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PURIFICATION OF HEMOGLOBIN

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(57) Claim

1. A method for producing a partially purified hemoglobin solution substantially free of host cell proteins comprising:

   (a) contacting a hemoglobin-containing cell lysate with an immobilized metal affinity chromatography (IMAC) resin charged with a divalent metal ion;

   (b) washing the IMAC resin with at least one IMAC wash solution; and

   (c) eluting the IMAC resin with an eluting solution at a pH greater than 7.0 to obtain the hemoglobin solution.

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(54) Title: PURIFICATION OF HEMOGLOBIN

(57) Abstract

This invention relates to methods for the production of pure hemoglobin solutions, particularly pure hemoglobin solutions derived from expression of hemoglobin in recombinant systems, and compositions thereof. Methods are also disclosed for removal of contaminant hemoglobins using rapid heating to result in substantially protoporphyrin IX-free hemoglobin solutions, and for removal of high amounts of bacterial proteins and simultaneous purification of crude hemoglobin-containing starting materials to result in partially purified hemoglobin solutions. Methods for the further purification of partially purified hemoglobin solution and the removal of metal contamination in hemoglobin solutions, if required, are also disclosed.

PURIFICATION OF HEMOGLOBIN

This application is a continuation-in-part of Chivers and Belval, Serial Number 08/097,273, filed July 23, 1993 and Ryland, Matthews, Ernst, Houk, Traylor, Williams, Mitchell, Chivers and Belval, Serial Number 08/153,071, filed November 15, 1993, both owned by Somatogen, Inc.

FIELD OF THE INVENTION

This invention relates to methods for the purification of hemoglobin, especially recombinant hemoglobin.

BACKGROUND OF THE INVENTION

Severe blood loss requires both replacement of the volume of fluid that is lost and replacement of oxygen carrying capacity. This is typically accomplished by transfusing red blood cells, either as packed RBC's or as units of whole blood. However, it is not always possible, practical or desirable to transfuse a patient with donated blood. In situations where human blood is not available, volume can be replaced utilizing plasma expanders such as colloid and crystalloid solutions. However, none of the volume replacement therapies currently approved for human use can transport oxygen. In these situations, use of a red blood cell substitute, such as a hemoglobin solution that transports oxygen as effectively as red blood cells, is desirable. Administration of a hemoglobin solution can increase and/or maintain plasma volume and decrease blood viscosity in the same manner as conventional plasma expanders, but, in addition, administration of a hemoglobin-based red blood cell substitute should be able to support adequate transport of oxygen from the lungs to peripheral tissues. The only current therapy with this capability is human blood transfusion.

Human blood transfusions, however, are associated with many risks and limitations, only a few of which are listed below:

1) Risk of infectious disease transmission (i.e., human immunodeficiency virus (HIV), non-A and non-B hepatitis,

hepatitis B, *Yersinia enterocolitica*, cytomegalovirus, human T-cell leukemia virus 1)

2) Risk of immunologic reaction (i.e., mild hemolytic to fatal transfusion reaction, immunosuppresion, graft versus host reaction)

3) Requirement for typing and cross-matching prior to administration

4) Limited availability

5) Limited stability (shelf life of 42 days or less; cannot be frozen)

The oxygen carrying component of the red blood cell is the protein molecule hemoglobin. Human hemoglobin A_o (also known as naturally occurring or native hemoglobin) is a tetrameric protein molecule composed of two identical alpha globin subunits (α_1, α_2) and two identical beta globin subunits (β_1, β_2). A heme molecule is incorporated into each of the alpha and beta globins. Heme is a large organic molecule coordinated around an iron atom. In a hemoglobin tetramer, each alpha globin is associated with a beta globin to form two stable alpha/beta dimers, which in turn associate to form the tetramer. The subunits are noncovalently associated through Van der Waals forces, hydrogen bonds and salt bridges.

In the deoxygenated ("deoxy", or "T" for "tense") state, the four subunits form a tetrahedron. During ligand binding, the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces remain relatively fixed while the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces exhibit considerable movement. When the hemoglobin molecule is oxygenated, the intersubunit distances are increased relative to the deoxygenated distances, and the molecule assumes the "R" configuration (relaxed state) which is the predominant form of the molecule when a ligand is bound to the heme.

Genetic engineering techniques have allowed the expression of heterologous proteins in a number of biological expression systems, such as insect cells, plant cells, transgenic cells, yeast systems and bacterial systems. Because the sequences of alpha and beta globin of hemoglobin are known, and efficient expression criteria have been determined, it is possible that any suitable biological protein expression system can be utilized to produce large quantities of recombinant hemoglobin. Indeed, hemoglobin has been expressed in a number of biological systems, including bacteria (Hoffman et al., WO 90/13645), yeast (De Angelo et al., WO 93/08831 and WO 91/16349;

Hoffman et al., WO 90/13645) and transgenic mammals (Logan et al., WO 92/22646; Townes, T.M and McCune, S.L., WO 92/11283). Although heterologous expression of hemoglobin in these systems can be achieved at high levels, purification of the final product to the extreme level of purity required for pharmaceutical use of hemoglobin remains difficult.

Nevertheless, hemoglobin has been purified from some of these expression systems as well as from outdated human and mammalian red blood cells. Purification of hemoglobin generally requires at least some lytic step to liberate the hemoglobin from the cellular matrix, a low resolution fractionation step to remove contaminating soluble and insoluble proteins, lipids, membranes, etc. (e.g., filtration, centrifugation, pH dependent precipitation, heating) followed by some form of chromatographic final purification steps. For example, hemoglobin has been isolated and purified from outdated human red blood cells by hemolysis of erythrocytes followed by cation exchange chromatography (Bonhard, K., *et al.*, U.S. Patent 4,439,357), anion exchange chromatography (Tayot, J.L. *et al.*, EP Publication 0 132 178; Shorr, et al., US Patent 5,264,555), affinity chromatography (Hsia, J.C., EP Patent 0 231 236 B1), filtering through microporous membranes (Rabiner, S.F. *et al.*, (1967) J. Exp. Med. *126:* 1127-1142), slowly heating a deoxygenated solution of semi-purified hemoglobin to precipitate residual contaminants (Estep, T.N., PCT publication PCT/US89/014890, Estep, T.N., U.S. Patent 4,861,867), precipitating contaminants by the addition of polyvalent ions and polysulfates (Simmonds, R.S and Owen, W.P., U.S. Patent 4,401,652) or precipitating the hemoglobin itself with zinc followed by resuspension (Tye, R.W., U.S. Patent 4,473,494). Hemoglobin has also been purified from other sources, e.g. bovine blood, and treated by any of the methods above or by microporous filtration, ultrafiltration and finally ion exchange chromatography (Rausch, C.W. and Feola, M., EP 0 277 289 B1, Rausch, C.W. and Feola, M., US Patent 5,084,558) or by ultrafiltration alone (Kothe, N. and Eichentopf, B., U.S. Patent 4,562,715). Recombinant hemoglobins produced in transgenic animals have been purified by chromatofocusing (Townes, T.M. and McCune, S. L., PCT publication PCT/US/09624).

These techniques, however, are generally associated with purification of hemoglobin from erythrocytic starting materials and are not suited for the purification of material from recombinant sources such as yeast and bacterial cells. Purification of recombinant hemoglobin produced in microbial expression systems poses unique problems due to the extraordinarily large contamination of the expressed protein with microbial proteins, cellular

components, and especially bacterial lipopolysaccharides (endotoxins) upon lysis of the microbial cells. All these non-hemoglobin components can elicit pyrogenic responses upon administration in even minute amounts to mammals, and may even lead to sepsis and death (Rietschel, E.T. and Brade, H. (1992) Scientific American *267*: 54-61; Suffredini, A.F. et al., (1989) New Eng. J. Med., *321*: 280-287). The necessity for removal of any bacterial contaminants from hemoglobin is even more pressing in light of the observation that hemoglobin and endotoxin co-administration result in an observed enhancement of the lethality of endotoxin compared to the toxicity of endotoxin alone (White, C.T. et al., (1986) J. Lab. Clin. Med. *108*: 132-137; Chang, T.M.S. et al., (1990) Biomat., Art. Cells, Art. Org, *18(2)*: vii-viii).

The concern with contamination from bacterial components is clearly illustrated in US patent 5,084,558 to C.W. Rausch and M. Feola. They teach that the starting material (bovine erythrocytes) used for extra or ultrapure hemoglobin blood substitute solutions must be relatively free of bacterial contamination and state "Avoiding the introduction of bacteria and the maintenance of endotoxin-free or low endotoxin level material is important" (column 13, lines 29-31). They further state that "If the endotoxin level [of the blood] is higher than 6-7 EU [Endotoxin Units] per ml the blood is discarded" (column 13, lines 57-58). Starting levels of somewhat greater than 250 EU/ml have been removed utilizing chelation in the presence of a detergent (Römisch, J. and Heimburger, N., US patent 5,136,026). However, when heterologous proteins are expressed in microbial systems, especially bacterial systems, and the cells are lysed to release the expressed protein, the endotoxin contamination of the resultant lysate is millions of EU per ml. Thus any purification technique that would be utilized for the purification of hemoglobin expressed in microbial systems must be able to reduce enormous quantities of endotoxin contamination to low, pharmaceutically acceptable levels. In addition, for commercial use the purification technique must be scalable and economical. Further, lysis of microbial systems typically yields a reducing environment in the resultant solution, and thus any purification system developed for the purification of proteins from lysed microbial cells must be utilizable in a reducing environment.

A number of purification systems have been developed to reduce the amount of contaminating bacterial components in protein solutions. For example, heating of bacterial cell lysates, particularly *E. coli* lysates, is a common technique utilized in the purification of proteins derived from recombinant technology. However, heating of the material in solution after

lysis of bacterial cells has generally been restricted to purification of known heat-stable proteins. This technique exploits the differences in thermal stability between most bacterial proteins and the heterologous protein. For example, Tanaka and co-workers (Tanaka et al., (1981) Biochemistry *89*: 677-682) expressed 3-isopropylmalate dehydrogenase from a thermophilic bacterium in *E. coli*, and purified this enzyme by heating the crude lysate for 10 minutes at 70°C. They note that this was a simple and effective procedure for rapidly purifying protein, and further state that "the enzymes of extreme thermophiles are stable in conditions where most of the proteins of *E. coli* cells used as host are heat denatured and precipitated.....these observations suggest that any thermophilic enzyme can be purified with relative ease by cloning the genes in question into *E. coli*.

Tsukagoshi and co-workers (Tsukagoshi et al., (1984) Mol. Gen. Genet. *193*: 58-63) also purified a heat stable protein expressed in *E. coli*, however, they found that the thermal stability of the α-amylase that they were purifying was ligand dependent. The thermal stability in the absence of Ca^{++} was approximately 10°C lower than in the presence of Ca^{++} (see Figure 5, page 61). As a result, these workers added Ca^{++} to the medium prior to heating to enhance stability of the enzyme and to recover greater activity. Moreover, this paper also demonstrates that the media conditions can be manipulated in order that the protein of interest is, or becomes, more thermostable than the contaminating *E. coli* proteins. It is of note that these systems require a significant difference between the thermal stability of most of the contaminating proteins and the protein of interest.

Immobilized metal affinity chromatography (IMAC) has also been used extensively in the field of protein purification. For example, recombinant human interleukin-4 has been purified from a crude fermentation broth by passing the broth at pH 7.2 over a zinc charged IMAC column wherein the interleukin-4 is bound, and eluting with 0.5 M NaCl or 50 mM EDTA (Tang, J.C.T. et al., US Patent 5,077,388). Interleukin-2 and interferon gamma expressed in mammalian systems have been purified using cation exchange chromatography followed by zinc-charged IMAC (Georgiades, J. A. and Gumulka, J. US Patent 4,723,000). A relatively pure interleukin-2, interferon gamma solution was loaded onto an IMAC column and contaminants were removed from the stream by binding to the column material while the interleukin and interferon flowed through the column. No elution was necessary. Recombinantly produced soluble CD4 receptors have also been purified using IMAC (Staples, M.A. and Pargellis, C.A., US Patent 5,169,936).

Contaminants were removed from the partially purified starting solution by eluting with salt and a higher concentration of a weak ligand to the metal charging the IMAC. IMAC has also been used to separate mixtures of relative pure proteins into individual components (Kato, Y. et al., (1986) J. Chrom. *354*: 511-517). Aqueous two-phase metal affinity extraction has been used to purify hemoglobin by complexing a soluble copper-charged, polyethylene glycol charged bidentate chelator with a soluble hemoglobin and complexing the hemoglobin to the soluble chelator to produce a partitioned, two phase system (Wuenschell, G.E. ,et al., (1990) Bioprocess Eng. *5*: 199-202). However, immobilized metal affinity chromatography has not been used in the purification of any hemoglobin, and especially not recombinantly produced hemoglobin. Moreover, IMAC has not been used to achieve greater than 1000 fold removal of *E. coli* proteins while simultaneously purifying hemoglobin from a lysate to greater than 99% purity.

Metals can contaminate hemoglobin solutions (Marshall et al. (1993) in *Blood Substitutes and Oxygen Carriers*, Chang (ed.), Marcel Dekker, Inc., New York, pp. 267-270). This kind of contamination may be removed using a number of different methodologies, but the success of a particular method for a particular solution is unpredictable. For example, various chelating resins have been used to separate various metals from a solution, including nickel, although many times such separation is effective only at very low pH and is thus unsuitable for use with hemoglobin solutions (Figura and McDuffie, (1977) Anal. Chem. *49*: 1950-1953; Darnall et al., (1986) Envir. Sci. Tech. *20*: 206-208; Vernon, (1977) Chem. and Industry *15*: 634-637; Moyers and Fritz, (1977) Anal. Chem. *49*: 418-423; Yip et al., (1989) Anal. Biochem. *183*: 159-171; Yalpani, M. and Abdel-Malik, M.M., U.S. Patent 4,952,684). Metal chelators, such as ethylenediamine tetraacetate (EDTA) have also been used to remove metal contamination from solution. However, these chelators can be toxic at the concentrations required to remove all metal contamination (Heindorff, K. et al., (1983) Mutation Res. *115*: 149-173), and may enhance oxidation of the hemoglobin molecule (K ugawa, K., et al., (1981) Chem. Pharm. Bull. *29*: 1382-1389).

The present invention provides for efficient production of hemoglobin to pharmaceutically acceptable levels of purity. Current purification techniques that are suitable for use in the purification of erythrocyte derived hemoglobin, for example anion exchange chromatography, may remove small quantities of *E. coli* derived material, but are not effective for the removal of the large quantities of bacterial contamination that are encountered during the

purification of recombinantly produced hemoglobin. Likewise, techniques that have been developed for the purification of recombinantly produced proteins are not applicable to hemoglobin both because of the need to provide extraordinary levels of purification to ensure adequate bacterial contaminant removal and the difficulty in economically scaling these processes to produce suitable amounts of hemoglobin.

SUMMARY OF THE INVENTION

The present invention relates to a method for the production of a partially purified hemoglobin solution comprising the following steps:

a) contacting a hemoglobin-containing lysate at pH greater than 7.0 with an immobilized metal affinity chromatography resin charged with a divalent metal ion,

b) washing the resin with a first suitable buffer,

c) washing the resin with a second suitable buffer,

and

c) eluting the partially purified hemoglobin solution from the resin with a solution of pH higher than the second suitable buffer, a chelating agent, or a competitive ligand.

The hemoglobin-containing lysate is preferably a clarified hemoglobin-containing lysate, most preferably a substantially protoporphyrin IX-free hemoglobin solution; the lysate or solutions are preferably at pH between about 7.5 and 8.5, preferably 8.0-8.35; the immobilized metal chromatography resin is preferably charged with zinc; the resin is washed with a first suitable buffer having a pH greater than about 7.5 and a conductivity greater than about 25 mS/cm, preferably Tris/NaCl, more preferably 20 mM Tris/0.5 M - 0.75 M NaCl, pH 7.5-8.5 having a conductivity of about 25 - 50 mS/cm, most preferably 20 mM Tris/500 mM NaCl, pH 8.3 having a conductivity of about 46 mS/cm or 20 mM Tris/750 mM NaCl, pH 8.0 having a conductivity of about 35 mS/cm; the second suitable buffer is the same or different from the first suitable buffer and preferably having a conductivity lower than the first suitable buffer, more preferably having a conductivity between 2 - 6 mS/cm and a pH greater than 7.6, more preferably between 7.6 and 8.5; more preferably having a conductivity of about 2.5 - 5 mS/cm , most preferably the second wash buffer is 10 mM Tris, 25 to 50 mM NaCl, pH about 8.0 - 8.3; preferably the partially purified hemoglobin solution is eluted from the column with a chelating agent, more preferably EDTA, still more preferably 5

-30 mM EDTA, most preferably 10-20 mM EDTA, and the chelating agent or competitive ligand is at pH greater than 7, more preferably at pH greater than 8, most preferably about pH 8.5.

Note that for the purposes of the instant invention, all conductivity and pH measurements have been normalized to pH and conductivity at 8°C.

The present invention further comprises methods for the production of a substantially protoporphyrin IX-free hemoglobin solution comprising:

(a) lysing hemoglobin containing cells to produce a crude hemoglobin-containing lysate,

(b) converting the hemoglobin in the crude hemoglobin-containing lysate to a thermally stable state,

(c) heating the crude hemoglobin-containing lysate for a sufficient time and at a sufficient temperature to kill most remaining bacterial cells, precipitate microbial contaminants and cell debris, precipitate contaminating hemoglobins, and especially precipitate protoporphyrin IX-containing hemoglobin,

and

d) mechanically removing the precipitated microbial contaminants and cell debris, precipitated contaminating hemoglobin and especially protoporphyrin IX-containing hemoglobin from the crude hemoglobin-containing lysate, to produce a substantially protoporphyrin IX-free hemoglobin solution.

