12mS/cm. This sample was loaded onto a 25 ml SP Sepharose 4FF column (15 x 1.6cm) equilibrated with Phosphate buffer pH 2.5. Bound proteins were eluted with a 0.5M sodium chloride, 0.3M of L-arginine & 5% mannitol in 50 mM Phosphate buffer pH 8 and 40% acetonitrile in 50mM phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. No protein was detected in flow through. 67% of protein was recovered in the elution. As the triton interferes with the purity analysis, estimating purity for these experiments was not possible.

TABLE: 14

	Volume (mL)	Protein(mg)	Yield (%)
Load	185	242	100
FT	1000	0	
Elution	140	160	67

EXAMPLE 15:

CAPTURE OF THE PROTEIN FROM SUPERNATANT

The Pichia pastoris supernatant containing hGH was incubated with 2% Triton X 100 and 3M urea at pH 2.5 for 2 hours at room temperature and diluted with water to get conductivity less than 12mS/cm. This sample was loaded onto a 250 ml SP Sepharose 4FF column (130 x 50mm) equilibrated with Phosphate buffer pH 2.5. Bound proteins were eluted with a 0.5M sodium chloride, 0.3M of L-arginine & 5% mannitol in 50 mM Phosphate buffer pH 8 and 40% acetonitrile in 50mM phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. No protein was detected in flow through. 67% of protein was recovered in the elution.

TABLE: 15

	Components	pH	Conductivity (mS/cm)	Volume (mL)	Purity by RP HPLC	Protein (mg)	Recovery (%)
Eq buffer	Water + o-PA+ NaOH	2.5	4.1	1375	0	0	
Load	Sample + water	2.5	7.5-8.0	4200	85.6	6174	
Wash	Eq buffer	2.5	4.1	1375	0	0	
Elution 1	0.5 M NaCl+ 0.3 M Arginine + 5% Mannitol + phosphate buffer pH 8	8	44.9	1600	85.6	4121	67
Elution 2	40 % acetonitrile + phosphate buffer pH 8	8	1.9				

EXAMPLE 16:

BUFFER EXCHANGE OF SP -SEPHAROSE ELUTION

The SP-Sepharose elution containing hGH was loaded on Sephadex G-25 column for removal of salt components. This sample was loaded onto a 500 ml Sephadex G-25 column (260mm x 50mm) equilibrated with 10mM Phosphate buffer pH 8. The protein front and salt fronts were collected separately. Total operation was done at 150cm/h of linear flow rates. Load & Elution fractions were analyzed by C4 RPHPLC method. 90% of protein was recovered in the elution.

TABLE: 16

	Components	pH	Conductivity (mS/cm)	Volume (mL)	Purity by RP HPLC	Protein (mg)	Recovery (%)
Load	SP sepharose elution	8	25	1600	85.6	4121	
Elution		8	2.9	5682	79.1	3709	90.0024266

EXAMPLE 17:

PURIFICATION BY ANION EXCHANGE CHROMATOGRAPHY

The SP Sepharose elution containing hGH was buffer exchanged to 10mM Phosphate buffer pH 8 & 2% tween 20 was added to the load. This sample was loaded onto a 500 ml Toyopearl Super Q 650 M column (500mm x 50mm) equilibrated with 10 mM phosphate 2% tween 20 pH 8. Bound proteins were eluted with a 0.5M sodium chloride in 10 mM Phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 67% of loaded protein was found in elution.

TABLE: 17

	Components	pH	Conductivity (mS/cm)	Volume (mL)	Purity by RP HPLC	Protein (mg)	Recovery (%)
Eq buffer	10 mM Phosphate buffer	8	2	2000	0	0	
Load	Sephadex G-25 elution	8	2	5682	79.1	3709	
Wash 1	2 % Tween in Eq buffer	8	2	2000	0	0	
Wash 2	Eq buffer	8	2	2000	0	0	
Elution 1	10 mM Phosphate buffer	8	1.6	1600	90.87	2468	66.54084659
Elution 2	125 mM NaCl + 10 mM Phosphate buffer	8	15.5				

EXAMPLE 18:

PURIFICATION BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

The elution from anion exchange column was adjusted to conductivity of 0.4M sodium sulfate. This sample was loaded onto a 500 ml Toyopearl Super Butyl 550C column (260mm x 50mm) equilibrated with 0.4M Sodium sulfate pH 8. Bound proteins were eluted with a decreasing salt gradient. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. The elution contained 77% of loaded protein.

