EP 1 394 179 reports a method for producing erythropoietin free from animal proteins. The production of erythropoietin by endogenous gene activation with viral promoters is the subject matter of EP 0 986 644. A method for producing recombinant glycoproteins and in particular erythropoietin is reported in EP 1 428 878. A method for producing a desired profile of erythropoietin glyco-isoforms is reported in EP 1 492 878.

Summary ~~of the Invention~~

The production and purification of recombinant erythropoietin requires several steps, in particular chromatography steps.

The disclosure relates to~~A first aspect of the invention is~~ a method for producing erythropoietin comprising at least one chromatography step with a stationary phase containing hydroxyapatite comprising the steps of

i) bringing an erythropoietin containing solution containing Calcium-ions at a concentration of from 0.5 mM to 20 mM into contact with a stationary phase containing hydroxyapatite which has been equilibrated with a solution containing Calcium-ions at the same concentration of from 0.5 mM to 20 mM,

ii) passing a solution which contains Calcium-ions at a concentration of less than 0.5 mM over the stationary phase containing hydroxyapatite, and

iii) passing a solution which contains Calcium-ions at a concentration of less than 0.5 mM over the stationary phase containing hydroxyapatite and thereby producing erythropoietin.

In a first aspect, there is provided a method for purifying erythropoietin comprising

a) providing a solution containing i) erythropoietin and ii) Calcium-ions at a first concentration comprising 20 mM TRIS-HCl, 5 mM $CaCl_2$, pH 6.9 ± 0.2,
b) providing a chromatography column containing a ceramic hydroxyapatite-containing stationary phase,
c) applying a solution containing Calcium-ions at a second concentration to the column of b) comprising 20 mM TRIS-HCl, 5 mM $CaCl_2$, 0.25 M NaCl, 9% (v/v) 2-propanol, pH 6.9 ± 0.2,
d) applying the solution of a) to the column obtained in c),
e) applying a solution containing Calcium-ions at a third concentration to the column obtained in d) comprising 20 mM TRIS-HCl, 0.25 M NaCl, 9% (v/v) 2-propanol, pH 6.9 ± 0.2, and
f) recovering purified erythropoietin by applying a solution containing Calcium-ions at a fourth concentration to the column obtained in e) comprising 10 mM TRIS-HCl, pH 6.9 ± 0.2,

wherein said first and second Calcium-ion concentrations are the same and said third and fourth Calcium-ion concentrations are the same, lower than said first and second Calcium-ion concentration,
wherein said third and fourth Calcium-ion concentration is 0.1 mM or less.

In a second aspect, there is provided a method for producing erythropoietin using a sequence of chromatography steps as follows:

i) affinity chromatography,
ii) hydrophobic interaction chromatography,
iii) chromatography on a stationary phase containing ceramic hydroxyapatite,

wherein the chromatography on a stationary phase containing hydroxyapatite, is carried out as defined above.

In a third aspect, there is provided a method for producing erythropoietin comprising culturing cells containing an EPO-encoding nucleic acid and isolating erythropoietin from the cells or the culture medium, characterized in that the isolation of erythropoietin comprises the following steps:

i) applying the cell supernatant to an affinity chromatography material and recovering/collecting the fractions containing erythropoietin,
ii) optionally applying the fractions containing erythropoietin from i) to a hydrophobic interaction chromatography material and recovering/collecting the fractions containing erythropoietin,
iii) applying the fractions containing erythropoietin from i) or ii) to a stationary phase containing ceramic hydroxyapatite and recovering/collecting the fractions containing erythropoietin using a method as defined above.

In a fourth aspect, there is provided a method for producing mono-PEGylated erythropoietin comprising the following steps:

i) purifying erythropoietin with a method as defined above,
ii) PEGylating erythropoietin using an activated PEG having a molecular weight between 20 kDa and 40 kDa,
iii) purifying the PEGylated erythropoietin obtained in step ii) with two successive cation exchange chromatography steps using the same stationary phase,
iv) recovering and thereby producing mono PEGylated erythropoietin from the second stationary phase.