The hemoglobin containing cells are preferably non-erythrocyte cells, more preferably bacterial cells, most preferably *E. coli* cells, the hemoglobin in the crude hemoglobin containing lysate is converted to the R state or T state, most preferably to the R state, preferably by adding a liganding gas selected from the group consisting of oxygen, nitric oxide and carbon monoxide, most preferably carbon monoxide, and precipitated microbial contaminants, cell debris, precipitated contaminated hemoglobins and especially precipitated protoporphyrin IX-containing hemoglobin are mechanically removed from the crude hemoglobin containing lysate by chromatography or solid-liquid separation techniques, more preferably by filtration, most preferably by rotary drum vacuum filtration.

The present invention further provides a method for the production of a substantially purified hemoglobin solution comprising:

a) buffer exchanging a partially purified hemoglobin solution into a first suitable buffer to produce a buffer exchanged, partially purified hemoglobin solution,

b) loading the buffer exchanged, partially purified hemoglobin solution onto an anion exchange resin,

c) washing the anion exchange resin loaded with the buffer exchanged, partially purified hemoglobin solution with the first suitable buffer,

c) washing the anion exchange resin loaded with partially purified hemoglobin solution with a wash buffer of lower pH than the first suitable buffer,

d) eluting the buffer exchanged, partially purified hemoglobin solution from the anion exchange resin with an eluting buffer of still lower pH than the wash buffer to produce a substantially purified hemoglobin solution.

Preferably the suitable buffer is a suitable cationic buffer, preferably Tris buffer of pH 8.5-9.5 and conductivity of 200-800 uS/cm, most preferably 20 mM Tris buffer, pH about 8.9, conductivity of approximately 400 uS/cm; preferably the anion exchange resin is a strong anion exchange resin, preferably a Sepharose Q Fast Flow resin; preferably the wash buffer is a suitable cation exchange buffer, preferably Tris buffer of pH less than 8.5, more preferably pH 7.6-7.9, preferably 10-15 mM Tris buffer, most preferably 12 mM Tris, pH 7.7, preferably having a conductivity of between about 600 to 800 uS/cm, most preferably having a conductivity approximately 700 uS/cm; preferably the partially purified hemoglobin solution is eluted with an eluting buffer of lower pH than the wash buffer, more preferably Tris buffer, more preferably 10 -15 mM Tris buffer pH 7.4-7.7 having a conductivity between 550-1200 uS/cm , more preferably 12 mM Tris buffer, pH 7.5 having a conductivity between 550 and 850 uS/cm, most preferably having a conductivity of 700 uS/cm.

Note that the ligand on the substantially purified hemoglobin can then be exchanged after the above step to produce an oxygenated, substantially purified hemoglobin solution.

The present invention still further comprises methods for the production of a pure hemoglobin solution by the removal of any trace metal, especially nickel, that may have been introduced during any manufacturing or purification process comprising:

a) adding a chelating agent to the substantially purified hemoglobin solution by batch addition or ultrafiltration,

b) buffer exchanging the substantially purified hemoglobin solution by any suitable technique into a suitable formulation buffer to produce a pure hemoglobin solution.

The addition of the chelating agent to the substantially purified hemoglobin solution is most preferably by batch addition of the chelating agent, wherein the chelating agent is selected from the group consisting of EDTA and diethylamine triamine pentaacetic acid (DTPA also known as pentaacetic acid); preferably the substantially purified hemoglobin solution is buffer exchanged by ultrafiltration. Preferably a suitable formulation is 150 mM NaCl, 5 mM sodium phosphate, about 0.025 to 0.035% Tween, 1 mM ascorbate or less than 2.5 mM dithionite, less than 50 mM carbohydrate, less than 2% of a polyethylene glycol, pH about 7.

The present invention also further comprises methods for the production of a pure hemoglobin solution comprising:

a) lysing hemoglobin-containing cells *E. coli* cells to produce a crude hemoglobin-containing lysate,

b) converting the hemoglobin in the crude hemoglobin-containing lysate to R state hemoglobin by adding carbon monoxide,

c) heating the crude hemoglobin-containing lysate for a sufficient time and at sufficient temperature to kill most remaining bacterial cells, precipitate microbial contaminants and cell debris, precipitate contaminating hemoglobin, and especially precipitate protoporphyrin IX-containing hemoglobin,

d) mechanically removing the precipitated microbial contaminants and cell debris, precipitated contaminating hemoglobin and especially precipitated protoporphyrin IX-containing hemoglobin from the crude hemoglobin lysate to produce a substantially protoporphyrin IX-free hemoglobin solution,

e) binding the substantially protoporphyrin IX-free hemoglobin solution at pH approximately 8.0 to 8.3 to an immobilized metal affinity resin that is charged with zinc,

f) washing the substantially protoporphyrin IX-free hemoglobin solution bound to the immobilized metal affinity resin with at least four column volumes of about 20 mM Tris/500-750 mM NaCl, pH about 8.0-8.3, conductivity of about 35-50 mS/cm,

g) washing the substantially protoporphyrin IX-free hemoglobin solution bound to the immobilized metal affinity resin with at least four column volumes of about 10 mM Tris/25 - 50 mM NaCl, pH about 8.0 - 8.3, conductivity of about 2.5 - 4.5 mS/cm,

h) eluting the substantially protoporphyrin IX-free hemoglobin solution bound to the immobilized metal affinity resin with about 15 mM EDTA at pH about 8.5 to produce a partially purified hemoglobin solution,

i) buffer exchanging the partially purified hemoglobin solution into 20 mM Tris at about pH 8.9 to produce a buffer exchanged, partially purified hemoglobin solution,

j) loading the buffer exchanged, partially purified hemoglobin solution onto an anion exchange resin,

k) washing the anion exchange resin loaded with buffer exchanged, partially purified hemoglobin solution with 20 mM Tris buffer, pH about 8.9, conductivity of approximately 400 uS/cm,

l) washing the anion exchange resin loaded with buffer exchanged, partially purified hemoglobin solution with about 12 mM Tris buffer, pH about 7.7, conductivity of approximately 700 uS/cm ,

m) eluting the anion exchange resin with 12 mM Tris buffer, pH 7.5 having a conductivity between 550 and 800 uS/cm to produce a substantially purified hemoglobin solution,

n) oxygenating the substantially purified hemoglobin solution by introducing oxygen under pressure,

o) removing any metal contamination in the purified hemoglobin solution by batch addition of a chelating agent selected from the group consisting of EDTA and DTPA, if such metal removal is necessary,

p) removing the chelating agent if such removal is necessary and simultaneously buffer exchanging the purified hemoglobin solution into a suitable formulation buffer.

Another aspect of the present invention relates to essentially pure hemoglobin solutions and pharmaceutical compositions, preferably such solutions obtained from purification of recombinant hemoglobin and particularly such recombinant hemoglobins as obtained by the methods of the present invention.

To assist in the interpretation of the present patent, the following terms shall have the following meaning throughout this patent, including the claims appended hereto, unless otherwise indicated.

"Hemoglobin" or "hemoglobin-like protein" comprises one or more tetramers composed of (a) two alpha-like globins and two beta-like globins, (b) one di-alpha-like globin and two beta-like globins, (c) two alpha-like globins

and one di-beta-like globin, (d) one di-alpha-like globin and one di-beta-like globin, (e) one fused alpha-like/beta-like globin and separate alpha-like and beta-like globins, or (f) two fused alpha-like/beta-like globins. A globin of one tetramer may be crosslinked or genetically fused to a globin of another tetramer. In hemoglobin or a hemoglobin-like protein, whether derived from natural or recombinant sources, in either the R or the T state, each alpha-like globin and beta-like globin may contain a heme or protoporphyrin IX prosthetic group.

"Genetically fused hemoglobin" means a hemoglobin-like protein comprising at least one "genetically fused globin-like polypeptide", the latter comprising two or more globin-like domains, for example di-alpha-like globin or beta-like globin, which may be the same or different.

A "di-alpha-like globin" is one which consists essentially of two alpha-like globin sequences (domains) connected by peptide bonds between the C-terminus of the first alpha-like globin (domain) and the N-terminus of the second alpha-like globin (domain). An alpha-like globin (or domains thereof) has at least about 75% sequence identity with native human alpha globin. However, a polypeptide of lesser sequence identity may still be considered substantially homologous with alpha globin, and thus may be an alpha-like globin, if it has a greater sequence identity than would be expected from chance and also has the characteristic higher structure of alpha globin and similar biological activity. Likewise, a beta-like globin (or domains thereof) has at least about 75% sequence identity with native human beta globin. However, a polypeptide of lesser sequence identity may still be considered substantially homologous with beta globin, and thus may be a beta-like globin, if it has a greater sequence identity than would be expected from chance and also has the characteristic higher structure of beta globin and similar biological activity.

In a di-alpha-like globin, the two alpha-like globin sequences may be directly connected, or connected through a peptide linker of one or more amino acids; the term "peptide bonds" is intended to embrace both possibilities. Alpha-like globin chains crosslinked at the N- and C-termini other than by peptide bonds (e.g., by 4,4'-diisothiocyanatostilbene-2,2'-disulfonates, DIDS) are not di-alpha-like globins. The di-alpha-like globin preferably can fold together with beta globin and all globins in the protein can incorporate heme to form a functional hemoglobin-like protein. The "di-beta globin-like polypeptide" is analogously defined.

"rHb1.1" means one di-alpha-like globin and two beta-like globins, wherein the two alpha-like globins are connected by a single glycine between

the C terminus of a first alpha-like globin and the N terminus of a second alpha-like globin, the beta-like globins contain the Presbyterian mutation, βN108->K, and both of the beta-like globins as well as the di-alpha-like globin contain a val->met mutation at the N terminus.

"Recombinant hemoglobin" means hemoglobin, whether native or mutant, comprising alpha-like globin proteins and beta-like globin proteins, at least one of which is obtained by expression of a globin gene carried by a recombinant DNA molecule in a cell other than the cell in which that hemoglobin gene and/or hemoglobin protein is naturally found, i.e., the hemoglobin gene is heterologous to the host in which it is expressed. Therefore, the expression of any human hemoglobin gene in any cell other than a human red blood cell would be considered to be a recombinant hemoglobin. Moreover, the expression of a vertebrate hemoglobin in any species of invertebrate, or any vertebrate other than the vertebrate where the hemoglobin to be expressed is naturally occurring, would be considered a recombinant hemoglobin. The expression of any naturally occurring hemoglobin mutant in any species other than the species in which it is naturally occurring, would be considered a recombinant hemoglobin. The expression of any non-naturally occurring mutant hemoglobin in any species would be considered a recombinant hemoglobin. The expression of a naturally occurring mutant hemoglobin in any individual organism, regardless of species, other than the individual organism in which said mutant is naturally expressed, would be considered a recombinant hemoglobin.

"Liganded hemoglobin" means hemoglobin to which any ligand is bound. Common ligands include, but are not limited to O_2, CO_2, NO, CO, HCN, and the like. Preferably the ligand is one that binds in the heme pocket. Common preferred ligands include, but are not limited to O_2, CO, NO and the like.

"Oxyhemoglobin" means hemoglobin in which each of the functional oxygen binding sites has bound to it an oxygen molecule.

"Deoxyhemoglobin" or "unliganded hemoglobin" means any hemoglobin to which no ligand is bound to the alpha globin, the beta globin, and/or any functional heme prosthetic group.

"R-state hemoglobin" is the high affinity state of hemoglobin and is the dominant form of hemoglobin when a ligand is bound at the heme pockets. The ligand is typically oxygen, thus this state is known as the "oxy" or "R" (for relaxed) state. In the R state, intersubunit distances are increased relative to the distances in T-state hemoglobin.

"T-state hemoglobin" is the low affinity state of hemoglobin in which the subunits form a tetrahedron and is the dominant form of hemoglobin when it is deoxygenated ("deoxy", or "T" for "tense").

"Contaminating hemoglobin(s)" means any hemoglobin that is not the substantially pure hemoglobin of the instant invention, and can include protoporphyrin IX-containing hemoglobin, hemoglobin isoforms, methemoglobin, aggregated hemoglobin, acetylated hemoglobin, methylated hemoglobin, glycated hemoglobin, and the like.

"Protoporphyrin IX-containing hemoglobin" means any hemoglobin in which one or more heme prosthetic groups does not contain an iron atom.

"Hemoglobin-containing lysate" means a hemoglobin solution derived from erythrocyte or non-erythrocyte cells wherein hemoglobin is no longer contained in those cells and may be either crude or clarified hemoglobin lysate.

"Crude hemoglobin-containing lysate" means a hemoglobin solution, whether erythrocyte or non-erythrocyte derived, that has not been processed except to release the hemoglobin from the cells that expressed the hemoglobin. Contaminating material may be in solution or may be precipitated, but the precipitated contaminating material has not be removed from the solution.

"Clarified hemoglobin-containing lysate" means a hemoglobin solution derived from erythrocyte or non-erythrocyte cells that is substantially free of solids, such as contaminating cell membranes, precipitated non-hemoglobin proteins, and precipitated contaminating hemoglobins, especially protoporphyrin IX-containing hemoglobin and the like.

"Substantially protoporphyrin IX-free hemoglobin solution" is one wherein the amount of protoporphyrin IX-containing hemoglobin in the substantially protoporphyrin IX-free hemoglobin solution is less than about ten percent (10%) of the total hemoglobin, more preferably, less than about six percent (6%) of the total hemoglobin, more preferably less than about one percent (1%) of the total hemoglobin. Most preferably, the protoporphyrin IX-containing hemoglobin in a substantially protoporphyrin IX-free hemoglobin solution is below the detection limit for protoporphyrin IX in the measurement technique described in Example 6.

"Partially purified hemoglobin solution" means a hemoglobin solution that contains 99% by weight hemoglobin relative to other proteins in the solution, and has at least 100 fold less, more preferably 500 fold less, most preferably 1000 fold less *E. coli* proteins (*E. coli* proteins as measured using the techniques specified in Example 11) than clarified hemoglobin-containing lysate.

"Substantially purified hemoglobin solution" means hemoglobin that meets the following specifications:

Methemoglobin	< 10% (wt/wt)
Carbonmonoxyhemoglobin	< 5% (wt/wt)
E. coli proteins (ECP's)	< 50 ppm
LAL Endotoxin	< 0.5 EU/ml
Bioburden	1 CFU/ml
Protoporphyrin IX	< 2 % (wt/wt heme)
EDTA	< 5 mg/L

"Pure hemoglobin solution" means a hemoglobin solution that meets the following purity specifications:

Methemoglobin	< 10% (wt/wt)
Carbonmonoxyhemoglobin	< 5% (wt/wt)
E. coli proteins (ECP's)	< 50 ppm
LAL Endotoxin	< 0.5 EU/ml
Bioburden	1 CFU/ml
Protoporphyrin IX	< 2 % (wt/wt heme)
Nickel	< 100 ug/L
EDTA	< 5 mg/L

DESCRIPTION OF THE FIGURES

Figures 1A - C describe a configuration for the process of the instant patent. Figure 1A refers to the fermentation process, Figure 1B refers to the first portion of a purification process flow and Figure 1C shows the final steps of a purification process flow.

Figure 2 shows residence times in seconds (Y-axis) required to achieve a 25-log kill of *E. coli* at various temperatures (°C - X-axis).

Figure 3 shows a summary of fully functional hemoglobin yield in percent (gray bars with scale indicated on the left Y-axis) and protoporphyrin IX remaining (• with scale indicated on the right Y-axis) in a protoporphyrin IX-containing hemoglobin solution after heating by steam injection as described in Example 4. Note 0.4% and 0.5% values are at PIX limit of quantitation. Values are presented for a number of temperatures (X-axis). Heating retention times are noted on the gray bars.

Figure 4 shows a plasmid map of pSGE705, a plasmid used in the recombinant expression of a mutant hemoglobin, rHb1.1. The plasmid map includes relevant restriction sites.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a process for the purification of hemoglobin, especially recombinant hemoglobin. In particular, this invention provides for a surprisingly high degree of purification using a single chromatographic step, immobilized metal affinity chromatography (IMAC), to result in a partially purified hemoglobin solution. Moreover, this invention provides methods for the removal of contaminating proteins, particularly protoporphyrin IX-containing hemoglobin, by rapidly heating a crude hemoglobin lysate wherein the hemoglobin is stabilized in the R state or the T state to result in a protoporphyrin IX-free hemoglobin solution. The invention further provides for the purification of a partially purified hemoglobin solution using anion exchange chromatography to produce a substantially purified hemoglobin solution, and if needed, the invention provides for removal of metals that may have been introduced during the purification process by addition and then removal of a suitable chelating agent. Each of these processes can be used alone, or they can be combined as required.

Hemoglobin can be purified to produce a clarified hemoglobin-containing lysate from a number of sources well known to the art, including but not limited to outdated human red blood cells, bovine red blood cells and a number of non-red blood cell systems including, but not limited to, bacterial, yeast, plant, and mammalian cells. In all these systems, one of the usual initial steps in the purification of hemoglobin from the cellular matrix is removal of contaminating cellular components. This is important whether or not the source material is red blood cells or bacterial expression systems. The special potentiation of the toxic effects of endotoxin by hemoglobin requires particular attention to the removal of endotoxin contaminants or the prevention of bacterial contamination of the crude starting material (Rausch, C.W. and Feola, M., US Patent 5,084,558). The problem of removal of cellular contamination, particularly bacterial contamination, is especially acute in the setting of expression of recombinant hemoglobin in a bacterial expression system, since, prior to the instant invention, initial high levels of bacterial contamination could not be removed, especially at large commercial scale, without risking the quality of the final product hemoglobin.