TABLE 18

	Components	pH	Conductivity (mS/cm)	Volume (mL)	Purity by RP HPLC	Protein (mg)	Recovery (%)
Eq buffer	0.4 M sodium sulphate + 50 mM phosphate buffer	8	48.1	2000	0	0	
Load	Super Q EL + 0.4 M sodium sulphate + 50 mM phospahte buffer	8	50.9	1912	90.58	2274	
Wash	Eq buffer	8	48.1	2000	0	0	
Elution A	10 mM Phosphate buffer+0.4M sodium sulfate	8	1.6	2800	94.69	1772	77.9
Elution B	10 mM Phosphate buffer	8	15.5				

EXAMPLE 19:

BUFFER EXCHANGE OF TOYOPEARL SUPER BUTYL ELUTION

The Toyopearl Super Butyl Elution elution containing hGH was loaded on Sephadex G-25 column for removal of salt components. This sample was loaded onto a 500 ml Sephadex G-25 column (260mm x 50mm) equilibrated with 10mM Phosphate buffer pH 8. The protein front and salt fronts were collected separately. Total operation was done at 150cm/h of linear flow rates. Load & Elution fractions were analyzed by C4 RPHPLC method. 90% of protein was recovered in the elution.

TABLE 19

	Components	pH	Conductivity (mS/cm)	Volume (mL)	Purity by RP HPLC	Protein (mg)	Recovery (%)
Load	Butyl Toyopearyl elution	8	30	2800	94.69	1772	
Elution	10 mM Phosphate buffer	8	1.6	7240	94.69	1595	90.0

EXAMPLE 20

POLISHING BY ANION EXCHANGE CHROMATOGRAPHY

The Toyopearl Super Butyl elution containing hGH was buffer exchanged to 10mM Phosphate buffer pH 8 & 2% tween 20 was added to the load. This sample was loaded onto a 500 ml Toyopearl Super Q 650 M column (500mm x 50mm) equilibrated with 10 mM phosphate 2% tween 20 pH 8. Bound proteins were eluted with a 0.5M sodium chloride in 10 mM Phosphate buffer pH 8. Total operation was done at 150cm/h of linear flow rates. Load, flow through & Elution fractions were analyzed by C4 RPHPLC method. 60% of loaded protein was found in elution.

	Components	pH	Conductivity (mS/cm)	Volume (mL)	Purity by RP HPLC	Protein (mg)	Recovery (%)
Eq buffer	10 mM Phosphate buffer	8	2	2000	0	0	
Load	G-25 elution	8	2	7240	94.69	1595	
Wash 2	Eq buffer	8	2	2000	0	0	
Elution A	10 mM Phosphate buffer	7	1.6	1200	97.9	945	59.2
Elution B	125 mM NaCl + 10 mM Phosphate buffer	7	15.1				

We claim

1) A method of purifying human growth hormone from a mixture containing at least one related impurity, said impurity being a pigment impurity comprising contacting the mixture with a detergent and a chaotrope for a sufficient contact time under conditions that enable breakage of interaction between the human growth hormone and the pigment.

2) The method as claimed in claim 1, wherein the breakage of interaction is afforded by incubation of the mixture with at least 2% of the detergent and at least 2M of the chaotrope.

3) The method as claimed in claim 1, wherein the detergent is selected from a group comprising Triton and Tween.

4) The method as claimed in claim 1, wherein the chaotrope is selected from a group comprising guanidine hydrochloride, guanidinium hydrochloride, sodium thiocyanate and urea.

5) The method as claimed in claim 1, wherein pH is maintained at 2.

6) The method as claimed in claim 1, wherein the mixture is incubated for at least 2 hours.

7) The method as claimed in claim 1, wherein the mixture is subsequently subjected to one or more chromatography steps.

8) A method of purifying human growth hormone from a mixture containing at least one related impurity, said impurity being a pigment impurity comprising contacting the mixture with a detergent and a chaotrope for a sufficient contact time under conditions that enable breakage of interaction between the human growth hormone and the pigment thereby subjecting mixture to chromatography comprising steps:

   a) packing the chromatographic column with a silica based polymer resin equilibrated with a buffer;

b) loading the mixture on the column at a flow rate of at least about 150cm/h cm/hr; and

c) eluting purified product from the column with 0.5M sodium chloride, 0.3M of L-arginine and 5% mannitol in 50 mM Phosphate buffer.

9) The method as claimed in any of the preceding claims, wherein the yield is at least 90%.

10) The method as claimed in any of the preceding claims, wherein the human growth hormone obtained has a purity of at least 80%.

