EXHIBIT SS (part 1)

PATENT

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

Application of	)	"PRODUCTION OF ERYTHROPOIETIN"
FU-KUEN LIN	)	
Serial No. 113,178	)	Group Art Unit 183
Filed: October 23, 1987	)	Examiner - H. E. Schain

DECLARATION PURSUANT TO 37 C.F.R. 51.132

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, THOMAS WAYNE STRICKLAND, solemnly declare as follows:

1. Since April, 1984, I have been employed by Amgen Inc., Thousand Oaks, California and currently hold the position of Research Scientist.

2. From February, 1981 to March 1984, I held the position of Assistant Research Biological Chemist, Department of Biological Chemistry, University of California, Los Angeles. My research activities included studies on biosynthesis and protein folding of glycoprotein hormones; chemical modification, isolation and receptor cross-linking of glycoprotein hormones.

3. I have read United States Patent Application Serial No. 113,178 ("USSN 113,178") entitled "Production of Erythropoietin".

4. On page 19, lines 26-32 of USSN 113,178, it is stated that:

DEF'S EXHIBIT 200 AM 27 033198
FOR I.D. 11-30-99
RUTH C. MOORE, CSR #8444
WITNESS Strickland

TRIAL EXHIBIT
2108
97-10814 WGY

> "Novel glycoprotein products of the invention include those having a primary structural conformation sufficiently duplicative of that of a naturally-occurring (e.g., human) erythropoietin to allow possession of one or more of the biological properties thereof and having an average carbohydrate composition which differs from that of naturally-occurring (e.g., human) erythropoietin."

5. A series of experimental procedures were conducted under my direction to illustrate some of the differences between the carbohydrate composition, specifically differences relating to the carbohydrate structure, of recombinant human erythropoietin ("r-HuEPO") prepared according to the procedures of USSN 113,178 and naturally-occurring human erythropoietin isolated from urine ("u-EPO").

6. The r-HuEPO for use in the experimental procedures was prepared in accordance with the general procedures described in Example 10 of USSN 113,178, purified in accordance with the procedures described in my U.S. Patent 4,667,016 (Exhibit "A"), and the specific samples were drawn from two GMP lots (Nos. 514 and 516) of material prepared for FDA clinical trial applications.

7. The u-EPO employed in the procedures of paragraphs 8 and 9 hereof was purified in 1984 from urine of aplastic anemia patients using a modification of the procedure of Miyake et al., JBC., 252, 5558, 1977 (Exhibit "B") as follows and was stored until use at -70°C following purification:

(a) Concentrated urine was treated with phenol/-p-aminosalicylate essentially as described by Chiba et al., Biochem.Biophys.Res.Comm., 47,, 1372 (1972) (Exhibit "C");

(b) Treated urine was subjected to ethanol precipitation essentially as described by Miyake et al. (1977);

(c) The 75-90% ETOH precipitation fraction was subjected to DEAE-Agarose chromatography essentially as described by Miyake et al. (1977);

(d) The fraction from the DEAE-Agarose at 10mM Tris·HCl, 30mM $CaCl_2$ was dialyzed against 5mM calcium acetate, pH 7.2, adjusted to pH 4.5 with 1% acetic acid, and subjected to chromatography on sulfopropyl-Sephadex essentially as described by Miyake et al. (1977), except that the column was developed (in succession) with 5mM calcium acetate, pH 4.5 (equilibration buffer), 15mM calcium acetate/20% ethylene glycol, pH 5.5, and 0.1M calcium acetate/20% ethylene glycol, pH 7.0 instead of the solutions described by Miyake et al. (1977);

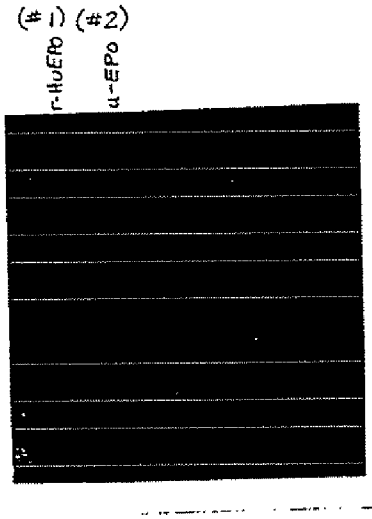
(e) The fraction eluting from the sulfopropyl-Sephadex column at 15mM calcium acetate/20% ethylene glycol, pH 5.5 was solvent exchanged into a buffer consisting of 0.15M NaCl/10mM Tris·HCl, pH 7.0/5mM $CaCl_2$ and applied to a column of wheat germ agglutinin agarose (WGA-agarose) equilibrated with 0.15M NaCl/10mM Tris·HCl, pH 7.0/5mM $CaCl_2$, and the unbound fraction was washed through the column with the equilibration buffer and the column was developed with a 10 mg/ml solution of chitotriose (oligo-N-acetyl-glucosamine) in 0.15M NaCl/10mM Tris·HCl, pH 7.0/5mM $CaCl_2$;