An additional step that may be taken is the removal of contaminant hemoglobins such as hemoglobin isoforms, methemoglobin, aggregated hemoglobins, and especially protoporphyrin IX-containing hemoglobins ("contaminating hemoglobins"). Such contaminating hemoglobins may be produced as a result of incorporation of one or more inactive heme groups into a given hemoglobin molecule or a result of oxidation of hemoglobin products during initial production or purification steps. Removal of these contaminating hemoglobins, including protoporphyrin IX-containing hemoglobin, is desirable to maximize product purity and stability.

Hemoglobin-containing cells suitable as starting material for the present invention are readily available from a number of sources. For example, slaughter houses produce very large quantities of hemoglobin-containing cells. If a particular species or breed of animal produces a hemoglobin-containing cell especially suitable for a particular use, those creatures may be specifically bred for this purpose in order to supply the needed blood. Also, transgenic animals may be produced that can express a recombinant hemoglobin in hemoglobin-containing cells. Human blood banks must discard human blood, including hemoglobin-containing cells, after a certain expiration date.

Moreover, the genes encoding subunits of a desired hemoglobin may be cloned, placed in a suitable expression vector and inserted into microorganism, animal, plant or other organism, or inserted into cultured animal or plant cells or tissues. These organisms, cells or tissues may be produced using standard recombinant DNA techniques and may be grown in cell culture or in fermentations (Figure 1A). Human alpha and beta globin genes have been cloned and sequenced by Liebhaber et al. (Proc. Natl. Acad. Sci. USA (1980) 77: 7054-7058) and Marotta et al. (J. Biol. Chem. (1977) 252: 5040-5053) respectively. Techniques for expression of both native and mutant alpha and beta globins and their assembly into hemoglobin are set forth in U.S. Patent 5,028,588 to S.J. Hoffman; K. Nagai and Hoffman, S.J. et al., PCT/US90/ 02654; Townes, T.M. and McCune, S.L., PCT/US91/09624; and De Angelo, J. et al., PCT/US91/ 02568 and PCT/US91/08108.

In most cases, the first step in the preparation of a pure hemoglobin solution is to get the hemoglobin outside of the hemoglobin-containing cell that has expressed it to produce a crude hemoglobin-containing lysate. This can usually be accomplished by breaking open the cells, e.g., by sonication, homogenization, enzymatic lysis or other cell breakage technique known in the art. Alternatively, hemoglobin can be released from hemoglobin containing cells by dilution at a controlled rate with a hypotonic buffer so that some

contamination with cellular components can be avoided (Shorr et al., US Patent 5,264,555). In addition, cells may be engineered to secrete the globins. After or concurrent with this first step, a large amount of the various contaminating cellular components and contaminant hemoglobins, including protoporphyrin IX-containing hemoglobin, can be removed if needed as prescribed in the present invention by heating the crude hemoglobin-containing lysate and mechanically removing precipitated material to produce a clarified hemoglobin-containing lysate (Figure 1B). This is especially true for hemoglobin derived from recombinant expression systems. However, if removal of contaminating cellular components and contaminating hemoglobins is not required, then this step may be omitted, and the hemoglobin solution may be clarified of cellular contaminants by any method known in the art such as settling and centrifugation as described below.

Heating of the crude hemoglobin-containing lysate can be achieved by any suitable means known to those skilled in the art, which include, but are not limited to, convection/conduction heat exchangers such as tube and shell heat exchangers (e.g. Process Engineers Inc., Hayward, California) and plate and frame heat exchangers (e.g., APV Crepaco Inc., Rosemont, Illinois); steam injection heating, microwave heating (Charm, U.S. Patent 4,975,246) and the like. Most preferably the crude hemoglobin-containing lysate is heated by a means that heats the solutions extremely rapidly, particularly steam injection (Figure 1B). Steam injection, for example, can occur by combining a steam stream with a stream of crude hemoglobin-containing lysate. Such steam injection can be accomplished using known engineering techniques, such as an in-line static mixer, Venturi mixer or sudden expansion mixer, although the sudden expansion mixer is preferred because of the advantages it affords in avoiding fouling of the fluid stream line. Others are known to those in the art, e.g., *Chemical Engineering Handbook*, 5th edition, McGraw-Hill, New York (1973) pages 6-29 to 6-32. Prior to the introduction of the high heat for the rapid heating, the crude hemoglobin-containing lysate may be prewarmed using suitable heat exchangers known in the art as listed above, most preferably by using a plate and frame heat exchanger (e.g., APV Crepaco Inc., Rosemont, Illinois).

Heating of the crude hemoglobin-containing lysate must occur for a sufficient time and at a sufficient temperature to achieve significant precipitation of contaminating hemoglobins, especially protoporphyrin IX-containing hemoglobin. Heating may also occur for a sufficient time and at a sufficient temperature to ensure that living microorganisms are killed. The

present invention shows that when crude hemoglobin-containing lysates are exposed to heat for a surprisingly short period of time, significant protoporphyrin IX-containing hemoglobin is removed and adequate kill of recombinant organisms is achieved. Preferably, the heating time is less than about five minutes, more preferably less than about three minutes, most preferably less than about two minutes.

In order to achieve bacterial kill and removal of contaminating hemoglobins from a crude hemoglobin-containing lysate in such a short time, it is necessary to heat the crude hemoglobin-containing lysate at a relatively high temperature. Figure 2 shows times and temperatures to achieve a 25 log reduction in living *E. coli*. Such relatively high temperatures are temperatures above those to which hemoglobin is exposed in its natural environment, i.e., 37°C, preferably a temperature of at least about 55°C, more preferably at least about 65°C, more preferably at least about 70°C, even more preferably from about 70°C to about 85°C. Figure 3 indicates that reduction of contaminating hemoglobins in the form of protoporphyrin IX-containing hemoglobin to levels below the preferred one percent level usually occurs only at longer retention times (greater than about 5 seconds) and at higher temperatures (greater than about 70°C). The most preferred combination of temperature and time is about 82 ± 2°C and about 10 to 12 seconds. However, the selection of the most preferred combination of time and temperature is based on maximizing the amount of precipitation of contaminating hemoglobins, especially protoporphyrin IX-containing hemoglobins, while minimizing loss of the hemoglobin of the present invention. It is desirable to reduce the protoporphyrin IX hemoglobin to below levels detectable by the measurement technique described herein, 0.4%.

During heating, the hemoglobin in the crude hemoglobin-containing lysate may be in either the liganded or unliganded state, but preferably is in either the fully liganded or fully unliganded state for selective removal of contaminants without substantial loss of the product hemoglobin.

To ensure that the hemoglobin is either completely in the T state or completely in the R state, the crude hemoglobin-containing lysate can be first either deoxygenated (favoring the unliganded or T state) and/or treated with a suitable liganding gas (favoring the liganded or R state).

Deoxygenation can be accomplished by addition of an exogenous chemical reducing agent to the solution, such as dithionite or bisulfite, or by treating the solution with an inert gas such as nitrogen. Preferably, deoxygenation can occur by isolating the crude hemoglobin-containing lysate

from contact with the atmosphere and allowing the reducing equivalents in a crude hemoglobin-containing lysate to consume any available oxygen. This latter method is the preferred method of deoxygenation and is particularly suited to crude hemoglobin-containing lysates that are obtained as a result of production of recombinant hemoglobin since the interior cell environment of most suitable host cells, particularly bacterial and yeast cells, is highly reducing. Therefore, a crude hemoglobin-containing lysate derived from the lysis of bacterial or yeast cells, which is essentially a crude solution of reducing cell components, will provide a reducing environment without the need of exogenous chemical reducing agents.

Hemoglobin can be liganded with oxygen or non-oxygen ligands by mixing or sparging a crude hemoglobin-containing lysate with a suitable gas mixture. Non-oxygen ligands that can bind to hemoglobin include those recognized by Antonini and Brunori, *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North Holland Publishing Company, Amsterdam (1971) 436 pages. Non-oxygen ligands are preferred because complete oxygen binding to hemoglobin to produce a fully liganded hemoglobin is difficult to achieve in the reducing environment that is present in the crude hemoglobin-containing lysate. Preferably, the non-oxygen ligands are gases that bind to hemoglobin at the heme pocket. Those non-oxygen gases that bind at the heme pocket facilitate the transition to the R state. Examples of such preferred non-oxygen gases that bind at the heme pocket include, but are not limited to, carbon monoxide and nitric oxide. Preferably, the mixing of the crude hemoglobin-containing lysate with a non-oxygen gas occurs by sparging a crude hemoglobin-containing lysate with the non-oxygen gas after the hemoglobin-containing cells have been broken but prior to the heating (Figure 1B). Alternatively, the non-oxygen gas can be mixed with the hemoglobin-containing cells prior to harvesting of the cells (Figure 1A). The most preferred non-oxygen gas is carbon monoxide (CO), which can be essentially pure CO or mixtures of CO with other gases such as air, nitrogen, argon, helium or hydrogen (Scott Specialty Gases, Plumsteadville, Pennsylvania). Preferably, the CO is essentially pure CO. The rate of mixing or sparging can be any rate that results in saturation of the hemoglobin in the solution with CO or other non-oxygen gas. Therefore, the rate of sparging will be a function of the concentration of CO in the sparge gas and can be a specified flow rate of gas (e.g., 0.1-100 standard cubic liters per minute [sclm]) or, alternatively, sparging can continue until a specified amount of the hemoglobin is carbonmonoxy-hemoglobin (also known as carbonylhemoglobin or HbCO).

The amount of carbonmonoxyhemoglobin can be measured using a variety of analytical techniques (Evelyn, et al., (1938) J. Biol. Chem. *126:* 655; Collison et al., (1968) Clin. Chem. *14:* 162; Johansson and Wollmer, (1989) Clin. Physiol. *9:* 581; Rodkey et al., (1979) Clin. Chem. *25:* 1388). The complexity of reported methods range from simple two wavelength analyses (Commins and Lawther (1965) Brit. J. Ind. Med. *22:* 139; Small (1971) J. Appl. Physiol. *31(1):* 154-160) to multiple wavelength measurements requiring extensive calculations (Fogh-Andersen et al., (1987) Clin. Chim. Acta *166:* 283-289).

An approach developed and preferred for the present invention is to employ multiple wavelength measurements in a fashion analogous to commercially available Hemoximeter instruments (Fogh-Andersen, et al., (1987) Clin. Chim. Acta *166:* 283-289) with the exception that extinction coefficients for various mutant hemoglobins can be used when the mutant hemoglobins, such as rHb1.1, are the desired hemoglobin to be purified from crude hemoglobin-containing lysates. This method uses application of a pseudoinverse matrix derived from extinction coefficients of the hemoglobin species of interest at the selected wavelengths to the measured absorbances of a given sample (see Example 5 for further details).

The methods of the present invention can be used to remove contaminating hemoglobins from a crude hemoglobin-containing lysate to result in a substantially protoporphyrin IX-free hemoglobin solution substantially free of contaminating cell membranes, precipitated non-hemoglobin proteins and contaminating hemoglobins, particularly protoporphyrin IX-containing hemoglobins. Cellular debris and precipitated contaminants from either a heated or a non-heated crude hemoglobin-containing lysate can be removed by a number of mechanical means suited to solid-liquid separations, including but not limited to sedimentation techniques such as centrifugation and settling; direct capture techniques such as expanded bed or flow through big bead chromatography; and filtration methods, such as vacuum filtration, pressure filtration, tangential flow or cross flow filtration, most preferably rotary drum vacuum filtration. Note that expanded bed or flow through big bead resins can also be immobilized metal affinity chromatography (IMAC) resins, and thus removal of precipitated contaminating hemoglobins and cellular debris can be combined with IMAC purification (see below) in one step.

Flocculant aids, such as polyethyleneimine, DEAE cellulose, other poly-cationic flocculants (for example, Magnafloc 573™, Cytec Industries, Indianapolis, IN) or diatomaceous earth (Eagle-Picher Minerals, Inc.) may be

added to aid in the precipitation of cellular debris and contaminating material. After mechanical removal of cellular debris and precipitated contaminating material, a substantially protoporphyrin IX-free hemoglobin solution is available for further manipulation. This material may then be used for further applications, such as for example, chemical modifications of hemoglobin to alter oxygen affinity or to cause formation of polymers, or can be further purified as taught in the instant invention.

The substantially protoporphyrin IX-free hemoglobin solution may be treated again with the liganding gas to ensure that all the hemoglobin in the solution is in the appropriate conformation. Most preferably, the hemoglobin solution is sparged again with carbon monoxide as described above. In addition, chelating agents, for example EDTA or DTPA, can be added to prevent oxidative damage of the hemoglobin in the solution. The substantially protoporphyrin IX-free hemoglobin solution can then be subjected to immobilized metal affinity chromatography to further remove other hemoglobin and non-hemoglobin contaminants as well as a surprisingly large amount of *E. coli* proteins. Immobilized metal affinity chromatography utilizes an immobilized metal affinity resin or membrane sheet linked to a bidentate chelator (such as, for example iminodiacetic acid). Suitable IMAC resins include but are not limited to ToyoPearl AF-Chelate 650M (TosoHaas, Inc., Philadelphia, PA), Flow Through Big Bead Resin modified for IMAC (Sterogene, Inc., Arcadia, CA), Chelating Sepharose Big Bead, Chelating Sepharose 6B™ (both Pharmacia, Piscataway, NJ), most preferably Chelating Sepharose Fast Flow (Pharmacia, Piscataway, NJ). Suitable membrane sheets include but are not limited to Acti-Mode Separation™ (FMC, Inc., Natick, MA). The IMAC resin or membrane can be charged with any divalent metal ion, including nickel, copper, cobalt and zinc. Preferably the divalent metal ion used to charge the IMAC resin or sheet is zinc in the form of zinc acetate. Zinc is added to the clarified hemoglobin-containing lysate that is a substantially protoporphyrin IX-free hemoglobin solution to a final zinc concentration of 2 - 4 mM using, for example, 1 M zinc acetate. After zinc addition, the solution is brought to high pH, preferably greater than 7.0, more preferably pH 8.0 to 8.5, most preferably pH 8.0 to 8.3 with 0.5 N NaOH. The solution should be maintained between 6 and 20°C. Prior to loading onto the IMAC resin or membrane the solution should be filtered through a filtration device, preferably a depth filter, preferably a CUNO filtration device (Cuno, Inc., Meriden, CT) to remove any material that might have been precipitated by the addition of the divalent metal. The IMAC resin or membrane should be

maintained at a suitable temperature, preferably less than 25°C, more preferably between 4-15°C, more preferably between 4 and 10°C, and charged with a divalent metal ion as mentioned above. Charging of the resin or membrane can be accomplished by passing a solution of the selected metal over the resin or column in accordance with the manufacturer's recommendations such that all possible metal binding sites are loaded. Most preferably this occurs by passing at least two column volumes of a 20 mM zinc acetate solution over a Chelating Sepharose Fast Flow column. The charged IMAC resin or membrane should then be equilibrated with at least two column volumes of a salt solution, the salt solution preferably less than 500 mM NaCl, most preferably 200 mM NaCl. After equilibration, the substantially protoporphyrin IX-free hemoglobin solution can be loaded onto the charged IMAC resin or membrane, at a load of between 5-100 grams of hemoglobin/liter of resin, most preferably 15-30 g/l.

Contaminating proteins, especially E. *coli* proteins, can be removed from the IMAC resin by washing the resin with sufficient volumes of a suitable buffer or buffer/salt solution. Such buffers can include Tris, HEPES, MOPS, triethylamine, triethanolamine, bicarbonate and phosphate. Preferably a first wash solution comprises a higher concentration salt solution than the load solution, and preferably the resin is washed with at least four column volumes. Preferably the wash solution is a Tris/NaCl solution, more preferably 20 mM Tris and 0.5 M - 0.75 M NaCl (most preferably 0.5 M NaCl) , pH 7.5 - 8.5 (most preferably pH 8.0 - 8.3) with a conductivity between 25 and 50 mS/cm, most preferably about 35 mS/cm to 46 mS/cm.. A second wash may be performed using a second wash solution comprising a buffer and a salt, the buffers including Tris, HEPES, MOPS, triethylamine, triethanolamine, bicarbonate and phosphate, and having still lower conductivity than the first wash buffer, preferably between 2 and 6 mS/cm, most preferably having a conductivity of 2.5 - 4.5 mS/cm. A preferred solution can be 10 mM Tris, 25-50 mM NaCl, pH 7.5-8.5, most preferably pH 8.0-8.3.

The hemoglobin of the present invention can then be eluted from the column (or membrane sheet if an IMAC membrane sheet is used) by increasing the pH or by eluting with a chelating agent or a suitable competitive ligand to produce a partially purified hemoglobin solution. Suitable competitive ligands include histidine, imidazole, Tris, or glycine. Suitable chelating agents include but are not limited to ethylenediamine tetraacetic acid (EDTA) and diethylamine triamine pentaacetic acid (DTPA, also called pentaacetic acid). Most preferably, the hemoglobin of the invention is eluted using a sufficient

amount of EDTA in the elution buffer to elute the hemoglobin of the invention, preferably 10 - 45 mM EDTA at pH > 8.0, most preferably at least four column volumes of 15 mM EDTA at pH 8.5. Elution can occur utilizing any suitable elution scheme, for example by isocratic elution, stepwise elution, stepwise gradient elution or gradient elution. Most preferably elution occurs by isocratic elution.