In one embodiment the produced erythropoietin is depleted of de-O-EPO species and de-N-EPO species compared to the erythropoietin containing solution in i). In a further embodiment the method comprises at least one additional chromatography step in addition to the chromatography step with a stationary phase containing hydroxyapatite. In a further embodiment all chromatography steps are each based on different chromatographic principles, whereby each chromatographic principle is used only once in the method. In a further embodiment the chromatographic principle(s) is/are selected from affinity chromatography, hydrophobic interaction chromatography, reversed phase chromatography, gel permeation chromatography, cation exchange chromatography, and/or anion exchange chromatography. In a further embodiment the sequence of chromatography steps are affinity

chromatography, hydrophobic interaction chromatography, chromatography on a stationary phase containing hydroxyapatite, optionally reversed phase chromatography, and anion exchange chromatography. In one embodiment the method according to the disclosure~~invention~~ comprises optional intermediate steps such as salt precipitation, concentration, diafiltration, ultrafiltration, dialysis, and/or ethanol precipitation in addition to the chromatography steps.

The disclosure relates to~~One aspect of the invention is~~ an erythropoietin composition which has been obtained using a method according to the disclosure~~invention~~.

The disclosure also relates to~~A further aspect of the invention is~~ a method for depleting Chinese hamster ovary (CHO) cell protein from an erythropoietin preparation comprising a chromatography step with a stationary phase containing hydroxyapatite according to the current disclosure~~invention~~ wherein the collection of fractions containing erythropoietin is controlled by the light absorption at 280 nm and begins at an absorption of 15 mAU to 75 mAU and is terminated after the peak maximum has passed through at an absorption of 200 mAU to 40 mAU.

In one embodiment the stationary phase containing hydroxyapatite is a pure crystalline hydroxyapatite. In another embodiment the solution used in the chromatography in step ii) and/or iii) contains a concentration of phosphate ions of approximately 5 mM, in another embodiment 5 mM sodium - or potassium - phosphate.

The disclosure also relates to~~Another aspect of the invention is~~ a method for preparing an erythropoietin composition which is a mixture of erythropoietin isoforms which have a number of more than 6 and less than 17 sialic acid residues per erythropoietin molecule and which have a ratio of tetraantennary to triantennary to biantennary glycosyl forms in a range of from 83:14:2 to 74:21:6. In one embodiment the ratio is linear in the range.

Thus, the disclosure relates to~~a further aspect of the invention is~~ an erythropoietin composition which is a mixture of erythropoietin isoforms which have a number of more than 6 and less than 17 sialic acid residues per erythropoietin molecule and which have a ratio of tetraantennary to triantennary to biantennary glycosyl forms in a range of from 83:14:2 to 74:21:6. In one embodiment the ratio is linear in the range.

In one embodiment the erythropoietin composition has a ratio of tetraantennary glycosyl forms to tetraantennary glycosyl forms with one repeat to tetraantennary

glycosyl forms with two repeats to triantennary glycosyl forms to triantennary glycosyl forms with one repeat to and biantennary glycosyl forms in a range of from 43:28:12:8:6:2 to 45:21:7:14:7:6. In one embodiment the ratio is linear in the range.

Thus, the disclosure also relates to~~a further aspect of the invention is~~ an erythropoietin composition having a ratio of tetraantennary glycosyl forms to tetraantennary glycosyl forms with one repeat to tetraantennary glycosyl forms with two repeats to triantennary glycosyl forms to triantennary glycosyl forms with one repeat to biantennary glycosyl forms in a range of from 43:28:12:8:6:2 to 45:21:7:14:7:6. In one embodiment the ratio is linear in the range.

The disclosure also relates to~~A further aspect of the invention is~~ an erythropoietin composition which is a mixture of erythropoietin isoforms which have a number of more than 6 and less than 17 sialic acid residues per erythropoietin molecule and which has been obtained with a method according to the invention.

The disclosure also relates to~~Yet a further aspect of the present invention is~~ a method for producing recombinant erythropoietin comprising cultivating host cells, which contain an erythropoietin-encoding nucleic acid, in a suitable medium under suitable conditions to produce erythropoietin, and isolating the erythropoietin from the culture medium or the cells. The cells are cultured in suspension in one embodiment. In a further embodiment the cells are cultured in a fermenter, and in particular in a fermenter with a volume of 10 l to 50,000 l.

The isolation of erythropoietin from the culture medium comprises the following steps:

(a) applying the cell culture supernatant to an affinity chromatography material and recovering/collecting the fractions containing erythropoietin,

(b) optionally applying the fractions of (a) to a hydrophobic interaction chromatography material and recovering/collecting the fractions containing erythropoietin,

(c) applying the fractions of (a) or (b) to a stationary phase containing hydroxyapatite and recovering/collecting the fractions containing erythropoietin using a method according to the present invention, and

(d) concentrating or/and passing the fractions of (c) over a reversed phase HPLC material.