(f) The fraction eluting from WGA-agarose with the chitotriose solution was dialyzed against 0.25mM potassium phosphate, pH 7.5 and subjected to hydroxylapatite chromatography essentially as described by Miyaké et al. (1977), except that the column was developed (successively)

with 0.25mM potassium phosphate (equilibration buffer), 0.75mM potassium phosphate pH 7.5, 6mM potassium phosphate/10% ethylene glycol, pH 9.0, and 20mM potassium phosphate/10% ethylene glycol, pH 9.0 instead of the solutions described by Miyake et al. (1977); and,

(g) The material eluting from the hydroxylapatite column at 0.75mM potassium phosphate was concentrated using a stirred cell ultrafiltration device to yield u-EPO.

8. Separate aliquots of r-HuEPO and u-EPO, each containing approximately 5 ug of the respective protein, were subjected to isoelectric focusing in a polyacrylamide gel in the presence of 5M urea essentially in accordance with the procedure described by LKB Technical Bulletin #2217 (Exhibit "D"). The nominal pH range was from about 3 to 5 and a Coomassie Blue stain was used to stain the gel. A photograph of the gel was taken under visible light and a copy of the photograph is set out immediately below.



AM 27 033201

The r-HuEPO is present in lane #1 designated "r-HuEPO" and the u-EPO is present in lane #2 marked "u-EPO". The pH values measured on the gel using a surface electrode are indicated on the right side of the photograph.

Analysis of the isoforms of r-HuEPO and u-EPO in lane #1 and lane #2, respectively, demonstrates that the u-EPO isoforms exhibit lower (more acidic) isoelectric points.

9. To determine whether the more acidic nature of u-EPO is due to differences in the protein (amino acid) or carbohydrate composition of the molecule, r-HuEPO and u-EPO were subjected to enzymatic digestion with N-glycanase or a mixture of N-glycanase and sialidase as follows:

(a) r-HuEPO and u-EPO samples that were analyzed and reagents employed were prepared as follows:

(1) r-HuEPO

GMP Lot 516 solvent exchanged into H_2O, A_{280}=3.8, make 1:20 dilution into H_2O and dry 24 µl (0.0045 A_{280}) aliquots in Speed Vac without heating.

(2) u-EPO

6/19/84 preparation as in paragraph 7(a) through (g), dry 28 µl (0.005 A_{280}) aliquots in Speed Vac without heating.

(3) N-glycanase

Genzyme Lot #12761, 250 U/ml.

(4) Sialidase

1 U vial of Arthrobacter ureafaciens sialidase (Calbiochem) reconstituted to 1 ml H_2O

(b) the following incubation mixtures were prepared:

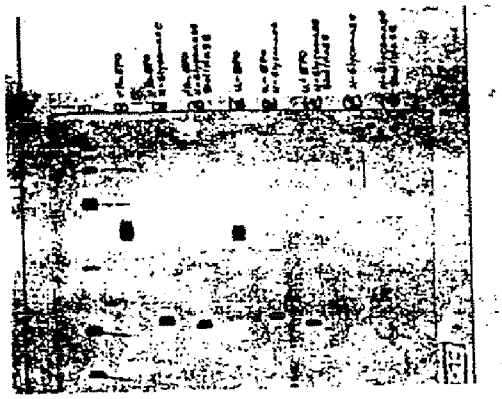
Incubation Mixture	Sample	H_2O	N-glycanase	Sialidase	75 mM CHAPS
r-HuEPO	r-HuEPO	10 µl	--	--	1 µl
r-HuEPO/N-glycanase	r-HuEPO	--	10 µl	--	1 µl
r-HuEPO/N-glycanase/sialidase	r-HuEPO	--	10 µl	1 µl	1 µl
u-EPO	u-EPO	10 µl	--	--	1 µl
u-EPO/N-glycanase	u-EPO	--	10 µl	1 µl	1 µl
u-EPO/N-glycanase/sialidase	u-EPO	--	10 µl	--	1 µl
N-glycanase	--	--	10 µl	1 µl	1 µl
N-glycanase/sialidase	--	--	10 µl		