The partially purified hemoglobin solution can then be further purified by anion exchange chromatography. However, prior to anion exchange chromatography, the solution may be brought to the proper pH and ionic conditions for loading onto the desired anion exchange resin. This can be accomplished by dialysis or ultrafiltration against a suitable cationic buffer. Most preferably this is accomplished by ultrafiltration against the buffer used to equilibrate the anion exchange resin. Suitable buffers include alkylamines, aminoethyl alcohol, triethanolamine, ethylenediamine, Tris and pyridine. Preferably these buffers are Tris or triethanolamine, more preferably this buffer is Tris, most preferably this buffer is 20 mM Tris, pH about 8.9. Ultrafiltration can be performed in any suitable ultrafiltration apparatus equipped with a suitable ultrafilter, preferably an ultrafilter of <50,000 nominal molecular weight cutoff (NMCO), more preferably <30,000, most preferably <10,000.

Suitable anion exchange resins are well known in the art, and include but are not limited to Q Sepharose Fast Flow, DEAE Sephadex A-50 (both from Pharmacia, Inc., Piscataway, NJ), Dowex 1-X8 resin, and AG MP-1 resin (Bio-Rad, Richmond, CA). Most preferably Q Sepharose Fast Flow is used to further purify the partially purified hemoglobin solution. After or during the preparation of the partially purified hemoglobin solution for loading onto a Q Sepharose Fast Flow resin, the resin itself should be equilibrated by washing with the same buffer as was used to prepare the partially purified hemoglobin solution for anion exchange chromatography. As above, this buffer can be any suitable cationic buffer. Suitable buffers include alkylamines, aminoethyl alcohol, triethanolamine, ethylenediamine, Tris and pyridine. Preferably these buffers are Tris or triethanolamine, more preferably this buffer is Tris, most preferably this buffer is 20 mM Tris, pH 8.9. The partially purified hemoglobin solution can be loaded onto the resin to a charge of 5-50 grams of hemoglobin per liter of resin, most preferably 20 grams of hemoglobin per liter of resin. The loaded resin can be washed with a suitable cationic buffer such as the equilibration buffer, more preferably 15 - 25 mM Tris, pH 7.5 - 9.5, conductivity of 200 - 800 uS/cm, most preferably 20 mM Tris, pH 8.9, conductivity of 400 uS/cm. The loaded resin can be further washed with a suitable cationic buffer,

more preferably 10 - 15 mM Tris, pH 7.5 - 8.9, conductivity of 200 - 800 uS/cm, most preferably 12 mM Tris, pH 7.7, conductivity of 700 uS/cm. Elution of the hemoglobin to create a substantially purified hemoglobin-containing solution can be accomplished by lowering the pH to 7.4 - 7.6, most preferably to pH 7.5, using a suitable buffer as described above, most preferably 12 mM Tris. Elution can occur utilizing any suitable elution strategy, for example, isocratic elution, stepwise elution, stepwise gradient or gradient elution. Most preferably elution occurs by isocratic elution. Conductivity of the elution buffer may be between 550 and 1200 uS/cm, more preferably between 550 and 850 uS/cm, most preferably 700 uS/cm.

At this point in the purification, the hemoglobin ligand that was previously added can be removed to produce deoxygenated hemoglobin or can be exchanged with another ligand, preferably oxygen or nitric oxide, most preferably oxygen. This can be accomplished using a number of techniques, including photolysis (Di Iorio, E. E., (1981) in *Methods in Enzymology*, E. Antonini, L Rossi-Bernardi and E. Chiancone, (eds.) Academic Press, NY, pp 57-72); and techniques designed to increase gas mass transfer that are well known in the art (for an example listing of methods see *Chemical Engineering Handbook*, 5th edition, McGraw-Hill, New York (1973) chapter 18). These methods include such techniques as flowing the carbonmonoxyhemoglobin against an oxygen-containing gas stream in a hollow fiber membrane or gas exchange apparatus; diafiltering and sparging the exchanging buffer with oxygen as is diafiltered through an ultrafilter; using a thin-film flow apparatus equipped with a pressurized gas sweep or that allows light mediated carbon monoxide removal; sparging a slow flow trickle-bed with oxygen; sparging packed beds with oxygen, and most preferably, sparging the solution in a pressurized holding tank with oxygen and removing released CO.

Finally, if contaminating metals have been introduced into the substantially purified hemoglobin solution during the production or processing of the hemoglobin, they can be removed in a further aspect of the invention. This removal can be accomplished by addition of any suitable chelating agent, preferably EDTA or DTPA, most preferably EDTA, to the substantially purified hemoglobin solution and subsequent removal of the added chelating agent, or by diafiltration against a controlled amount of chelating agent, preferably EDTA or DTPA, most preferably EDTA as further described in co-pending application Serial Number 08/097,273, filed July 23, 1993, to result in a pure hemoglobin solution. Whether metal removal is necessary or not, the substantially purified hemoglobin solution must be

diafiltered into a suitable formulation buffers. Such buffers are described in Hoffman and Nagai, U.S. Patent 5,028,588, Chivers and Belval, U.S. Serial Number 08/097,273, filed July 23, 1993 and Rosenthal and Gerber, U.S. Serial Number 08/208,740, filed March 8, 1994, and further can include about 150 mM NaCl, about 5 mM sodium phosphate, about 0.025 to 0.035% Tween, less than 1 mM ascorbate, less than 2.5 mM dithionite, less than 50 mM carbohydrate, and less than 2% of a polyethylene glycol, pH about 7.

For the purposes of the present invention, a pure hemoglobin solution is any hemoglobin solution substantially free of protoporphyrin IX-containing hemoglobin contaminants, endotoxins and contaminating metals, especially nickel, that has the functionality necessary for a given utility. The pure hemoglobin solution can be used as, for example, a source of bio-available iron in dietary supplementation, a highly purified molecular weight marker for laboratory applications, a volume expander and most preferably, as a modifier of the oxygen content of a solution, such as in the case of the use of hemoglobin as an oxygen carrying solution that modifies the oxygen content of blood or the use of hemoglobin to change the oxygen content of a tissue or cell culture. The pure hemoglobin solution from which contaminants are removed can be either naturally occurring human hemoglobin or any of a variety of hemoglobins from other species, mutant hemoglobins, or hemoglobin-like molecules. The pure hemoglobin can be used alone in solution or can be part of a suitable pharmaceutical composition such as those described in Hoffman and Nagai, U.S. Patent 5,028,588, Chivers and Belval, U.S. Serial Number 08/097,273, filed July 23, 1993 and Rosenthal and Gerber, U.S. Serial Number 08/208,740, filed March 8, 1994.

It will be appreciated from the methods and descriptions described herein that the present invention can also be used to remove other contaminants besides bacterial contaminants from hemoglobin solutions, for example, the same process can be used to purify hemoglobin expressed in yeast expression systems.

The foregoing description of the specific embodiments reveals the general nature of the invention so that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

All references cited herein to books, journals, articles, patents and patent applications are hereby incorporated by reference for their relevant teachings.

EXAMPLES

The following examples are provided by way of describing specific and preferred embodiments of the present invention without intending to limit the scope of the invention in any way.

Example 1

Production of Protein Solution Containing Hemoglobin

A. Construction of a Bacterial System for the of rHb1.1

Hemoglobin was produced by fermentation of one of the strains listed in Table 1, utilizing either plasmid pSGE1.1E4 or pSGE705. The level of expression of rHb1.1 from the two plasmids was approximately the same, independent of the strain used under the same fermentation conditions. Plasmid pSGE1.1E4 is described in Hoffman et al., WO 90/13645. Construction of pSGE705 is described below.

Strain SGE127 carrying the plasmid pSGE1.1E4 is referred to as SGE128. Strain SGE800 carrying pSGE705 is SGE1353. Strain SGE1661 carrying the plasmid pSGE705 is referred to as SGE1662.

Table 1. Bacterial Strains

STRAIN	GENOTYPE
SGE127	F' traD36 laci$^q\Delta$ (lacZ)M15 proBA+ /ϕ1A^R ϕ2A^R recA1 thi gyrA96(NalR) endA Δ(lac-proBA) hsdR17 relA1 supE44
SGE800	gyrA96(Nal^R) endA hsdR17 relA1 supE44,ϕ1A^R, ϕ2A^R, ϕ 3A^R recJ
SGE1661	gyrA96(Nal^R) endA hsdR17 relA1 supE44, ϕ1A^R, ϕ2A^R, ϕ 3A^R, ϕ 4A^R, recJ

ϕ1A, ϕ2A, ϕ3A, and ϕ4A are phage isolated from the fermentation area. ϕ1A appears to be T5. ϕ2A, ϕ3A, and ϕ4A have not yet been identified but are not T phage.

Materials. pBR322, pUC19 and pNEB193 were purchased from New England Biolabs (Beverly, MA). Most plasmids used for the preparation of pSGE705 are described in Table 2. Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer Model 392 (Foster City, CA). The oligonucleotides used in preparing pSGE705 are listed in Table 3. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and used according to manufacturer's specifications. T4 DNA ligase was purchased from either New England Biolabs (Beverly, MA) or Gibco-BRL (Gaithersburg, MD) and used according to manufacturer's specifications. Pfu polymerase was purchased from Stratagene (La Jolla, CA) and used according to manufacturer's specifications.

Media used are described in J. H. Miller, *Experiments in Molecular Genetics*. (Cold Spring Harbor Press, (1972) Cold Spring Harbor, NY) and J. H. Miller, *A Short Course in Bacterial Genetics* (Cold Spring Harbor Press, (1992) Cold Spring Harbor, NY). Acridine orange, ampicillin and kanamycin sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Tetracycline was purchased from Aldrich Chemicals (Milwaukee, WI).

Genetic and Molecular Biological Procedures. Standard bacterial genetic procedures are described in J. H. Miller, *Experiments in Molecular Genetics*. (Cold Spring Harbor Press, (1972) Cold Spring Harbor, NY) and J. H. Miller, *A Short Course in Bacterial Genetics* (Cold Spring Harbor Press, (1992) Cold Spring Harbor, NY). Standard molecular biology procedures were performed as described by Sambrook (Sambrook et al., *Molecular Cloning*, (1989) Cold Spring Harbor Press, Cold Spring Harbor, NY).

Plasmid DNA Transformation. DNA transformations were performed by the procedure described by Wensick (Wensick et al., (1974) Cell 3: 315-325). Briefly, cells were grown to mid log phase and then pelleted, resuspended in an equal volume of 10 mM $MgSO_4$ and incubated on ice for 30 minutes. The cells were centrifuged and the pellet resuspended in 1/2 original volume of 50 mM $CaCl_2$ and placed on ice for 20 minutes. The cells were centrifuged again and then resuspended in 1/10 original volume of 50 mM $CaCl_2$. Plasmid DNA was added to the competent cells in a solution of 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ and 10 mM $CaCl_2$. The mixture was incubated on ice for 15 minutes and then incubated at 37°C for 5 minutes. One milliliter of LB medium was added and the mixture incubated with shaking for 30-60 minutes. The culture was then centrifuged, resuspended in 0.1 ml of LB medium and

plated on the appropriate selective medium. SGE1662 was prepared by a one-step transformation method (Chung, C.T., et al., (1989) Proc. Natl. Acad. Sci. *86:* 2172-2175) which introduced pSGE705 into SGE1661.

Purification of DNA. DNA fragments were purified from an agarose gel using the Geneclean system (Bio 101, Inc. La Jolla, CA; method provided with product). PCR products were prepared and cleaved with restriction endonucleases using the Double Geneclean system (Bio 101, Inc. La Jolla, CA; method provided with product). Briefly, the PCR product was purified away from the PCR primers, then the PCR product was cleaved with restriction endonuclease(s) and purified from the restriction endonuclease and buffer. The PCR product was then ready for ligation reactions.

Table 2. Plasmids

PLASMID	DESCRIPTION
pSGE1.1E4	rHb1.1 expression plasmid containing di-alpha and beta genes
pSGE1.1E5	pSGE1.1E4 but ampicillin resistant instead of tetracycline resistant
pSGE490	pUC19 lacI on a Bam HI-Hind III fragment
pSGE491	pUC19 α on an Eco RI-Xba I fragment
pSGE492	pNEB193 Ptac- α
pSGE493	pUC19 β on an Xba I-Hind III fragment
pSGE500	pUC19 α β on a Bam HI-Hind III fragment
pSGE504	pSELECT-1 replace Sty I with a Pme I site
pSGE505	pSGE504 rrnB T1 transcriptional terminator in the Eco RI-Cla I sites
pSGE507	ColE1 ori and tet, 2213 bp
pSGE509	ColE1 ori tet lacI, 3425 bp
pSGE513	ColE1 ori tet lacI α β, 4386 bp
pSGE515	ColE1 ori tet lacI diα β , 4812 bp
pSGE700	pTZ18U + diα β from pSGE515
pSGE705	modified rHb1.1 expression plasmid, ColE1 ori, tet, lacI, diα and β genes
pTZ18U	a phagemid derivative of pUC19, for oligonucleotide directed mutagenesis
pDLII-91F	pGEM1 + α missing valine in 2nd position (Des-val)
pNEB193	pUC19 with more restriction sites in the multi cloning sites
pBR322	ColE1 ori tet amp
pRG1	pACYC177 lacI^q

Table 3. Oligonucleotides

OLIGO	SEQUENCE (5'-3')	DESCRIPTION
EV18 SEQ. ID #1	CGGGAATACGGTCTAGATCATTAACGGTATTTCGAAGTCAGAACG	C-term of α gene, Xba I site
EV27 SEQ. ID #2	GATCCGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGA ATTGTGACGGATAACAATTTCACACAGGAAATTAATTAATGCT GTCTCC	tac promoter sequence, Bam HI-Eag I sites
EV28 SEQ. ID #3	GGCCGGAGACAGCATTAATTAATTTCCTGTGTGAAATTGTTATC CGCTCACAATTCCACACATTATACGAGCCGATGATTAATTGTCA ACAGCTCG	tac promoter sequence, Bam HI-Eag I sites, complement of EV27
EV29 SEQ. ID #4	TCGGATTCGAATTCCAAGCTGTTGGATCCTTAGATTGAAC TGTCTCCGGCCGATAAAACCACCG	5' end of α with Eco RI, Bam HI and Eag I sites
EV30 SEQ. ID #5	CGGAAGCCCAATCTAGAGGAAATAATATATGCACCTGACTCCG GAAGAAAAATCC	5' end of β with Xba I site
EV31 SEQ. ID #6	CCCGAAACCAAGCTTCATTAGTGAGCTAGCGCGTTAGCAACACC	3' end of β with Hind III site
MW007 SEQ. ID #7	TTTAAGCTTCATTAGTGGTATTTGTGAGCTAGCGCGT	mutagenesis reverse primer, adds last 3 codons of β for pSGE515
MW008 SEQ. ID #8	CAGCATTAATTAACCTCCTTAGTGAAATTGTTATCCG	mutagenesis reverse primer to optimize α ribozyme binding site (RBS)
MW009 SEQ. ID #9	GGTGCATATATTTACCTCCTTATCTAGATCATTAACGGTATTTCG	mutagenesis reverse primer to optimize β RBS and remove second Bgl II site
TG14 SEQ. ID #10	GGTTTAAACC	Pme I linker
TG59 SEQ. ID #11	GGCGAATAAAAGCTTGCGGCCGCGTTGACACCATCGAATG GCGCAAAACCTTTCGCGG	Upstream of lacI gene, has a Hind III and a Not I site upstream of the promoter

Continuation of Table 3. Oligonucleotides

OLIGO	SEQUENCE (5'-3')	DESCRIPTION
TG60 SEQ. ID #12	GGGCAAATAGGATCCAAAAAAAAGCCCGCTCATTAGG CGGGCTTTATCACTGCCCGCTTTCCAGTCGGG	Downstream side of lacI gene with the trp transcriptional terminator and a Bam HI site
TG62 SEQ. ID #13	CCCCGAAAAGGATCCAAGTAGCCGGCGGCCGCGTTCCACTG AGCGTCAGACCCC	upstream primer for pBR322 ori positions 3170-3148 with a Bam HI and a Not I site
TG63 SEQ. ID #14	GGCGGTCCTGTTTAAACGCTGCGCTCGGTCGTTCGGCTGCGG	downstream primer for pBR322 ori positions 2380-2404 with Pme I site

Annealing of oligonucleotides. Complementary oligonucleotides were annealed according to the following procedure. Equimolar amounts of each oligonucleotide were mixed in 15-25 µl of 10 mM Tris-HCl pH 8.0/1 mM EDTA and incubated at 65°C for 30 minutes. The sample was transferred to a 37°C water bath for 30 minutes. Finally, the sample was incubated on ice for 60 minutes or overnight in the refrigerator.

Oligonucleotide directed mutagenesis. Oligonucleotide directed mutagenesis was performed with the Muta-gene phagemid *in vitro* mutagenesis kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions which are based on the method of Kunkel (Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. USA *82:* 488; Kunkel et al., (1987) Methods Enzymol. *154:* 367). The rHb1.1 region of pSGE515 was cloned into pTZ18U (Bio-Rad, Hercules, CA or U.S. Biochemical, Cleveland, OH) on a Bam HI-Hind III fragment to create pSGE700. Three oligonucleotides, MW007, MW008 and MW009 were used to simultaneously introduce multiple changes in a single reaction.

Preparation of pBR322 *ori*. PCR primers were designed to amplify the pBR322 origin of replication. These primers, TG62 and TG63, annealed to the positions 2380-2404 and 3170-3148 on the pBR322 DNA sequence (Sutcliffe, J. G. (1979) Cold Spring Harbor Symp. Quant. Biol. *43:* 77-90). The PCR product

was digested with Not I and Pme I. The DNA fragment was purified according to the Geneclean procedure.