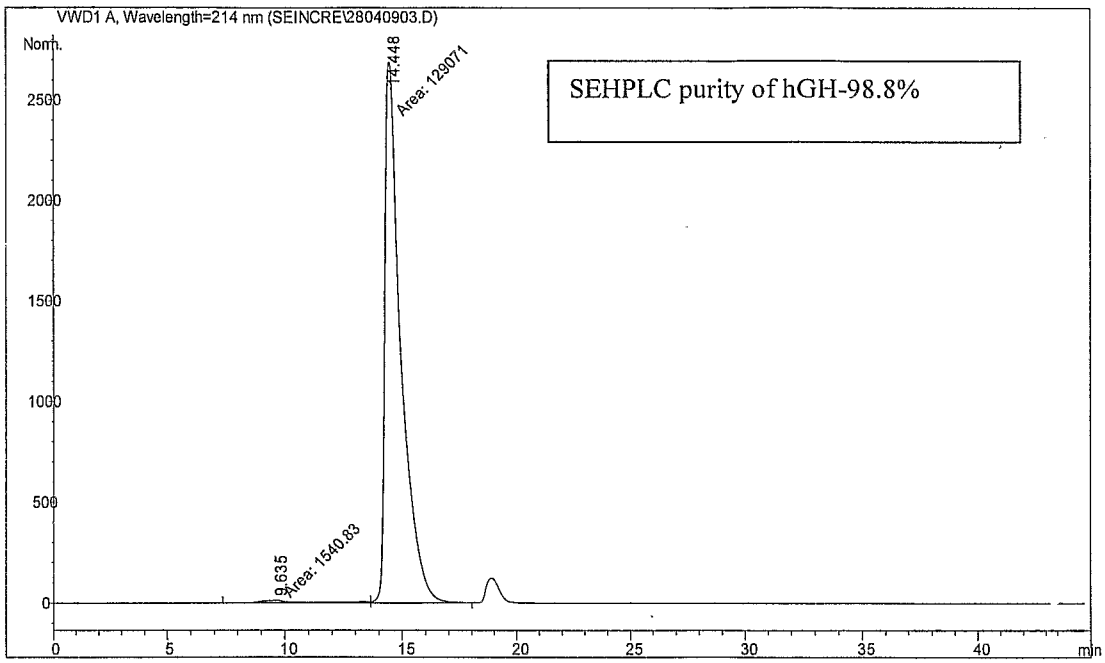
11) The method as claimed in any of the preceding claims, wherein the human growth hormone obtained has a purity of at least 85%.

12) The method as claimed in any of the preceding claims, wherein the human growth hormone obtained has a purity of at least 90%.

13) The method as claimed in any of the preceding claims, wherein the human growth hormone obtained has a purity of at least 95%.

14) The method as claimed in any of the preceding claims, wherein the human growth hormone obtained has a purity of 100%.

15) Purified growth hormone with purity of at least 85%.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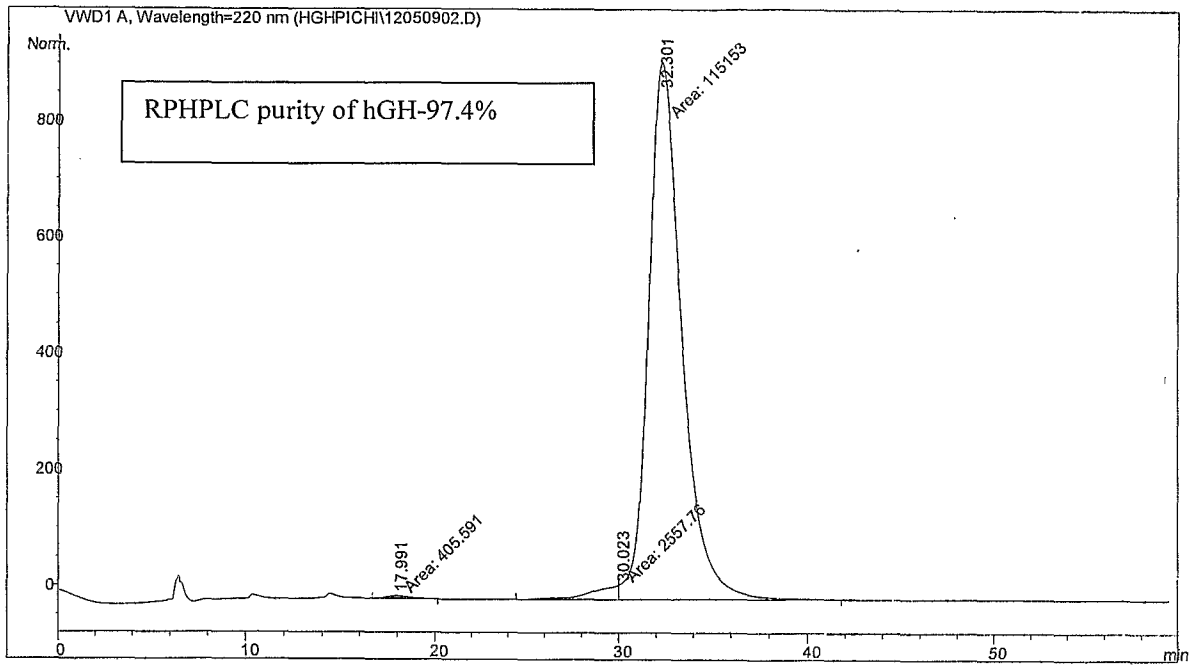
5
10
15
20
25
30
35
40
min

FIGURE 1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10
20
30
40
50
min

FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2009/000380

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 14/475 (2006.01) *C07K 17/08* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
COMPUTER DATABASE SEARCHED: SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN search of Medline, WPIDS, CA, Biosis (Keywords: purif?, chromatograph?, recombinant, eukaryot?, human growth hormone)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RIBELA, M. et al, "Synthesis and chromatographic purification of recombinant human pituitary hormones", J. Chrom. B, 790, 285-316 (2003) See p. 294 l. 7-19, '3.1. Human growth hormone' pp. 302-305 inter alia	

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 2009	13 NOV 2009
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **GAVIN THOMPSON** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No: (02) 6283 2240