(c) The mixtures were incubated overnight (14-16 hr.) at 37°C. A 1 microliter sample was removed from each incubation mixture and subjected to polyacrylamide gel electrophoresis in the presence of SDS to monitor for complete enzyme digestion. The samples were reduced with dithiothreitol and heated to 65°C for 5 minutes prior to application to the gel.

The standards and incubation mixtures that were loaded to each lane marked on the gel are indicated as follows:

Lane	Sample Loaded
(1)	Molecular weight standards
(2)	r-HuEPO
(3)	r-HuEPO/N-glycanase
(4)	r-HuEPO/N-glycanase/sialidase
(5)	u-EPO
(6)	u-EPO/N-glycanase
(7)	u-EPO/N-glycanase/sialidase
(8)	N-glycanase
(9)	N-glycanase/sialidase

The gel was stained by the silver staining method. A photograph of this SDS-PAGE gel was taken under visible light and a copy of the photograph is set out immediately following.



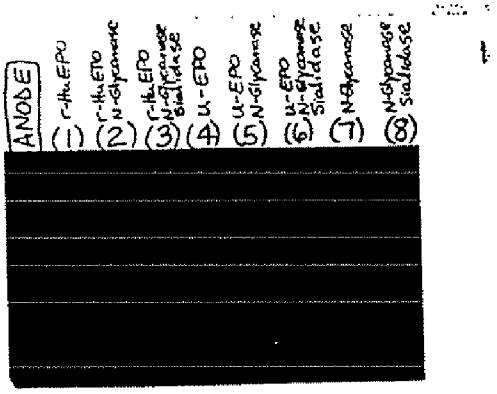
As shown by the photograph of the gel, there are substantial decreases in molecular weight of r-HuEPO and u-EPO upon digestion with N-glycanase (which gives rise to removal of N-linked oligosaccharides) and an additional further slight decrease in molecular weight when the N-glycanase-treated r-HuEPO and u-EPO samples are digested with sialidase (which gives rise to removal of sialic acid from O-linked carbohydrate). The gel demonstrates that the enzymatic reactions went to completion.

The remainder of each incubation mixture was loaded on a pH 3-10 range isoelectric focusing gel. The incubation mixtures were subjected to isoelectric focusing in a polyacrylamide gel in the presence of 5M urea essentially in accordance with the procedure described by LKB Technical Bulletin #2217.

The incubation mixtures were loaded to each lane marked on the gel are indicated as follows:

Lane	Sample Loaded
(1)	r-HuEPO
(2)	r-HuEPO/N-glycanase
(3)	r-HuEPO/N-glycanase/sialidase
(4)	u-EPO
(5)	u-EPO/N-glycanase
(6)	u-EPO/N-glycanase/sialidase
(7)	N-glycanase
(8)	N-glycanase/sialidase

The nominal pH range was from 3 to 10 and a Coomassie Blue stain was used to stain the gel. A photograph of the gel was taken under visible light and a copy of the photograph is set out immediately below.



As shown in the photograph of the gel, N-glycanase digestion of r-HuEPO [Lane (2)] results in two forms which are less acidic than the starting material. These two forms are most probably molecules with O-linked oligosaccharides containing one and two sialic acid residues. Sialidase digestion of the N-glycanase-treated r-HuEPO [Lane (3)] results in one major and one minor isoform. The less acidic nature of the major form is consistent with an O-linked oligosaccharide devoid of sialic acid. The minor form thus appears to contain a single sialic acid residue on its O-linked carbohydrate.