Preparation of tet gene fragment. The source for the tet gene was pSELECT-1 (Promega Corp., Madison, WI). This plasmid has a number of restriction endonuclease sites, such as Bam HI, Hind III, Sal I and Sph I removed from the tet gene (Lewis and Thompson (1993) Nucleic Acids Res. *18*: 3439-3443). A Pme I linker was inserted into the Sty I site of pSELECT-1. This plasmid was designated pSGE504. Oligonucleotides TG71 and TG72 were annealed and ligated to the Eco RI - Cla I fragment of pSGE504. This plasmid, pSGE505, was shown to have the expected restriction endonuclease sites and to have lost the sites present in the multicloning site of pSELECT-1. pSGE505 was digested with Not I and Pme I. The 1417 bp fragment was purified according to the Geneclean protocol.

Preparation of lacI gene. The lacI gene was isolated by amplifying the gene sequence from pRG1 (a gift from R. Garcia, Dana-Farber Cancer Inst., Boston, MA) that carried the lacI gene. The PCR primers, TG59 and TG60, were designed to generate a wild type lacI promoter (Farabaugh, P. J. (1978) Nature *274*: 765) upstream of the gene and to place the trp terminator sequence (Christie et al., (1981) Proc. Natl. Acad. Sci. USA *78*: 4180-4184) downstream of the gene. The same step could be carried out using Y1089 (Promega) or chromosomal DNA from any *E. coli* strain carrying the lac region, such as MM294 (ATCC 33625.) The PCR product was gel purified and isolated according to the Geneclean procedure and cloned into Bam HI-Hind III digested pUC19 DNA to make pSGE490.

Construction of pSGE515. PCR primers EV29 and EV18 were chosen to amplify the alpha gene from pDLII-91F (Hoffman et al., WO 90/13645). The purified PCR product was cleaved with the restriction endonucleases Eag I and Xba I.

To create a plasmid that contained P_{tac}-α, the alpha gene (from above) and the tac promoter, which was prepared by annealing EV27 and EV28, were mixed with Eco RI-Xba I cleaved pUC19 DNA. The mixture of the three DNA fragments, in approximately equimolar ratio, was treated with T4 DNA ligase. After incubation the ligation mixture was used to transform SGE476 (equivalent to MM294, ATCC 33625) and ampicillin resistant transformants were selected. (Transformation into Strain MM294 (ATCC 33625) would yield

equivalent results.) An isolate with the correct restriction endonuclease fragments (consistent with Figure 4) was designated pSGE492. The α gene and the tac promoter DNA sequences were verified by DNA sequencing.

Primers EV30 and EV31 were used to amplify the β gene from pSGE1.1E4 by PCR. The purified β gene fragment was digested with Xba I and Hind III and then mixed with Xba I -Hind III digested pUC19 DNA and treated with T4 DNA ligase. The ligation mixture was used to transform competent SGE476 and transformants were selected on LB + ampicillin (100 μg/ml) plates. An isolate that contained the appropriate restriction endonuclease fragments (consistent with Figure 4) was chosen and designated pSGE493. The β gene was confirmed by DNA sequencing.

The β gene was isolated from pSGE493 by restriction with Xba I and HindIII followed by purification according to the Geneclean method. This DNA fragment was then ligated to Xba I-Hind III restricted pSGE492 DNA and transformed into SGE713. (Any dam strain such as JM110 (ATCC 47013) or GM119 (ATCC 53339) could also be used.) An ampicillin resistant transformant that carried a plasmid that had the appropriate restriction fragments (consistent with Figure 4) was chosen and designated pUC19αβ (pSGE500).

The Bam HI-Hind III fragment that contained the α and β genes of pSGE500 was purified according to the Geneclean method. An Xho I fragment that carried a portion of the di-α gene containing the glycine linker region was gel purified from pSGE1.1E5. pSGE1.1E5 (described in Hoffman et al., US Serial Number 789,179, filed November 8, 1991) is a tetracycline sensitive analogue of pSGE1.1E4 (Hoffman et al., WO 90/13645), which could also have been used.

The pBR322 origin of replication region (pBR322 ori, above) was ligated to the tet gene fragment (above) and the ligation mixture was transformed into SGE476. (Transformation into MM294, above would yield equivalent results.) Tetracycline resistant transformants were selected and plasmid DNA was isolated and analyzed. An isolate that contained the appropriate restriction endonuclease fragments (consistent with Figure 4) was chosen and designated pSGE507.

Next, pSGE507 and pSGE490 were digested with Bam HI and Not I and the appropriate fragments (consistent with Figure 4) were purified. The two purified fragments were ligated together and the ligation mixture was used to transform competent SGE713. (Any dam strain could also be used; see above.) Tetracycline resistant transformants were selected, and plasmid DNA was

isolated and analyzed. A plasmid that had the appropriate restriction fragments (consistent with Figure 4) was chosen and designated pSGE509.

The purified Bam HI-Hind III fragment of pSGE500 that contained the α and β genes was ligated to Bam HI-Hind III digested pSGE509. The ligation mixture was used to transform pSGE713 (see above for equivalent strains) and tetracycline resistant transformants were selected and characterized. An isolate yielding the correct size plasmid with the expected restriction endonuclease fragments (consistent with Figure 4) was chosen and designated pSGE513.

The Xho I fragment of pSGE1.1E5 (described in Hoffman et al., US Serial Number 789,179, filed November 8, 1991) that contained the di-α glycine linker sequence was ligated to Xho I digested pSGE513 to create a plasmid that contained the di-α gene. SGE753 was transformed with the ligation mixture and tetracycline resistant transformants were selected. (Transformation into SGE800 would have yielded equivalent results.) Isolates were screened to identify those that contained the Xho I fragment inserted into pSGE513 in the correct orientation (consistent with Figure 4). An isolate that contained the correct configuration of the di-α gene, as determined by restriction endonuclease analysis with Eag I, was designated pSGE515.

Modification of pSGE515 to create pSGE705. The DNA sequence record used to design PCR primers for the amplification of the β gene did not contain the C-terminal three amino acids. Oligonucleotide directed mutagenesis was used to add these nine nucleotides to the DNA sequence of the β gene. In the same reactions, modifications were introduced to optimize the ribosome binding sites for the di-α and β genes, and to remove a Bgl II site near the end of the di-α gene.

In the construction of the plasmid, one of the last steps was the modification of the ribosome binding sites to optimize the sequences. The following are the changes that were made with the oligonucleotides MW008 and MW009.

di alpha

```
before - CAATTTCAC--AGGAAATTAATTAATGCTG    SEQ. ID #15
         |||||||||**||||**|||||||||||||
after  - CAATTTCACTAAGGAGGTTAATTAATGCTG    SEQ. ID #16
```

Four nucleotide changes, shown above, including the insertion of two nucleotides, were introduced with MW008 to optimize the ribosome binding site for di-alpha (| - indicates identity, * - indicates a change).

beta

```
before - TAAaGATCTAGA---GGAAATAA-TATATGCAC SEQ. ID #17
         |||*|||||||||***|||**|||*|||||||||
after  - TAATGATCTAGATAAGGAGGTAAATATATGCAC SEQ. ID #18
```

The six nucleotide changes shown above, including the insertion of four nucleotides, were introduced with MW009 to optimize the ribosome binding site for beta. The lower case "a" on the before strand was a T to A mutation in the construction of the alpha gene that introduced a Bgl II site into the sequence. This was removed so that there would only be a single Bgl II site in pSGE705 (|)- indicates identity, * - indicates a change).

End of Beta

```
before - CTCGCTCAC---------TAATGAA     SEQ. ID #19
         |||||||||*********|||||||
after  - CTCGCTCACAAATACCACTAATGAA     SEQ. ID #20
```

MW007 introduced the coding sequence for the last three amino acids of the beta gene as shown above (| - indicates identity, * - indicates a change).

Putative mutants were screened for loss of a Bgl II restriction endonuclease cleavage site (introduced by MW008). Seventeen of 24 had lost the site and were further characterized by DNA sequencing at the other two mutagenized sites. One of the 17 had incorporated all three modifications. These changes were verified by DNA sequencing and the rHb1.1 genes were cloned into Bam HI-Hind III digested pSGE509. An isolate that had the correct restriction endonuclease fragments was designated pSGE705.

A plasmid map of pSGE705 is shown in Figure 4. The plasmid map indicates many of the restriction endonuclease cleavage sites. pSGE705 is smaller than its counterpart, pSGE1.1E4, and the placement of its restriction sites facilitates modular alterations of the sequence. An unused antibiotic resistance marker was removed, and a promoter was added to the lacI gene that would allow tighter control of rHb1.1 expression.

A new sequence upstream of the α gene minimized the distance between the tac promoter (De Boer et al., (1983) Proc. Natl. Acad. Sci. *80:* 21-25) and the first codon of the alpha gene. The intergenic region between the di-α gene and the β gene was also designed to contain the minimum sequence that contained a restriction endonuclease site and the ribosome binding site for the β gene.

On November 10, 1993 *E. coli* strains SGE127 and SGE800 were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD (ATCC Accession Numbers 69485 and 69484, respectively); *E. coli* strain SGE1661 was deposited January 20, 1994 (ATCC Accession Number 55545). Deposits were made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organisms will be made available by the ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

B. Fermentations

The six hundred liter fermentation procedures described below were used to obtain material for purification and functionality determinations.

Seed Stock

Seed stock was grown up in LB broth containing 10 g/L BactoTryptone™, 5 g/L yeast extract, 5 g/L NaCl, 0.2 g/L NaOH, and 10 ug/ml tetracycline to an optical density of 1.5 - 1.7 at 600 nm. The solution was then made up to 10% glycerol and stored at -80°C until required.

Fermentor Inoculum (500 ml broth in 2 L shake flasks)

To prepare the fermentor inoculum, seed stock was thawed and 0.1-0.4 ml of seed stock were inoculated into 500 ml of a solution containing approximately:

4 g/L KH_2PO_4
7 g/L K_2HPO_4
2 g/L $(NH_4)_2SO_4$
1 g/L Na_3 Citrate·$2H_2O$
153 mg/L $MgSO_4 \cdot 7H_2O$

2.3 g/L of L-proline
2 g/L yeast extract
4.8-5 g/L glucose
75 mg/L thiamine HCl
12 mg/L tetracycline
81 mg/L $FeCl_3 \cdot 6H_2O$
4 mg/L $ZnCl_2$
6 mg/L $CoCl_2 \cdot 6H_2O$
6 mg/L $Na_2MoO_4 \cdot 2H_2O$
3.1 mg/L $CaCl_2 \cdot 2H_2O$
3.9 mg/L $Cu(II)SO_4 \cdot 5H_2O$
1.5 mg/L H_3BO_3
300 µl/L HCl

This culture was allowed to grow for 10 hours at 37°C on a shaker. Four flasks were combined and used to inoculate the Seed Fermentors.

Seed Fermentor (14 L volume in 20 L Fermentor)

The entire fermentor inoculum was then asceptically transferred to a 20-liter fermentor containing 10 liters of a solution described below. Note that masses of added reagents are calculated using the final volume of fermentation, 14 liters and are approximate within measurement error.

1.8 g/L KH_2PO_4
3.3 g/L K_2HPO_4
1.8 g/L $(NH_4)_2SO_4$
155 mg/L thiamine HCl
10.3 mg/L tetracycline
3.1 g/L proline
1.9 g/L $MgSO_4 \cdot 7H_2O$
1.9 g/L Na_3-citrate$\cdot 2H_2O$
133 mg/L $FeCl_3 \cdot 6H_2O$
6.4 mg/L $ZnCl_2$
9.9 mg/L $CoCl_2 \cdot 6H_2O$
9.9 mg/L $Na_2MoO_4 \cdot 2H_2O$
5 mg/L $CaCl_2 \cdot 2H_2O$
6.3 mg/L $Cu(II)SO_4 \cdot 5H_2O$
2.5 mg/L H_3BO_3
494 µl/L HCl

The pH was maintained at 6.8 to 6.95 by addition of 15% to 30% NH_4OH, dissolved oxygen was maintained at or above 20%, and 50 to 70% glucose was added throughout the growth period, sufficient to maintain low but adequate levels of glucose in the culture (0.1 g/L-10 g/L). Dissolved oxygen was maintained as close to 20% as possible. The culture was grown between 28 and 32°C for approximately 12 hours prior to transfer to the 600 liter fermentor.

Production Fermentor

The entire seed fermentor inoculum was then asceptically transferred to a 600-liter fermentor containing approximately 375 liters of the solution described below. Note the reagent additions are calculated with the final volume of the fermentation, 450 liters. All numbers are approximate.

1.8 g/L KH_2PO_4
3.3 g/L K_2HPO_4
1.8 g/L $(NH_4)_2SO_4$
3.3 ml/L polypropylene glycol-2000
220 g/L glucose
143 mg/L thiamine HCl
9.4 mg/L tetracycline
1.4 g/L $MgSO_4 \cdot 7H_2O$
1.4 g/L Na_3-citrate$\cdot 2H_2O$
2.9 g/L L-proline
99 mg/L $FeCl_3 \cdot 6H_2O$
4.8 mg/L $ZnCl_2$
7.3 mg/L $CoCl_2 \cdot 6H_2O$
7.3 mg/L $Na_2MoO_4 \cdot 2H_2O$
3.7 mg/L $CaCl_2 \cdot 2H_2O$
4.7 mg/L $Cu(II)SO_4 \cdot 5H_2O$
1.8 mg/L H_3BO_3
366 µl/L HCl

The pH was maintained at 6.8 - 6.95 by addition of 15% to 30% NH_4OH, dissolved oxygen was maintained at or above 20%, and 50-70% glucose was added throughout the growth period, sufficient to maintain low but adequate levels of glucose in the culture (0.1 g/L-10 g/L). The culture was grown between 25 and 30°C to an OD_{600} ~ 10-40 prior to induction with 10-1000 µM IPTG. Upon induction of hemoglobin synthesis, the *E. coli* heme biosynthesis was supplemented by addition of hemin dissolved in 1 N NaOH, either by

addition of the total mass of hemin required at induction, by continuous addition of hemin throughout the induction period, or by periodic addition of hemin dissolved in 50 mM to 1 M NaOH (e.g. one third of the total mass of hemin to be added to the fermentor was added at induction, another third was added after 1/4 of the total time after fermentation had elapsed, and the last third was added half-way through the induction period). Total hemin added ranged from 50 to 300 mg/L. The fermentor was allowed to continue for 8-12 hours post-induction. At the end of this period, several 1 ml aliquots were removed from the broth for determination of hemoglobin production and protoporphyrin IX content.

Although most of the material used for purification was produced by fermentation at the 600 liter scale, some was prepared in the 1000 liter scale. Fermentations at this scale differed little from 600 liter scale fermentations, except in the areas discussed below.

Fermentor inocula were grown in 2.5 liter final volumes rather than 0.5 liter final volumes. However, they were grown in the same medium as described for the 500 ml inoculum. Seed fermentations were performed using a 110 liter final volume rather than a 14 liter final volume, and the fermentations were performed using a slightly different medium:

2.6 g/L KH_2PO_4
4.6 g/L K_2HPO_4
2.6 g/L $(NH_4)_2SO_4$

All other components were as described for the 14 liter seed fermentor. Production fermentations were performed exactly as described for the 450 liter fermentations, except that the final volume of the fermentation was 1100 liters.

Example 2
Heating of Crude Deoxy Hemoglobin-Containing Lysates with a Tube and Shell Heat Exchanger

Fermentations were performed as described in Example 1 with *E. coli* strains SGE127 or SGE800 which contained the plasmids pSGE1.1E4 and pSGE705 respectively. The two strains produced the same mutant hemoglobin and the fermentation products were essentially the same. Unwashed *E. coli* cells (100-300L) were broken with a Niro homogenizer. The crude lysates were heated with a tube and shell design heat exchanger for 1.6-36 seconds at 70-90°C. 5 ml of 50% Magnafloc 573™ (Cytec Industries, Indianapolis, IN) solution/L of lysate, was then added and the lysate was clarified by centrifugation.

Protoporphyrin IX and heme could not be accurately quantitated in any lysate material, whether crude or clarified due to interference from other species (hemin and other fermentation products and components). Thus for protoporphyrin IX determinations, samples were prepared using immobilized metal affinity chromatography charged with zinc. Material that had been prepared using IMAC was suitable for protoporphyrin IX determination and reflected the same proportion of protoporphyrin IX that was present in the lysate (demonstrated by spike recovery experiments).

Immobilized metal affinity chromatography was performed using a chelating Sepharose Fast Flow 6B (Pharmacia, Inc., Piscataway, NJ) column charged with 2 column volumes of 20 mM $Zn(OAc)_2$. The column was then equilibrated with 2 column volumes of 200 mM NaCl. Clarified *E. coli* lysate prepared from unwashed cells was brought up to 1-2 mM $Zn(OAc)_2$, filtered, and then loaded onto the column. The column was washed with 4 column volumes of 500 mM NaCl/20 mM Tris, pH 8.3-8.5, and then further washed with 4 column volumes of 20 mM Tris, pH 8.3-8.5. Captured hemoglobin was eluted from the column with 15 mM EDTA, pH 8.5. The column was then cleaned with 2 column volumes of 200 mM NaCl followed by 2 column volumes of 0.5 N NaOH.