Step (a) of the purification method comprises passing the cell supernatant, which can optionally be pretreated, over an affinity chromatography material. In one embodiment the affinity chromatography material is one to which a blue dye has been coupled. An example is Blue Sepharose. In one embodiment, after elution from the affinity chromatography material, the eluate containing erythropoietin is passed over a hydrophobic interaction chromatography material if a culture medium with a serum content of ≥ 2% (v/v) was used for the fermentation/its production. In one embodiment the hydrophobic interaction chromatography material is butyl Sepharose. The eluate from step (a) or, if used, step (b) is passed over a stationary phase containing hydroxyapatite in step (c) and the eluate containing erythropoietin is isolated. In one embodiment the concentrating is by means of exclusion chromatography e.g. membrane filtration. In this case the use of e.g. a membrane with an exclusion size of 10 kDa has proven to be advantageous. The erythropoietin that can be obtained with the method according to the invention can contain α-2,3-linked or/and α-2,6-linked sialic acid residues.

The disclosure also relates to~~Another aspect of the invention is~~ a method for producing poly(ethyleneglycol) conjugated erythropoietin comprising the following steps:

a) covalently linking, i.e. PEGylating, erythropoietin using an activated poly(ethyleneglycol) reagent,

b) purifying the poly(ethyleneglycol) conjugated erythropoietin by two successive cation exchange chromatography steps and thereby producing poly(ethyleneglycol) conjugated erythropoietin,

in which the erythropoietin employed in step a) is obtained by a method according to the present disclosure~~invention~~.

The disclosure also relates to~~Another aspect of the present invention is~~ a pharmaceutical composition comprising erythropoietin which has been obtained with a method according to the disclosure~~invention~~. The composition according to the disclosure~~invention~~ can be formulated as a pharmaceutical preparation optionally together with conventional pharmaceutical diluents, excipients, auxiliary agents and carriers. The composition according to the disclosure~~invention~~, which can be used to produce a pharmaceutical preparation, has a purity of at least 99% or in another embodiment of at least 99.9% when determined by reversed phase HPLC (e.g. on a Vydac C4 column) or/and size exclusion chromatography (e.g. on a TSK 2000SW Ultrapac column).~~A further aspect of the~~

The disclosure also relates to~~invention is~~ a pharmaceutical composition comprising erythropoietin, which has been obtained using a method according to the disclosure~~invention~~, for treating anemia.

Detailed Description of the Invention

Whether different substances can be chromatographically separated from each another, depends above all on the conditions under which the chromatography is carried out, in addition on a good column packing and on the choice of the chromatography material (stationary phase). This includes the way in which impurities and the product are eluted in addition to the choice of the buffer system.

The present invention reports a method for purifying erythropoietin comprising at least one chromatography step with a stationary phase containing hydroxyapatite comprising the following steps:

i) bringing an erythropoietin solution to be purified containing Calcium-ions at a first concentration into contact with a stationary phase containing hydroxyapatite over which a volume of a solution containing Calcium-ions at a second concentration has been passed,

ii) passing a volume of a solution containing a lower concentration of Calcium-ions than the solutions from i), i.e. at a third concentration, over the stationary phase containing hydroxyapatite, whereby during this passing the erythropoietin remains bound to/is not detached from the stationary phase containing hydroxyapatite, and

iii) passing a volume of a solution containing less than 0.5 mM Calcium-ions, i.e. at a fourth concentration, over the stationary phase containing hydroxyapatite from ii), whereby the erythropoietin detaches and elutes from the stationary phase containing hydroxyapatite.

In one embodiment the first and second and/or third and fourth concentration are different. In another embodiment the third and fourth Calcium-ion concentration are 10% or less of the first and second Calcium-ion concentration.

Erythropoietin, which is also referred to as EPO in the following, is understood as a protein which has the biological ability to stimulate differentiation and division processes in erythroid precursor cells and, thus, to provide erythrocytes. This protein is preferably human erythropoietin and consists of 165 or 166 amino acids (SEQ ID NO: 1 and 2) with a molecular weight of about 34 – 38 kDa. The glycosyl residues account for about 40% of the molecular weight. Derivatives and fragments