N-glycanase digestion of u-EPO [Lane (5)] results in a major isoform which migrates with the r-HuEPO form believed to contain two sialic acid residues per O-linked carbohydrate and a small amount of a more acidic isoform which may represent a form containing 3 sialic acid residues per O-linked oligosaccharide. Additional evidence that r-HuEPO exists in two forms containing one or two sialic acid residues per O-linked oligosaccharide is demonstrated by the photograph of the SDS-PAGE gel of these reaction mixtures, shown previously, where two slightly different molecular weight forms can be discerned for N-glycanase-treated r-HuEPO, while only one molecular weight form is apparent for u-EPO. Sialidase digestion of N-glycanase-treated u-EPO [Lane (6)] results in a less acidic form which co-migrates with the major form of N-glycanase/sialidase-treated r-HuEPO [Lane (3)]. The equivalent isoelectric points of N-glycanase/sialidase treated r-HuEPO [Lane (3)] and u-EPO [Lane (6)] demonstrates that the more acidic isoelectric point of u-EPO is due to differences in the carbohydrate composition of the molecule and not the protein (amino acid) composition.

10. To determine whether the more negative isoelectric point of u-EPO is due to the different sialic acid linkages present in the glycoprotein [as reported by Takeuchi et al., J.Biol.Chem., 263, 3657 (1988) (Exhibit "E"), wherein it was disclosed that u-EPO contains both 2->3 and 2->6 sialic acid linkages while r-HuEPO produced according to the Amgen method contains only 2->3 sialic acid linkages] r-HuEPO and u-EPO were digested with sialidase alone. The sialidase used, (a sialidase obtained from Arthrobacter ureafaciens), removes both 2->3 and 2->6 linked sialic acids. The procedure employed is as follows:

(a) The r-HuEPO and u-EPO samples that were analyzed and reagents employed were prepared as follows:

(1) r-HuEPO

GMP Lot 516 solvent exchanged into H_2O, A_{280}=3.8, make 1:10 dilution into H_2O and dry 10 ul (0.0038 A_{280}) aliquots in Speed Vac without heating.

(2) u-EPO

Received from E. Goldwasser (prepared in 1976 by the authors and according to the procedure of Miyake et al.), 0.36 mg/ml, designated s-EPO, HT 7-27-76 (3), dry 10 µl aliquots in Speed Vac without heating.

(3) Sialidase

1 U vial of Arthrobacter ureafaciens sialidase (Calbiochem) reconstituted to 1 ml H_2O.

(b) The following incubation mixtures were employed:

Incubation Mixture	Dried Protein	H_2O	Sialidase	75 mM CHAPS
r-HuEPO	r-HuEPO	10 µl	--	1 µl
r-HuEPO/sialidase	r-HuEPO	10 µl	1 µl	1 µl
u-EPO/sialidase	u-EPO	10 µl	1 µl	1 µl
r-HuEPO/sialidase	r-HuEPO	10 µl	1 µl	1 µl

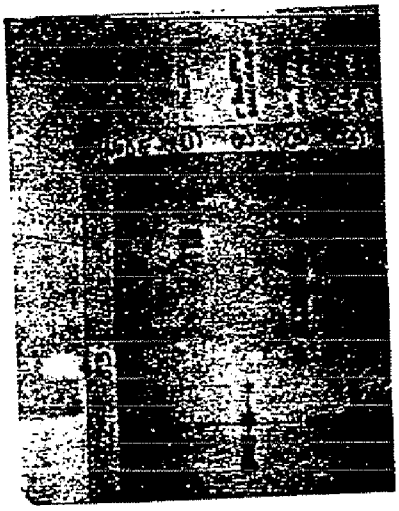
AM 27 033210

(c) The mixtures were incubated overnight (14-16 hrs.) at 37°C. The entire reaction mixture was then applied to an isoelectric focusing gel. The incubation mixtures were subjected to isoelectric focusing in a polyacrylamide gel in the presence of 5M urea essentially in accordance with the procedure described by LKB Technical Bulletin #2217.

The incubation mixtures that were loaded to each lane marked on the gel are indicated as follows:

Lane	Sample Loaded
(1)	r-HuEPO
(2)	r-HuEPO/sialidase
(3)	u-EPO/sialidase
(4)	r-HuEPO/sialidase

The nominal pH range was from 3 to 10 and a silver staining method was used. A photograph of the gel was taken under visible light and a copy of the photograph is set out immediately below.