Example 3

Effect of Temperature on the Efficiency of Protoporphyrin IX Removal with Rapid Heating under Deoxy Conditions

The cells from two fermentations with two different strains of *E. coli*, SGE127 and SGE800 containing the plasmids pSGE1.1E4 and pSGE705, respectively, were broken with the Niro homogenizer (40°C). No specific process steps were taken to ensure either deoxygenated or liganded conditions, but spectral analysis of the crude lysate solutions demonstrated that all the hemoglobin in the solutions was in the deoxygenated state. The crude *E. coli* lysate contained sufficient reducing power to maintain the solution in the deoxygenated state. Four portions of the crude solution were heated using a tube and shell heating apparatus (Process Engineers Inc., Hayward, California) for 6 seconds at 70, 80, 85, and 90°C.

Example 4

Heating of a Crude Hemoglobin-Containing Lysate -Liganded Conditions

Hemoglobin produced by fermentations as in Example 1 with both *E. coli* strains SGE127 and SGE800 containing the plasmids pSGE1.1E4 and

pSGE705, respectively, was sparged with 99.99% carbon monoxide at a flow rate of about 5 standard cubic liters per minute or alternatively, hemoglobin was produced by fermentations as in Example 1 with E. *coli* strain SGE1661 containing the plasmid pSGE705, and sparged with 99.99% carbon monoxide at a flow rate of about 300-500 mls/min so that there was a calculated stoichiometric excess of carbon monoxide relative to all available ligand binding sites in the solution. All sets of fermentations produced comparable results.

After sparging, the crude hemoglobin-containing lysate was preheated in a plate and frame apparatus (APV Crepaco Inc., Rosemont, Illinois) to a temperature of 55°C then heated for a length of time at a specific temperature by steam injection of the preheated crude hemoglobin-containing lysate as illustrated in the configuration of Figure 1B. Steam injection heating results in nearly instantaneous heating of the liquid. A variety of heating temperatures and retention times were examined.

All combinations of time and temperature and ligand choice described in Examples 2-4 resulted in substantially protoporphyrin IX free hemoglobin solutions with significantly reduced protoporphyrin IX-containing hemoglobin concentrations, as demonstrated in Figure 3. The final amount of protoporphyrin IX-containing hemoglobin in the lysate after heating decreased slowly from 40-80°C, and rapidly between 80-90°C (Figure 3). These data demonstrate that *E. coli* lysates heated between 80-90°C had the most significant decreases in protoporphyrin IX-containing hemoglobin. However, heating the lysate above 85-90°C resulted in a loss of rHb1.1 (Figure 3). These data show that the removal of protoporphyrin IX-containing hemoglobin is increased at higher temperatures or longer retention times. However, the data also show that removal of hemoglobin is also increased at higher temperatures and longer retention times, irrespective of the conditions under which the heating takes place.

Example 5
Spectrophotometric Method of Measuring Carbonmonoxyhemoglobin

A spectrophotometric method for the quantitation of hemoglobin species in aqueous solution was developed. Of particular importance was the ability to quantitate the various liganded forms of hemoglobin, e.g., methemoglobin (HbMet), carbonmonoxyhemoglobin (HbCO), oxyhemoglobin

(HbO_2), reduced hemoglobin (Hb) as well as the total hemoglobin content of the solution per se (HbTotal).

Multiple wavelength measurements in a fashion analogous to commercially available Hemoximeter instruments (Fogh-Andersen, et al., (1987) Clin. Chim. Acta *166:* 283-289) were developed, with the exception that extinction coefficients for rHb1.1 were used. This method used application of a pseudoinverse matrix, derived from extinction coefficients of the hemoglobin species of interest at the selected wavelengths, to the measured absorbances of a given sample. The assumptions made for this method were:

1. Only the defined hemoglobin species were responsible for absorption at the measured wavelengths.
2. Observed absorbances at all wavelengths were the sum of the absorbances for each species at that wavelength.
3. The absorbance of each species followed Beer's Law, i.e. absorption was a linear function of concentration.

Beer's Law may be represented as a series of simultaneous equations for several species:

$$\begin{bmatrix} A_1 \\ A_2 \\ \cdot \\ \cdot \\ \cdot \\ A_n \end{bmatrix} = \begin{bmatrix} \varepsilon_{11} & \varepsilon_{12} & \cdots & \varepsilon_{1m} \\ \varepsilon_{21} & \varepsilon_{22} & \cdots & \varepsilon_{2m} \\ \cdot & & & \\ \cdot & & & \\ \cdot & & & \\ \varepsilon_{n1} & \varepsilon_{n2} & \cdots & \varepsilon_{nm} \end{bmatrix} * \begin{bmatrix} c_1 \\ c_2 \\ \cdot \\ \cdot \\ \cdot \\ c_m \end{bmatrix}$$

Where A_1 is the absorption at wavelength 1, ε_{11} is the extinction coefficient of species 1 at wavelength 1, ε_{12} is the extinction coefficient of species 1 at wavelength 2, etc., and c_1 is the concentration of species 1.

If an extinction matrix is not square, it is not possible to generate a simple inverse matrix, instead a pseudoinverse matrix must be employed as follows:

$$A = \varepsilon * c$$

$$\varepsilon^T * A = (\varepsilon^T * \varepsilon) * c$$

$$[(\varepsilon^T * \varepsilon)^{-1} * \varepsilon^T] * A = I * c = c$$

where ε^T is the transposed extinction matrix, $(\varepsilon^T * \varepsilon)^{-1}$ is the inverse matrix of the product of matrices ^{T} and ε, and $[(\varepsilon^T * \varepsilon)^{-1} * \varepsilon^T]$ is the pseudoinverse matrix.

All spectrophotometric measurements were performed using a 2 nm resolution HP8452 diode array spectrophotometer (Hewlett Packard, Palo Alto, CA) blanked against air. The majority of spectra were collected using a 0.1 mm pathlength quartz cell, however some experiments required the use of 1 mm and 1 cm pathlength quartz cells. Spectra were collected over a 190 to 820 nm window with a 5 second integration time. Extinction coefficients were determined using the product from a fermentation of *E. coli* strain SGE127 containing the plasmid pSGE1.1E4, under conditions described below. No attempt was made to baseline correct the spectra for extinction coefficients at the time of collection. Subsequent sample spectra were collected employing the spectrophotometer's baseline correction routine specified to zero the average absorption between 700 nm and 800 nm.

Extinction Coefficient Determination:

In order to determine the pseudoinverse matrix for rHb1.1 at a given set of wavelengths, it was necessary to establish the extinction coefficients for the species of interest at those wavelengths. The following sequence was employed to calculate the extinction coefficients for HbMet, HbCO, HbO_2 and Hb.

1. A two-fold excess with respect to heme of potassium ferricyanide ($K_3Fe(CN)_6$) was added to an aliquot of the fermentation product and allowed to react for not less than 30 minutes prior to measurement. Spectra were then collected and assumed to be 100% HbMet. A diluted sample was measured with and without CO addition to determine the completion of oxidation.

2. A sample of recombinant hemoglobin was placed into a 5 ml syringe. The syringe was filled with 99% carbon monoxide, sealed and rotated for approximately 5 minutes. The gas was expelled and replaced with fresh carbon monoxide, sealed and rotated for approximately 5 minutes. The procedure was then repeated once more with carbon monoxide and the sample stored sealed with no headspace. Addition of carbon monoxide was assumed not to affect the HbMet content.

3. A sample of the fermentation product was processed as in sample 2 with the exception that oxygen was used in place of carbon monoxide. The addition of oxygen was assumed not to affect the HbMet or HbCO content.

4. A 100 fold excess of sodium dithionite ($Na_2S_2O_4$) based on hemes was added to an aliquot of the fermentation product and allowed to react for 5 minutes prior to measurement. The addition of the dithionite was assumed to

result in reduction of HbMet and HbO_2 to Hb but not to affect the HbCO content.

The original HbTotal concentration was taken from cyanomethemoglobin analysis as 50.79 g/L (for details of the cyanomethemoglobin analysis, see Tentori, T. and Salvati, A.M., (1981) in *Methods in Enzymology*, E. Antonini, L Rossi-Bernardi and E. Chiancone, (eds.) Academic Press, NY, pp 707-715). The original HbMet content was 5.64% as determined by the Evelyn-Malloy method. The original HbCO content was taken from CO gas chromatography analysis as 0.82%. Reduced hemoglobin content was assumed to be 0% for all samples except the dithionite reduction.

A total of 30 spectra were gathered for each of HbMet, HbCO and HbO_2, and 10 spectra were collected for Hb. In order to assure a zero baseline, the average absorption from either 700-800 nm or 790-810 nm for each spectrum was calculated and subtracted from all absorption in that spectrum. The rationale for evaluating two different correction windows was to ensure that no meaningful absorptions were lost due to the background correction. Subsequent calculations were performed in parallel to establish the effect of the correction window. All spectra, within a correction window set for a given species, were averaged at each wavelength. Accommodating for the dilution associated with the addition of $K_3Fe(CN)_6$ and $Na_2S_2O_4$ and the mixed nature of some samples the extinction coefficients for each species were calculated in units of OD*L/g*0.1mm and OD*L/4mmol*cm. Calculated values were then compared to literature values for native HbA_0 (Zijlstra et al., (1991) Clin. Chem. *37:* 1633; van Assendelfdt and Zijlstra (1975) Anal. Biochem. *69:* 43; Benesch et al., (1973) Anal. Biochem. *55:* 245).

Pseudoinverse matrices were prepared for the following extinction coefficient sets and for each correction window:

1. All wavelengths 500-640 nm including Hb (All Wave/Hb)
2. Six wavelengths including Hb (6 Wave/Hb)
3. All wavelengths 500-640 nm excluding Hb (All Wave/no Hb)
4. Six wavelengths excluding Hb (6 Wave/no Hb)

The six wavelengths were 504 nm, 538 nm, 554 nm, 562 nm, 580 nm, and 630 nm. Selection of these wavelengths was based upon the relative extinction coefficients of the four species of interest. Each wavelength is either a point of maximal separation of all species or an isosbestic point of two species with maximal separation of the remaining two species.

Initial experiments were designed to determine the effect of species concentration and distribution on resulting calculations. In all cases, multiple spectra were collected for each condition. Matrix multiplication was then performed with each pseudoinverse matrix described above to determine the most effective pseudoinverse matrix. Comparisons of pseudoinverse matrix performance were based upon average recovery of theoretical species concentration.

The All Wave/Hb matrix performed consistently better than other matrices when used with a pseudoinverse matrix solution for the determination of the concentration of hemoglobin species in rHb1.1 solutions. However, the total hemoglobin concentration appeared to have an effect on the extinction coefficients of constituent species. Therefore, at low concentrations (below 10g/L) a unique matrix obtained with dilute hemoglobin solutions must be employed to obtain accurate concentration and composition values.

Example 6
Method of Measuring Protoporphyrin IX Content

The determinations of the protoporphyrin IX (PIX) content in hemoglobin samples were accomplished by HPLC (high pressure liquid chromatography) analysis based on the separation of heme and protoporphyrin IX from globin on a reversed phase column. Samples were diluted to approximately 1 mg/ml hemoglobin prior to analysis. To ensure that all heme compounds were quantitated with the same color factor, all heme in the solution was oxidized to hemin before analysis. This was accomplished by mixing $K_3[Fe(CN)_6]$ with the hemoglobin sample just before injection of the sample onto the column to oxidize Fe^{2+} in heme to Fe^{3+}. Elution of heme, protoporphyrin IX, and globins was accomplished by an increasingly nonpolar buffer gradient (e.g., water/TFA to acetonitrile). Spectra of hemin and protoporphyrin IX are similar, with absorption maxima at 398 nm and 405 nm, respectively. At 396 nm, color factors for heme and protoporphyrin IX were almost equal, therefore the areas under each peak correspond directly to the relative content of each component. Levels of protoporphyrin IX less than 0.4% (protoporphyrin IX/heme + protoporphyrin IX) were considered to lie below the detection limit of the analytical methodology. Spectral measurements are made anywhere in the range of about 390-410 nm with similar results.

Example 7
Method of Producing a Substantially Protoporphyrin IX-free Hemoglobin Solution

Hemoglobin was produced as described in Example 1. The fermentor contents were then cooled to 10°C and adjusted to approximately pH 8.0. After

adjustment, the fermentor broth (containing the unbroken *E. coli* cells) was then directly fed to a Niro homogenizer set at a break pressure of 800 bar, with an inlet pressure of 17 psi at a flow rate of 5.7 - 6.3 liters/min. After one passage through the homogenizer, the stream of cellular debris and hemoglobin was sparged with 100% carbon monoxide at 400 cc/min and directed through a plate and frame pre-heater and warmed to 55°C. The effluent from the pre-heater was then directed to a sudden expansion mixer where protoporphyrin IX-containing hemoglobin and other bacterial contaminants and hemoglobin isoforms were removed by heating by steam injection to 82°C ± 2°C for approximately 11 seconds. Similar results may be obtained by heating for approximately 30 seconds at approximately 77°C or, alternatively, 90-120 seconds at 72°C. The crude cell lysate was then cooled to 25°C before further processing. After chilling, diatomaceous earth was added to the solution to a final concentration of 12% (wt/wt) and loaded onto a rotary drum vacuum filter that had been pre-coated with diatomaceous earth. Filtration of the lysate resulted in a substantially protoporphyrin-IX-free hemoglobin solution that was resparged inline with carbon monoxide prior to collection. Protoporphyrin IX content of the crude cell lysate was measured after cell breakage and prior to any processing as described in Examples 2 and 6, and then again after RDVF filtration using the same techniques. Results are shown in Figure 3 and demonstrate that a substantially protoporphyrin IX hemoglobin solution is produced at temperatures of 72°C or higher for retention times of 11 seconds or more.

Example 8

Method of Producing a Partially Purified Hemoglobin Solution

The substantially protoporphyrin-IX-free hemoglobin solution was produced as described in Example 7 and was then further processed as follows. Note that the solution was maintained between 6-20°C during all processing steps unless otherwise stated. One molar zinc acetate was added to the substantially protoporphyrin-IX-free hemoglobin solution to yield a final zinc concentration in the solution of 2 mM, and the pH was adjusted between 8.35 - 8.5. Because the addition of zinc and the change in pH resulted in precipitation of zinc-complexed material, the solution was re-clarified by depth filtration through a CUNO filtration device equipped with Zeta Plus 90LA filters (Cuno, Inc., Meriden, CT). The solution was then further processed using a chelating Sepharose Fast Flow 6B (Pharmacia, Inc., Piscataway, NJ) column charged with 2 column volumes of 20 mM $Zn(OAc)_2$. The column was then equilibrated

with 2 column volumes of 200 mM NaCl. The substantially protoporphyrin IX-free hemoglobin solution was then loaded onto the column at a load of approximately 20 grams hemoglobin/liter of resin. The column was washed with 4 column volumes of 20 mM Tris/750 mM NaCl, pH 8.35-8.5, and then further washed with 4 column volumes of 10 mM Tris/25 mM NaCl, pH 8.35-8.5. Captured hemoglobin was eluted from the column with 6 - 8 column volumes of 15 mM EDTA, pH 8.5. The column was then cleaned with 2 column volumes of 200 mM NaCl followed by 3 column volumes of 0.5 N NaOH. Linear flow rates for all steps were between 100-200 cm/hr; the column was maintained at 4-10°C. The partially purified hemoglobin solution was then characterized by protein analysis to determine the degree of purification across the IMAC separation step. Hemoglobin in the protoporphyrin IX-free hemoglobin solution was quantitated by centrifugation, filtration through a 0.2 um filter, immobilized metal (zinc) affinity chromatography as described in Example 2 for protoporphyrin IX determinations and detection at 412 nm. Hemoglobin in the partially purified hemoglobin solution was determined by absorbance at 540 nm. Total protein in the starting material (substantially protoporphyrin IX-free solution) was determined by a Bradford assay using bovine serum albumin as a standard (Bradford, M., (1976) Anal. Biochem. *72*: 248) . Total protein in the partially purified hemoglobin solution produced after immobilized metal affinity chromatography could not be determined because any remaining protein that was not hemoglobin was below the detection limits of the Bradford assay, thus a specific assay for *E. coli* proteins was used (see Example 11 for details). Three runs were compared for purification across the IMAC purification step with the following results:

Table 4. Hemoglobin Purity

Protoporphyrin IX-free Hemoglobin Solution			Partially Purified Hemoglobin Solution		
rHb1.1 (g/l)	Total Protein (g/L)	Purity (%) (rHb1.1/total protein)	rHb1.1 (g/L)	*E. coli* proteins (g/L)	Purity (%) (rHb1.1/total protein)
0.99 ± 0.15	2.73 ± 0.90	38.12 ± 8.8	9.70 ± 0.7	.00747 ± .0054	99.92 ± 0.05

Example 9
Method of Producing A Substantially Purified Hemoglobin Solution

A partially purified hemoglobin solution was produced as described in Examples 7 and 8. After IMAC separation, the partially purified hemoglobin solution was then ultrafiltered in a tangential flow filter fitted with 10,000 NMCO filters (Millipore, Inc., Bedford, MA). Ultrafiltration both concentrated the partially purified hemoglobin solution and allowed exchange of the buffer to 20 mM Tris, pH 8.9, which was the buffer required for loading onto the anion exchange resin. During the preparation of the partially purified hemoglobin solution for loading onto a Q Sepharose Fast Flow column (an anion exchange column), the column itself was equilibrated by washing with the same buffer as was used to prepare the partially purified hemoglobin solution, 20 mM Tris, pH 8.9. The partially purified hemoglobin solution was then loaded onto the column to a charge of 20 grams of hemoglobin per liter of resin. The loaded column was washed with 12 mM Tris, pH 7.7, conductivity of 700 uS/cm, and finally eluted by lowering the pH to pH 7.5, 12 mM Tris. The solution was then oxygenated by sparging the solution under pressure with oxygen while the solution was recirculated until HbCO was less than 3%.