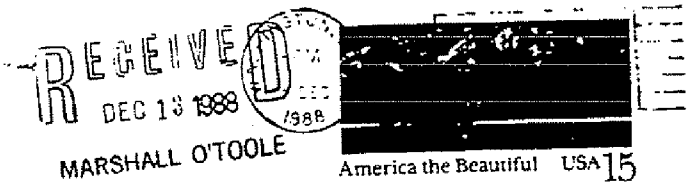
As shown by the photograph of the gel, sialidase digested r-HuEPO [Lanes (2), (4)] and u-EPO [Lane (3)] each contain several different isoforms. The sialidase-treated u-EPO isoforms [Lane (3)] are more acidic than the sialidase-treated r-HuEPO [Lanes (2), (4)] isoforms. The greater negativity of the sialidase-treated u-EPO isoforms [Lane (3)] when compared to the sialidase-treated r-HuEPO isoforms [Lanes (2), (4)] indicates that u-EPO contains sialidase resistant negative charges not found on r-HuEPO. These charges may be sulfate residues as suggested by Takeuchi et al., J.Biol.Chem., 263, 3657 (1988), or additional sialidase resistant sialic acid residues.

11. The above analysis of r-HuEPO and u-EPO demonstrate that the differences shown by the isoelectric focusing experiments, specifically, the more acidic nature of the u-EPO isoforms compared to the r-HuEPO isoforms, is due to the differences in carbohydrate composition, in particular carbohydrate structure, of r-HuEPO and u-EPO. This analysis indicates that recombinant erythropoietin as described by Serial No. 113,178 has a different carbohydrate composition than naturally occurring urinary erythropoietin.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

Thomas Wayne Strickland
THOMAS WAYNE STRICKLAND

Date: 11-30, 1988



MARSHALL, O'TOOLE, GERSTEIN, MURRAY & BICKNELL
SUITE 2100
TWO FIRST NATIONAL PLAZA
CHICAGO, ILLINOIS 60603

AM 27 033214

The Patent Office is hereby requested to acknowledge receipt of the following papers by stamping and returning this card.

Inventor: Fu-Kuen Lin
Serial No.: 113,178
Filed on October 23, 1987

PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. §1.136(a) WITH CERTIFICATE OF MAILING DATED DECEMBER 1, 1988
Fee: $390.00 (check# 11098 2-28/710)
APPLICANT'S AMENDMENT AND REPLY UNDER 37 C.F.R. §1.111 AND 1.115 AND DECLARATION OF THOMAS W. STRICKLAND UNDER 37 C.F.R. §1.132 WITH CERTIFICATE OF MAILING

AM 27 033215

MAIL ROOM DEC 5 1988 PAT. & TRADEMK.

United States Patent [19]

Lai et al.

[11] Patent Number: 4,667,016

[45] Date of Patent: May 19, 1987

[54] ERYTHROPOIETIN PURIFICATION

[75] Inventors: Por-Hsiung Lai, Westlake Village; Thomas W. Strickland, Camarillo, both of Calif.

[73] Assignee: Kirin-Amgen, Inc., Thousand Oaks, Calif.

[21] Appl. No.: 747,119

[22] Filed: Jun. 20, 1985

[51] Int. Cl.[4] A61K 37/24; A61K 35/22; A61K 37/36; C07K 15/14

[52] U.S. Cl. 530/397; 424/95; 424/99; 424/101; 435/68; 435/172.2; 435/172.3; 435/240; 435/241; 435/948; 514/6; 530/380; 530/395; 530/399; 530/416; 530/808; 530/809; 530/834; 935/109

[58] Field of Search ..... 260/112 R, 112 B; 424/95, 99, 101; 435/68, 172.3, 172.2, 240, 241; 514/6; 530/380, 395, 397, 399, 416, 808, 809, 834; 935/109

[56] References Cited

U.S. PATENT DOCUMENTS

3,033,753	5/1962	White et al.	424/101
3,109,774	11/1963	White et al.	424/101
3,111,458	11/1963	White et al.	424/101
4,254,095	3/1981	Fisher et al.	424/88 X
4,289,690	9/1981	Pestka et al.	260/112 R
4,303,650	12/1981	Takezawa et al.	514/21
4,465,624	8/1984	Chiba et al.	260/112 R
4,558,005	12/1985	Goldwasser et al	260/112 B X
4,568,488	2/1986	Lee-Huang	530/397

OTHER PUBLICATIONS

J. Biol. Chem. 255, No. 4, 1536–1541 (1980), Takagaki et al.

Advances in Chromatography, 20, (1982), Giddings editor, p. 43.