Example 10
Method of Producing A Pure Hemoglobin Solution

Contaminating metals, particularly nickel, were removed from the substantially purified hemoglobin solution produced using the methods of Examples 7, 8 and 9 by the following procedure. One millimolar EDTA was added to the oxygenation tank and the solution was allowed to incubate for 30 minutes prior to ultrafiltration. The oxygenated, EDTA treated hemoglobin solution was transferred to the Millipore ultrafiltration system described above and concentrated 5-6 fold. Diafiltration then began into formulation buffer (150 mM NaCl, 5 mM sodium phosphate, pH 7.4) and was continued until EDTA was less than approximately 5 ppm to produce a pure hemoglobin solution.

Ten batches of pure hemoglobin were prepared according to Examples 7, 8, 9 and 10 above and were characterized using the techniques described in Examples 6 and 11. The solutions had, on average, the following characteristics:

P_{50}	30.32 torr
Hill max (n_{max})	2.3
Methemoglobin	3.6%
Carbonmonoxyhemoglobin	3.5%
E. coli proteins (ECP's)	< 4 ppm *
LAL Endotoxin	< 0.03 EU/ml
Bioburden	0 CFU/ml
Protoporphyrin IX	< 0.4%**
Nickel	29 ug/L
EDTA	< 5 mg/L *

* Limit of quantitation of the assay

** 0.4% protoporphyrin IX was the limit of quantitation of the assay; 60% of the values were below this limit, the maximum protoporphyrin IX content obtained for the remaining 40% of the values was 0.58%.

Example 11

Purity and Functionality Determinations

Nickel was measured in the final solution by atomic adsorption spectroscopy as described by B. Welz in *Atomic Absorption Spectroscopy* (1985, Verlagsgesellschaft, Weinheim, Germany).

P_{50} and Hill coefficients were determined at 37°C as described in granted patent 5,028,588.

Protoporphyrin IX-containing hemoglobin was measured as described in Example 6.

HbCO and methemoglobin were measured as described in Example 5.

EDTA levels were determined chromatographically by taking a sample of purified hemoglobin and diluting the purified hemoglobin to a concentration of 10 mg/ml with formulation buffer (150 mM NaCl, 5 mM sodium phosphate, pH 7.4) prior to analysis. 100 ul of a 10mg/ml $FeCl_3 \cdot 6H_20$ solution was then added to 900 ul of the diluted sample. The iron-treated material was then ultrafiltered through a 30,000 NMCO filter (Centricon, Amicon, Boston, MA) and the permeate was analyzed on an ODS-Hypersil™ reverse phase chromatography column (5 um; 100 x 2.1 mm) (Hewlett Packard, Palo Alto, CA). Separations were achieved by isocratic elution with a buffer composed of 910 ml water, 160 ml methanol, 10 ml of 55% (wt/vol) tetrabutyl ammonium hydroxide, pH 6.0. The column was then cleaned with a buffer composed of 400 ml water, 600 ml methanol, 10 ml tetrabutyl ammonium

hydroxide, pH 6.0. EDTA elution was monitored at 254 nm and the peaks assigned to EDTA were quantitated against an EDTA standard.

Endotoxin was determined using the chromogenic LAL assay produced by Cape Cod and Associates (Falmouth, MA) according to the manufacturer's directions.

ECP's were measured using an ELISA double sandwich format immunoassay. The coating antibody was the IgG fraction of rabbit serum directed against a crude lysate of *E. coli* strain K-12 and is commercially available from Dakopatts, Inc. (Glostrup, Denmark). The detecting antibody was the same as the coating antibody conjugated to horseradish peroxidase. The enzyme substrate for this assay was TMB (3,3',5,5' tetramethylbenzidine). The ECP standard was generated by purification of an *E. coli* lysate through two cation exchange columns and is assumed to represent the ECP's removed by the final anion exchange step of the process.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention.

SEQUENCE LISTING

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(iii) **NUMBER OF SEQUENCES:** 20

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(C) **OPERATING SYSTEM:** System 7.0.1
(D) **SOFTWARE:** Microsoft Word 5.0a

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(2) **INFORMATION FOR SEQ ID NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 45
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** C-term of αgene, Xba I site

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:1:**

CGGGAATACG GTCTAGATCA TTAACGGTAT TTCGAAGTCA GAACG 45

(2) **INFORMATION FOR SEQ ID NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 95
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** tac promoter sequence, Bam HI-Eag I sites

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:2:**

GATCCGAGCT GTTGACAATT AATCATCGGC TCGTATAATG TGTGGAATTG 50

TGACGGATAA CAATTTCACA CAGGAAATTA ATTAATGCTG TCTCC 95

(2) **INFORMATION FOR SEQ ID NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 96
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** tac promoter, Bam HI - Eag I sites

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:3:**

GGCCGGAGAC AGCATTAATT AATTTCCTGT GTGAAATTGT TATCCGCTCA 50
CAATTCCACA CATTATACGA GCCGATGATT AATTGTCAAC AGCTCG 96

INFORMATION FOR SEQ ID NO:4:

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 64
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** 5' end of alpha gene,with EcoR1, BamH1 and Eag1 sites

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:4:**

TCGGATTCGA ATTCCAAGCT GTTGGATCCT TAGATTGAAC TGTCTCCGGC 50
CGATAAAACC ACCG 64

(2) **INFORMATION FOR SEQ ID NO:5:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 55
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** 5'end of beta with Xba I site

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:5:**

CGGAAGCCCA ATCTAGAGGA AATAATATAT GCACCTGACT CCGGAAGAAA 50
AATCC 55

(2) **INFORMATION FOR SEQ ID NO:6:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 44
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** 3' end of the beta gene with Hind III site

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:6:**

CCCGAAACCA AGCTTCATTA GTGAGCTAGC GCGTTAGCAA CACC 44

(2) **INFORMATION FOR SEQ ID NO:7:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 37
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** mutagenesis reverse primer

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:7:**

TTTAAGCTTC ATTAGTGGTA TTTGTGAGCT AGCGCGT 37

(2) **INFORMATION FOR SEQ ID NO:8:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 37
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** mutagenesis reverse primer

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:8:**

CAGCATTAAT TAACCTCCTT AGTGAAATTG TTATCCG 37

(2) **INFORMATION FOR SEQ ID NO:9:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 45
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** mutagenesis reverse primer

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:9:**

GGTGCATATA TTTACCTCCT TATCTAGATC ATTAACGGTA TTTCG 45

(2) **INFORMATION FOR SEQ ID NO:10:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 10
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** Pme I linker

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:10:**

GGTTTAAACC 10

(2) **INFORMATION FOR SEQ ID NO:11:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 58
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** oligonucleotide upstream of lacI gene

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:11:**

GGCGAATAAA AGCTTGCGGC CGCGTTGACA CCATCGAATG GCGCAAAACC 50
TTTCGCGG 58

(2) **INFORMATION FOR SEQ ID NO:12:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 69
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** downstream side of lacI gene

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:12:**

GGGCAAATAG GATCCAAAAA AAAGCCCGCT CATTAGGCGG GCTTTATCAC 50
TGCCCGCTTT CCAGTCGGG 69

(2) **INFORMATION FOR SEQ ID NO:13:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 54
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** primer for pBR322 ori positions 3170-3148

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:13:**

CCCCGAAAAG GATCCAAGTA GCCGGCGGCC GCGTTCCACT GAGCGTCAGA 50
CCCC 54

(2) **INFORMATION FOR SEQ ID NO:14:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 42
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** primer for pBR322 ori positions 2380-2404

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:14:**

GGCGGTCCTG TTTAAACGCT GCGCTCGGTC GTTCGGCTGC GG 42

(2) **INFORMATION FOR SEQ ID NO:15:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 28
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** dialpha gene fragment

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:15:**

CAATTTCACA GGAAATTAAT TAATGCTG 28

(2) **INFORMATION FOR SEQ ID NO:16:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 30
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** dialpha gene fragment

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:16:**

CAATTTCACT AAGGAGGTTA ATTAATGCTG 30

(2) **INFORMATION FOR SEQ ID NO:17:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 29
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** beta gene fragment

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:17:**

TAAAGATCTA GAGGAAATAA TATATGCAC 29

(2) **INFORMATION FOR SEQ ID NO:18:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 33
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** beta gene fragment

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:18:**

TAATGATCTA GATAAGGAGG TAAATATATG CAC 33

(2) **INFORMATION FOR SEQ ID NO:19:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 16
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** beta terminus

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:19:**

CTCGCTCACT AATGAA 16

(2) **INFORMATION FOR SEQ ID NO:20:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 25
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single
(D) **TOPOLOGY:** linear

(ii) **MOLECULE TYPE:** modified beta terminus

(iii) **HYPOTHETICAL:** no

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:20:**

CTCGCTCACA AATACCACTA ATGAA 25

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for producing a partially purified hemoglobin solution substantially free of host cell proteins comprising:

   (a) contacting a hemoglobin-containing cell lysate with an immobilized metal affinity chromatography (IMAC) resin charged with a divalent metal ion;

   (b) washing the IMAC resin with at least one IMAC wash solution; and

   (c) eluting the IMAC resin with an eluting solution at a pH greater than 7.0 to obtain the hemoglobin solution.

2. The method of claim 1, wherein said hemoglobin-containing cell lysate is obtained from non-erythrocyte cells.

3. The method of claim 2, wherein said non-erythrocyte cells are bacterial cells.

4. The method of claim 3, wherein said bacterial cells are *E.coli*.

5. The method of any one preceding claim, further comprising before step (a) converting haemoglobin in said haemoglobin-containing lysate to a thermally stable state.

6. The method of claim 5, wherein hemoglobin is converted to the thermally stable state by exposing the hemoglobin-containing lysate to a liganding gas to produce liganded hemoglobin.

7. The method of claim 6, wherein said liganding gas is oxygen, carbon monoxide or nitric oxide.

8. The method of claim 7, wherein said liganding gas is carbon monoxide.

9. The method of claim 1, wherein the divalent metal ion is nickel, copper, cobalt or zinc.

10. The method of claim 9, wherein the divalent metal ion is zinc.

11. The method of claim 1, wherein the hemoglobin-containing lysate is a clarified hemoglobin-containing lysate.

12. The method of claim 11, wherein the amount of protoporphyrin-IX containing hemoglobin in the clarified hemoglobin-containing lysate is less than about 10% of the total hemoglobin.

13. The method of any one preceding claim, wherein washing the IMAC resin with at least one IMAC wash solution in step (b) comprises:

washing the IMAC resin with a first IMAC wash solution; and

washing the IMAC resin with a second IMAC wash solution, wherein the first IMAC wash solution is the same or different than the second IMAC wash solution.

14. The method of claim 13, wherein the first and second IMAC wash solutions comprise Tris, a salt, and a pH greater than 7.5.

15. The method of claim 14, wherein the first IMAC wash solution comprises about 20 mM Tris, about 0.5 to about 0.75 M NaCl, and a pH greater than or equal to 7.5 and wherein the second IMAC wash solution comprises about 5 to about 19 mM Tris, about 0.25 to about 0.75 M NaCl, and a pH greater than or equal to 7.6.

16. The method of claim 15, wherein the second IMAC wash solution has a lower conductivity than the first IMAC wash solution.

17. The method of any one preceding claim, wherein the IMAC resin is eluted by a change in pH, a chelating agent or a competitive ligand.

18. The method of claim 17, wherein the IMAC resin is eluted by a chelating agent.

19. The method of claim 18, wherein the chelating agent is ethylenediamine tetraacetic acid in an eluting solution having a pH no less than about 8.0.

20 The method of any one preceding claim, wherein said haemoglobin-containing lysate is converted to a substantially contaminant-free haemoglobin solution prior to contact with the IMAC resin.

21. The method of claim 20 wherein before step (a), said hemoglobin-containing lysate is converted to the substantially contaminant-free hemoglobin solution by heating said hemoglobin-containing lysate for less than about 5 minutes at a sufficient temperature to reduce contaminants therein.

22. The method of claim 21 wherein said hemoglobin-containing lysate is heated for about 3.5 to 24.5 seconds.

23. The method of claim 21 or 22 wherein the sufficient temperature is in the range of about 70°C to about 90°C.

24. The method of any one preceding claim, wherein:

said divalent metal ion is zinc;

said first IMAC wash solution contains about 500 to 750 mM NaCl, about 20 mM Tris, pH of about 8.0 to about 8.3;

said second IMAC wash solution contains about 25 mM to 50 mM NaCl, about 20 mM Tris, pH of about 8.0 to about 8.3; and

said IMAC resin is eluted with about 15 mM ethylenediamine tetraacetic acid at a pH greater than about 8.0.

25. The method of any one preceding claim, further comprising:

(d) loading the hemoglobin solution of step (c) onto an anion exchange resin;

(e) washing the anion exchange resin; and

(f) eluting the anion exchange resin with a second eluting solution to obtain the hemoglobin solution.

26. The method of claim 25, further comprising before step (d) the buffer exchanging of the hemoglobin solution into an exchange solution.

27. The method of claim 26, wherein washing the anion exchange resin comprises:

washing the anion exchange resin with the exchange solution; and

washing the anion exchange resin with an anion exchange wash solution having a lower pH than the exchange solution.

28. The method of claim 27, wherein the second eluting solution has a lower pH than the anion exchange wash solution.

29. The method of claim 28, wherein the exchange solution, anion exchange wash solution and the second eluting solution are cationic.

30. The method of claim 29, wherein:

the exchange solution comprises about 10 to about 30 mM Tris with a pH of about 8.5 to about 9.5;

the anion exchange wash solution comprises about 10 to about 15 mM Tris with a pH of about 7.6 to about 7.9; and

the second eluting solution comprises about 10 to about 15 mM Tris with a pH of about 7.4 to less than about 7.6.

31. The method of claim 30, wherein the exchange solution is about 20 mM Tris with a pH of about 8.5, the anion exchange wash solution is about 12 mM Tris with a pH of about 7.7, and the second eluting solution is about 12 mM Tris with a pH of about 7.5.

32. The method of claim 6, further comprising removing said liganding gas by exposing said hemoglobin solution of step (f) to oxygen and simultaneously removing carbon monoxide gas.

33. The method of claim 32, further comprising: (g) adding a chelating agent to the hemoglobin solution of step (f).

34. The method of claim 33, further comprising: (h) removing said chelating agent from the hemoglobin solution of step (g).

35. The method of claim 34, wherein said chelating agent is ethylenediamine tetraacetic acid.

36. The method of any one preceding claim, wherein the haemoglobin-containing lysate contains recombinant haemoglobin.

37. A hemoglobin solution obtained according to the method of any preceding claim.

Dated this 20th day of November 1998

SOMATOGEN, INC

By their Patent Attorneys

GRIFFITH HACK

Fellows Institute of Patent

Attorneys of Australia

FIGURE 1A

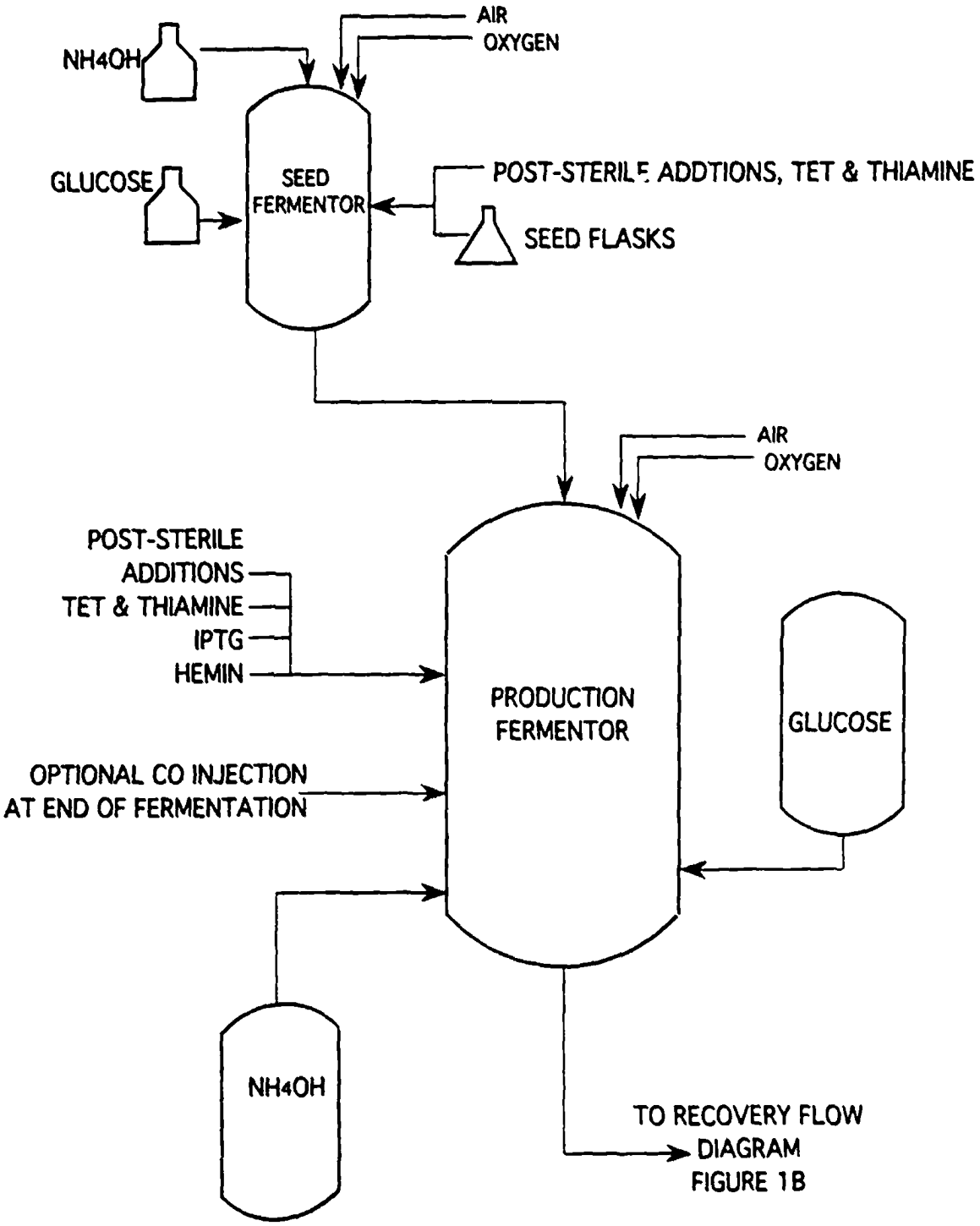

AIR
OXYGEN
NH4OH
GLUCOSE
SEED FERMENTOR
POST-STERILE ADDTIONS, TET & THIAMINE
SEED FLASKS
AIR
OXYGEN
POST-STERILE ADDITIONS
TET & THIAMINE
IPTG
HEMIN
PRODUCTION FERMENTOR
GLUCOSE
OPTIONAL CO INJECTION AT END OF FERMENTATION
NH4OH
TO RECOVERY FLOW DIAGRAM FIGURE 1B

FIGURE 1B

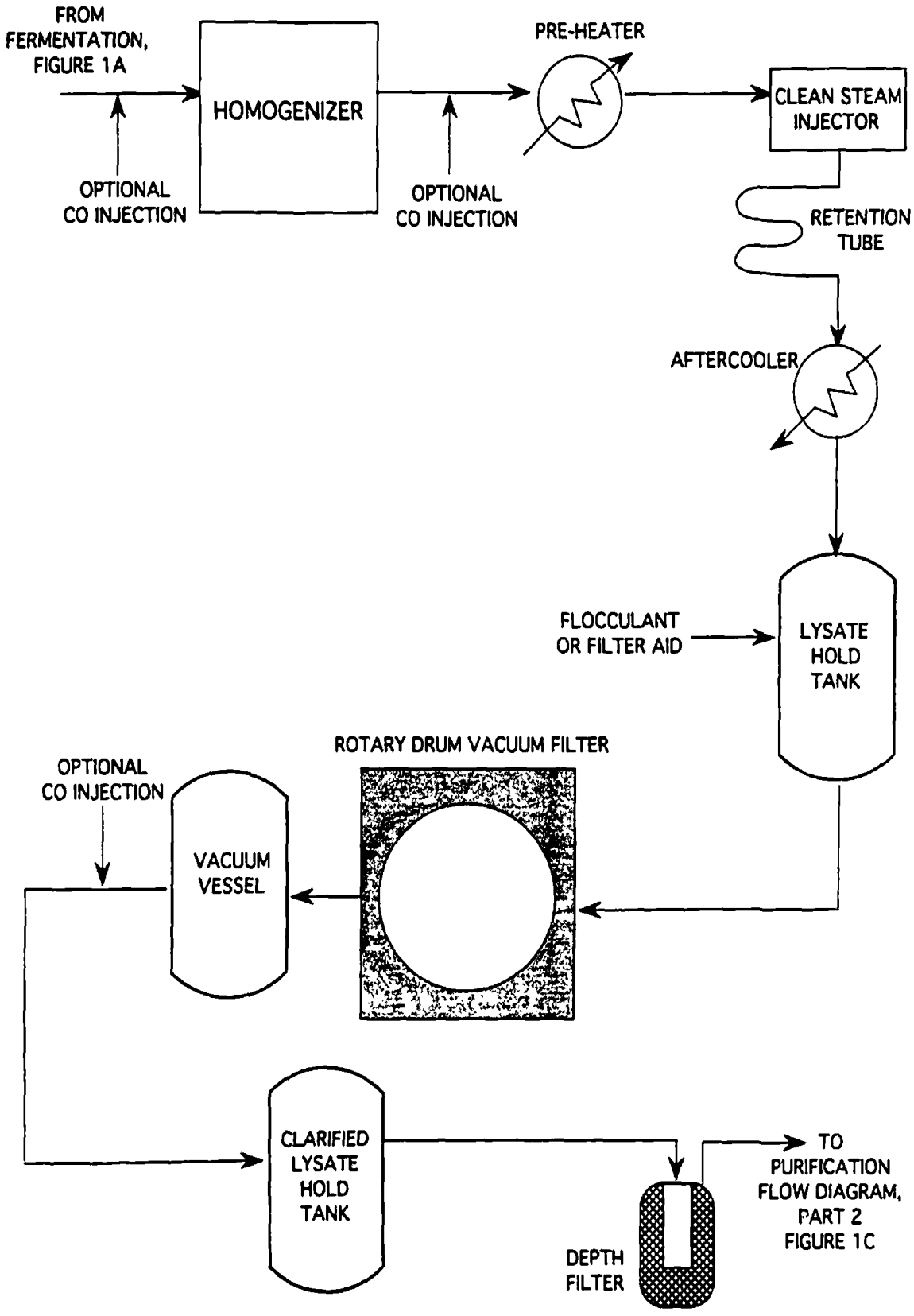

FROM FERMENTATION, FIGURE 1A
HOMOGENIZER
PRE-HEATER
CLEAN STEAM INJECTOR
OPTIONAL CO INJECTION
OPTIONAL CO INJECTION
RETENTION TUBE
AFTERCOOLER
FLOCCULANT OR FILTER AID
LYSATE HOLD TANK
ROTARY DRUM VACUUM FILTER
OPTIONAL CO INJECTION
VACUUM VESSEL
CLARIFIED LYSATE HOLD TANK
DEPTH FILTER
TO PURIFICATION FLOW DIAGRAM, PART 2 FIGURE 1C

FIGURE 1C

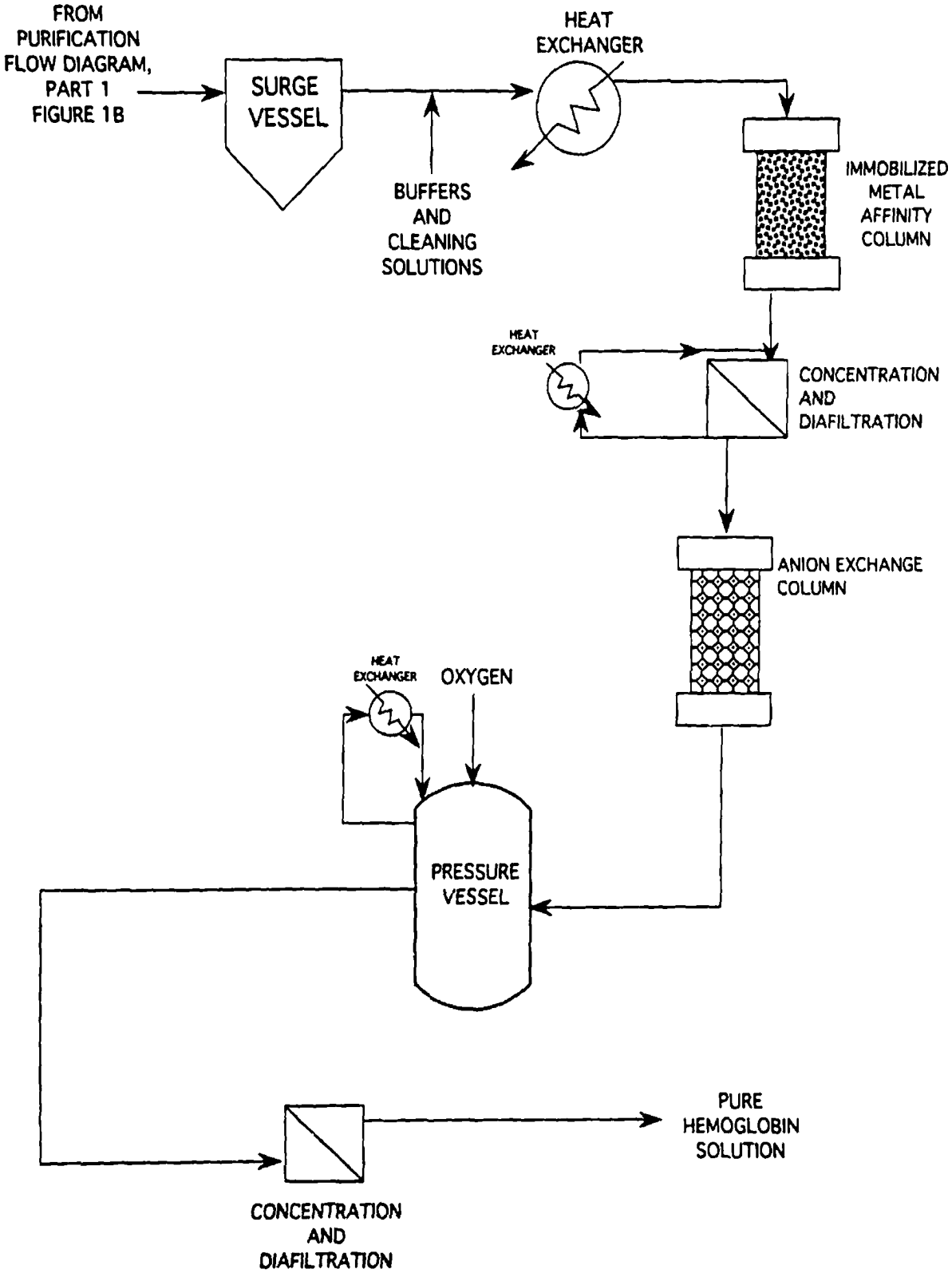

FROM PURIFICATION FLOW DIAGRAM, PART 1 FIGURE 1B
SURGE VESSEL
BUFFERS AND CLEANING SOLUTIONS
HEAT EXCHANGER
IMMOBILIZED METAL AFFINITY COLUMN
HEAT EXCHANGER
CONCENTRATION AND DIAFILTRATION
ANION EXCHANGE COLUMN
HEAT EXCHANGER
OXYGEN
PRESSURE VESSEL
PURE HEMOGLOBIN SOLUTION
CONCENTRATION AND DIAFILTRATION

FIGURE 2

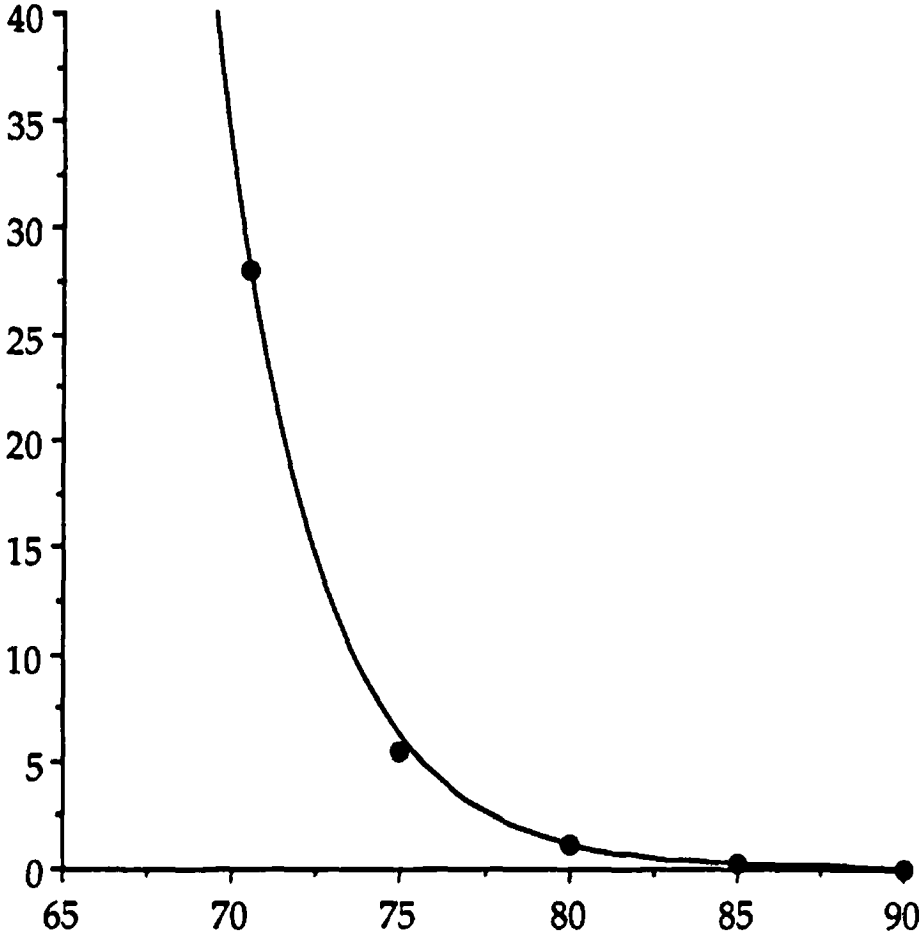
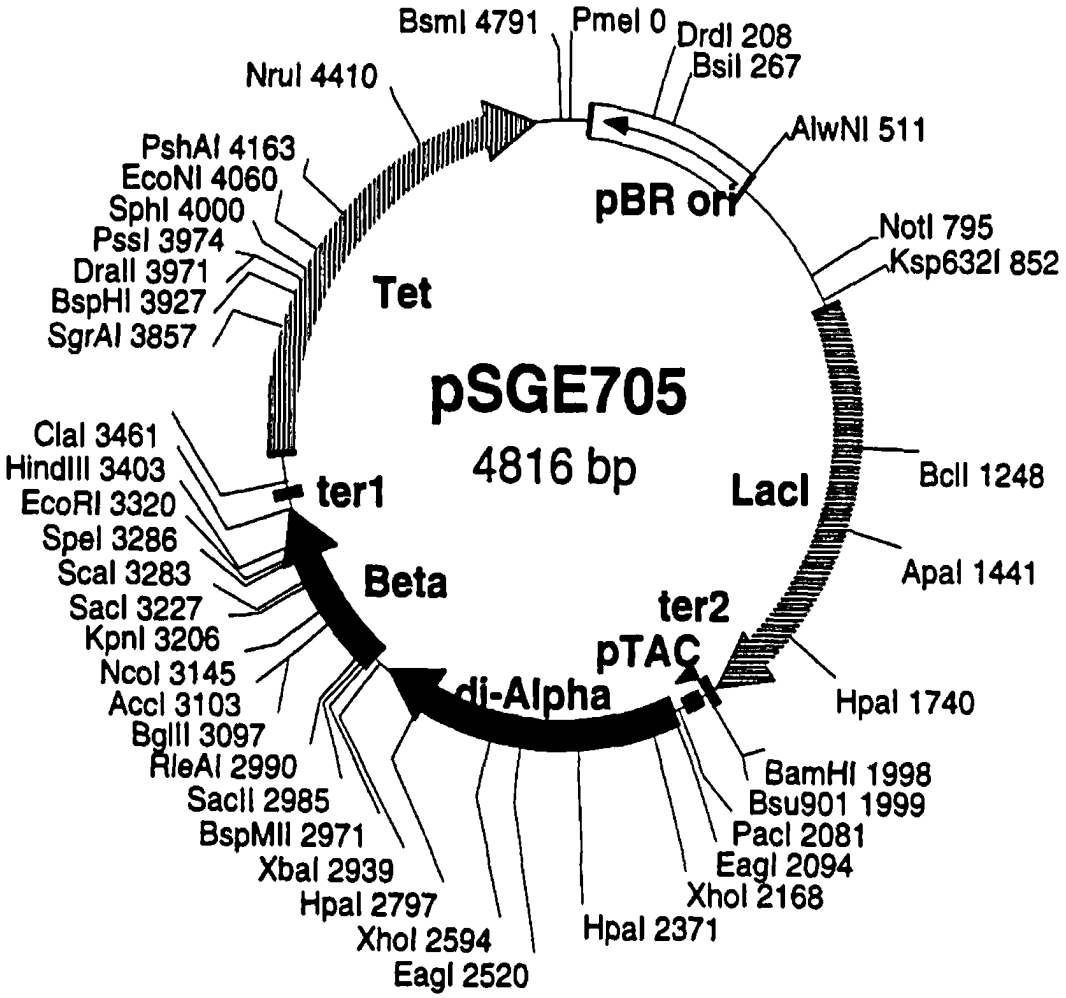


FIGURE 3

FIGURE 4


pSGE705
4816 bp
Tet
pBR ori
LacI
Beta
ter1
ter2
pTAC
di-Alpha
BsmI 4791
PmeI 0
DrdI 208
BsiI 267
NruI 4410
AlwNI 511
PshAI 4163
EcoNI 4060
SphI 4000
PssI 3974
DraII 3971
BspHI 3927
SgrAI 3857
NotI 795
Ksp632I 852
ClaI 3461
HindIII 3403
EcoRI 3320
SpeI 3286
ScaI 3283
SacI 3227
KpnI 3206
NcoI 3145
AccI 3103
BglII 3097
RleAI 2990
SacII 2985
BspMII 2971
XbaI 2939
HpaI 2797
XhoI 2594
EagI 2520
BclI 1248
ApaI 1441
HpaI 1740
BamHI 1998
Bsu901 1999
PacI 2081
EagI 2094
XhoI 2168
HpaI 2371