High Performance Liquid Chromatography of Proteins & Peptides, Proceedings of the 1st International Symposium, Hearn editor, (1981), pp. 161–165, O'Hare et al.

Analytical Biochemistry, 99, 1–21 (1979), Brown et al.

Endocrinology, 114, (6), 2223–2227 (1984), Parsons et al.

Vydac TM Comprehensive Guide to Reverse Phase Materials for HPLC, Harrison et al. (1984), pp. 1–12.

Primary Examiner—Howard E. Schain

Attorney, Agent, or Firm—Marshall, O'Toole, Gerstein, Murray & Bicknell

[57] ABSTRACT

Chromatographic procedures are individually and jointly applied to the rapid and efficient isolation of biologically active proteins and especially glycoproteins such as recombinant erythropoietin present in the medium of growth of genetically transformed mammalian host cells. Illustratively, recombinant EPO is isolated from culture fluids by reverse phase chromatography employing a C_4 or C_6 column and elution with ethanol. Recombinant erythropoietin may also be purified by anion exchange chromatography employing, e.g., a DEAE resin, with preliminary selective elution of contaminant materials having a lower pKa than erythropoietin from the resin under conditions mitigating against acid activated protease degradation. Practiced serially, the two chromatographic procedures allow for high yields of biologically active recombinant erythropoietin from mammalian cell culture media.

11 Claims, No Drawings

EXHIBIT A

AM 27 033216

ERYTHROPOIETIN PURIFICATION

BACKGROUND

The present invention relates generally to protein purification employing chromatographic techniques. More particularly, the present invention relates to procedures for the rapid and efficient isolation of biologically active proteins such as glycoproteins and especially glycoproteins having high sialic acid content (e.g., erythropoietic factors such as erythropoietin) from natural (e.g., blood fractions and urine) and recombinant (e.g., genetically transformed mammalian cell culture fluid) sources.

Numerous techniques have in the past been applied in preparative separations of biochemically significant materials. Commonly employed preparative separatory techniques include: ultrafiltration, column electrofocusing, flat-bed electrofocusing, gel filtration, electrophoresis, isotachophoresis and various forms of chromatography. Among the commonly employed chromatographic techniques are ion exchange chromatography and adsorption chromatography. The former process is a separatory method wherein fluid components with differing net charges are distinguished and isolated by means of elution (stepwise or with a continuously applied gradient) with eluents of differing ionic strength. A gel matrix (resin) carrying either a positive or negative charge is employed to adsorb (bind) components of opposing net charge. During desorption (elution) charged sample components are exchanged by salt ions in the selected eluent, with specific sample components eluting at specific ionic strengths. Reverse phase adsorption chromatography involves separation of fluid sample components based on differing polarity. Sample components are adsorbed to a granulated gel matrix (resin) by non-covalent bonds. Thereafter, stepwise or continuous gradient elution results in selective desorption of components upon exchange with a non-polar solvent in the eluent.

While the numerous separatory techniques mentioned above are routinely employed in the separation of relatively small hydrophobic and hydrophylic molecules, they have somewhat limited applicability in preparative separations of relatively large molecules such as proteins, especially complex proteins such as lipoproteins, nucleoproteins and glycoproteins. Illustrative of the state of the art in protein separations are reviews by Brown, et al., *Analytical Biochemistry*, 99, 1-21 (1979) and Rubinstein, *Analytical Biochemistry*, 99, 1-7 (1979). See also, "VYDAC™ Comprehensive Guide to Reverse Phase Materials for HPLC", The Sep/A/Ra/-Tions Groups, Hesperia, Calif. and the publication of co-applicants Strickland and co-workers in Parsons, et al., *Endocrinology* 114, (6), 2223-2227 (1984). Further, to the extent that, for example, reverse phase HPLC procedures have been suggested or employed in isolations of proteins or polypeptides, non-polar solvents generally recommended have included reagents that are difficult to handle or to separate from the desired protein such as acetonitrile. See Parsons, et al., supra. Only a single reference is known to exist disclosing elution with ethanol, specifically aqueous ethanol/formic acid mixtures. See Takagaki, et al. *Journal of Biological Chemistry*, 5, (4), 1536-1541 (1980).

The apparent limited utility of the abovenoted techniques in preparatory separations of high molecular weight complex proteins is especially problematic in

view of recent intensive efforts directed toward isolation, purification and application to therapeutic, immunoprophylactic and diagnostic procedures of a wide variety of complex viral and eucaryotic proteins available in only minute quantities from natural sources wherein they are found in association with myriad other complex proteins. As one example, biochemically significant mammalian hematopoietic factors such as erythropoietin, thrombopoietin, granulopoietin and granulocytemarcophage colony stimulating factor are available in extremely small quantities from urine of aplastic anemia patients. Recovery procedures from urinary fluid sources have generally been very complex, costly and labor-intenstive and have generated relatively low yields of active product. A widely practiced method for obtaining biologically active preparations of urinary erythropoietin (a high molecular weight, high sialic acid content glycoprotein) may be found in Miyake, et al., *Journal of Biological Chemistry*, 252 (15), 5558-5564 (1979). The seven-step procedure includes ion exchange chromatography, ethanol precipitation, gel filtration, and adsorption chromatography and is reported to provide a 21% yield of glycoprotein with 70,400 Units/mg potency.

The extensive application of recombinant methodologies to the large scale preparation of eucaryotic proteins has substantially enhanced the prospects for obtaining the desired molecules in quantity and in some instances even simplified purification procedures needed to obtain biologically active materials. Illustratively, where the desired recombinant proteins need not be glycosylated to possess biological activity, large quantities of protein can often be produced in E.coli recombinant hosts in the form of insoluble "inclusion bodies" which contain few proteinaceous contaminants, proteases, or the like. Where glycosylation and/or host membrane processing to develop proper secondary and tertiary conformation are required for biological activity, however, eucaryotic hosts such as yeast and mammalian cells in culture (e.g., COS-1 and CHO cells) provide more suitable recombinant hosts. Use of such hosts, however, generally gives rise to increased difficulty in recovery of biologically active forms of proteins in good yield. Host cell lysates frequently include proteinaceous constituents of sufficiently similar molecular weight, charge, polarity and solubility characteristics (vis-a-vis the recombinant protein) to make ready separation difficult. Further proteolytic enzymes endogenous to the host provide a relatively chronic source of biological activity loss for the desired protein. Where recombinant products are secreted into media supernatants by the host cells, similar problems attend isolation from, e.g., culture media from growth of transformed mammalian cell cultures owing principally to the complexity of the media employed.

There thus continues to exist a need in the art for rapid and efficient preparative separatory procedures suitable for recovery of biologically active proteins from fluid sources and most especially for recovery of complex recombinant proteins such as recombinant erythropoietin from variously "contaminated" fluids such as mammalian cell culture supernatants.

The disclosures of co-owned, co-pending U.S. patent application Ser. No. 675,298, entitled "Production of Erythropoietin", filed Nov. 30, 1984, by FuKuen Lin (corresponding to PCT No. US84/02021, filed Dec. 11, 1984, scheduled for publication June 20, 1985 as No.

AM 27 033217

WO85/02610) are specifically incorporated by reference herein for the purpose of relating the background of the present invention, especially with respect to the state of the art regarding recombinant methodologies applied to large scale production of mammalian erythropoietin.

BRIEF SUMMARY

The present invention provides novel chromatographic separatory procedures individually and jointly suitable for use in the isolation of proteins and specifically applicable to the isolation of erythropoietin, especially recombinant erythropoietin, in biologically active form from fluids, especially mammalian host cell culture supernatants.

According to one of its aspects, the present invention provides for the rapid and efficient recovery of erythropoietin from a fluid by means of a reverse phase liquid chromatographic separation involving selective binding of the desired compound to a C_4 or C_6 resin followed by elution with aqueous ethanol in about 50 to 80 percent solution at a pH of from about 4.5 to 8.0. In a highly preferred mode of practice of this aspect of the invention, high yields of biologically active recombinant erythropoietin are recovered from mammalian host cell culture supernatants through use of a C_4 resin and elution at pH 7.0 employing, stepwise or with a continuous gradient, an eluent comprising about 60 percent aqueous ethanol. Culture supernatants are preferably concentrated before chromatographic treatment and suitable steps are taken to remove ethanol from collected eluent fractions containing erythropoietin. The above elegantly simply separatory procedure reproducibly allows for isolation of erythropoietin having high specific activity in yields approaching 50 percent or more.

In another of its aspects, the present invention provides for rapid and efficient recovery of erythropoietin from a fluid by means of anion exchange chromatography involving selective binding of erythropoietin to a selected cationic resin, treatment of bound materials to guard against acid activation of proteases present, selective elution of bound materials having pKa's greater than that of erythropoietin with aqueous acid at pH's of from about 4.0 to about 6.0, and then elution with aqueous salt at about pH 7.0. Erythropoietin-containing eluent fractions are enriched with biologically active material but may optionally be subject to further processing, e.g., by gel filtration upon ethanol removal. In a presently highly preferred mode of practice of this aspect of the invention, high yields of biologically active recombinant erythropoietin are recovered from mammalian host cell culture supernatants through anion exchange chromatography employing a DEAE agarose resin. Following loading of the DEAE column, urea is added to protect against subsequent acid activation of proteases present and bound fluid components having pKa's greater than erythropoietin are eluted by washings with aqueous acid at about pH 4.3. Thereafter, the pH is adjusted to about 7.0 and aqueous salt is applied stepwise or in a continuous gradient to selectively elute biologically active erythropoietin.

In still another of its aspects, the invention provides for an erythropoietin recovery procedure involving serial application of the ion exchange and reverse phase liquid chromatographic procedures previously described. More specifically, erythropoietin (especially recombinant erythropoietin) is recovered from a fluid (such as a mammalian host cell culture supernatant) in the following stepwise manner. Culture supernatant pools (preferably preliminarily diafiltered and concentrated) are loaded on an anionic exchange column at about pH 7.0 and erythropoietin selectively binds to the cationic resin (preferably DEAE agarose). Bound materials are stabilized against acid activated protease degradation (preferably by addition of urea) and bound materials having pKa's greater than erythropoietin are eluted by one or more aqueous acid washed at from about pH 4.0 to pH 6.0 (preferably about pH 4.3). Thereafter, biologically active erythropoietin is eluted with aqueous salt at about pH 7.0. The erythropoietin-containing eluent fractions are than subjected to reverse phase liquid chromatography on a C_6 or, preferably, C_4 resin to selectively bind erythropoietin. Bound biologically active erythropoietin is then eluted at from about pH 4.5 to about pH 8.0 (preferably about pH 7.0) with an aqueous ethanol solution of from about 50 to 80 (preferably about 60) percent. The desired erythropoietin is isolated within erythropoietin-containing eluent fractions (as determined by absorbance at 280 nm. Ethanol may then be removed and the product may be subjected to gel filtration (e.g., using a Sephacryl S-200 column) with development using, e.g., a pharmaceutical formulation buffer such as 20 mm sodium citrate/100 mm sodium chloride, pH 6.8 to 7.0.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of the practice of preferred embodiments thereof.

DETAILED DESCRIPTION

Practice of the present invention is believed to be suitably illustrated by the following examples practiced on pooled CHO cell supernatants prepared in the manner described in Example 10 of the aforementioned U.S. patent application Ser. No. 675,298. More specifically, the treated supernatants were derived from cell strain CHO pDSVL-gHuEPO "amplified" by means of MTX and grown in roller bottles in serum-free medium as described at page 62 of the application. Example 1 generally refers to recovery of biologically active recombinant human erythropoietin by means of a reverse phase liquid chromatography. Example 2 relates to a composite recovery procedure practiced on the same supernatant material. Example 3 relates to RIA and in vivo assays performed on the resulting purified materials.

EXAMPLE 1

Unconcentrated culture supernatant obtained by pooling first and second (7-day) cycle supernatants was loaded on a closed (high pressure configuration) column laboratory packed with C_4 matrix (VYDAC TM 214TP-B). A 0.45×10 mm column was employed with a flowrate of 1 ml/min. Following sample application, biologically active recombinant erythropoietin was eluted with a linear gradient from 10 mm Tris, pH 7.0 to 80% EtOH/10 mm Tris, pH 7.0. Protein concentration was UVmonitored at 230 nm and the fractions of eluent at about 60% EtOH were pooled

EXAMPLE 2

The composite recovery procedure of this example consisted of serial practice of ion exchange and reverse phase chromatographic procedures performed on a larger fraction of supernatant than in Example 1. The chromatographic procedures were preceded by con-