

(19)



(11)

EP 4 269 578 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
05.06.2024 Bulletin 2024/23

(51) International Patent Classification (IPC):
C12N 9/26 (2006.01)

(21) Application number: **23191930.9**

(52) Cooperative Patent Classification (CPC):
C12N 9/2408; C12Y 302/01035; C12Y 302/01036

(22) Date of filing: **06.03.2009**

(54) **SOLUBLE HYALURONIDASE COMPOSITION**

LÖSLICHE HYALURONIDASE-ZUSAMMENSETZUNG

COMPOSITION D'HYALURONIDASE SOLUBLE

(84) Designated Contracting States:
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR
HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL
PT RO SE SI SK TR**
Designated Extension States:
AL BA RS

(30) Priority: **06.03.2008 US 6862208 P**

(43) Date of publication of application:
01.11.2023 Bulletin 2023/44

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:
**21189086.8 / 3 960 854
20186922.9 / 3 760 715
13184202.3 / 2 674 487
09718114.3 / 2 268 805**

(73) Proprietor: **Halozyme, Inc.
San Diego, CA 92121 (US)**

(72) Inventors:
• **Baker, David
San Diego, CA, 92128 (US)**
• **Bookbinder, Louis
San Diego, CA,, 92128 (US)**

(74) Representative: **Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastraße 30
81925 München (DE)**

(56) References cited:
US-A1- 2004 268 425

Remarks:

The file contains technical information submitted after
the application was filed and not included in this
specification

EP 4 269 578 B1

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Processed by Luminess, 75001 PARIS (FR)

Description**FIELD OF THE INVENTION**

5 [0001] Provided are methods for large-scale production of a recombinant human protein.

BACKGROUND

10 [0002] Hyaluronidases are a family of enzymes that degrade hyaluronic acid (also known as hyaluronan or hyaluronate), an essential component of the extracellular matrix and a major constituent of the interstitial barrier. By catalyzing the hydrolysis of hyaluronic acid, hyaluronidase lowers the viscosity of hyaluronic acid, thereby increasing tissue permeability. As such, hyaluronidases have been used, for example, as a spreading or dispersing agent in conjunction with other agents, drugs and proteins to enhance their dispersion and delivery. Hyaluronidases also have other therapeutic and cosmetic uses. Because of the increasing use of hyaluronidases for therapeutic and cosmetic uses, there is a need for large-scale quantities of purified hyaluronidase. Therefore, among the objects herein, it is an object to provide methods for the production and purification of hyaluronidases.

15 [0003] US 2004/268425 A1 discloses a soluble hyaluronidase glycoprotein (sHASEGP), process for preparing the same, uses and pharmaceutical compositions comprising thereof.

SUMMARY

[0004] The invention is defined in the claims.

[0005] In particular, the present invention relates to:

20 A composition comprising a mixture of polypeptides, said mixture comprising the polypeptides set forth in SEQ ID NOS: 4-8, wherein the composition is obtainable by the following method:

a) inoculating cell medium in a bioreactor with an inoculum of cells that encode soluble recombinant human PH20 (rHuPH20) to produce a cell culture, wherein:

30 the cells comprise between 150 and 300 copies of nucleic acid encoding soluble rHuPH20;
the bioreactor contains at least 100 liters of cell culture;
 10^{10} - 10^{11} cells are inoculated per 100 liters cell culture; and
the cells are cultured at a set temperature;

35 b) feeding the cells with a first feed medium containing glucose, L-alanyl-L-glutamine, human insulin and yeast extract in amounts sufficient to increase cell growth and peak cell density, and to increase soluble rHuPH20 synthesis, wherein the feed medium is added to the culture at a volume of 0.5 % to 20% of the cell culture volume;

40 c) feeding the cells with a second feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and induce cell cycle arrest; and
lowering the temperature compared to the temperature in step a) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; wherein:

45 the amount of L-alanyl-L-glutamine is decreased compared to the amount of L-alanyl-L-glutamine in step b);
the amount of yeast extract is increased compared to the amount of yeast extract in step b); and
the feed medium is added to the culture at a volume of 0.5 % to 20% of the cell culture volume;

d) feeding the cells with a third feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and increase cell cycle arrest, and lowering the temperature compared to the temperature in step c) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; wherein:

50 the amount of L-alanyl-L-glutamine is decreased compared to the amount of L-alanyl-L-glutamine in step c);
the amounts of yeast extract, glucose and sodium butyrate are increased compared to the amounts of yeast extract, glucose and sodium butyrate in step c); and
55 the feed medium is added to the culture at a volume of 0.5 % to 20 % of the cell culture volume;

e) feeding the cells with a fourth feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and increase cell cycle arrest, and

lowering the temperature compared to the temperature in step d) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; wherein:

the amount of L-alanyl-L-glutamine and glucose is decreased compared to the amount of L-alanyl-L-glutamine and glucose in step d);
the amount of sodium butyrate is decreased compared to the amount of sodium butyrate in step d); and
the feed medium is added to the culture at a volume of 0.5 % to 20% of the cell culture volume;

f) continuing to culture the cells until viability drops below at least 50%;

g) obtaining the harvest cell culture fluid; and

h) purifying the rHuPH20 from the harvest cell culture fluid,

wherein soluble rHuPH20 is a soluble form of human PH20 that is recombinantly expressed in Chinese Hamster Ovary (CHO) cells.

[0006] Provided herein are methods for the production and purification of soluble rHuPH20. Methods are not claimed as such. Also provided herein are cell medium and harvested cell culture fluid that contain soluble rHuPH20. The methods provided herein can be used to produce and purify any quantity of soluble rHuPH20. For example, the methods and steps described herein are amendable for scale-up or scale-down, as would be apparent to one of skill in the art.

[0007] The methods provided herein can be used to produce and purify large-scale quantities of soluble rHuPH20. The methods provided herein for producing soluble rHuPH20 can include a) inoculating cell medium in a bioreactor with an inoculum of cells that encode soluble rHuPH20 to produce a cell culture, wherein cells contain between 150 and 300 copies of nucleic acid encoding soluble rHuPH20, the bioreactor contains at least 100 liters of cell culture and about 10^{10} - 10^{11} cells are inoculated per 100 liters cell culture and cells are cultured at a set temperature; b) feeding the cells with a first feed medium containing glucose, L-alanyl-L-glutamine, human insulin and yeast extract in amounts sufficient to increase cell growth and peak cell density, and to increase soluble rHuPH20 synthesis, wherein the feed medium is added to the culture at a volume of 4% of the cell culture volume; c) feeding the cells with a second feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and induce cell cycle arrest, wherein the amount of L-alanyl-L-glutamine is decreased compared to the amount of L-alanyl-L-glutamine in the second step, and the amount of yeast extract is increased compared to the amount of yeast extract in step b), and the feed medium is added to the culture at a volume of 4% of the cell culture volume and the temperature is lowered compared to the temperature in the step a) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; d) feeding the cells with a third feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and increase cell cycle arrest, wherein the feed medium is added to the culture at a volume of 4% of the cell culture volume, the amount of L-alanyl-L-glutamine and glucose is decreased compared to the amount of L-alanyl-L-glutamine and glucose in step c), and the amount of yeast extract and sodium butyrate is increased compared to the amount of yeast extract and sodium butyrate in step c), and the temperature is lowered compared to the temperature in step c) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; e) feeding the cells with a fourth feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and increase cell cycle arrest, wherein the amount of L-alanyl-L-glutamine and glucose is decreased compared to the amount of L-alanyl-L-glutamine and glucose in step d), the amount of sodium butyrate is decreased compared to the amount of sodium butyrate in step d), the temperature is lowered compared to the temperature in step d) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase and feed medium is added to the culture at a volume of 4% of the cell culture volume; f) culturing the cells until the viability drops below at least or about 50%; g) obtaining the harvest cell culture fluid; and h) the soluble rHuPH20 is purified from the harvest culture fluid.

[0008] The harvest cell culture fluid can be filtered prior to purification. In some examples, the temperature in step a) is 37°C, the temperature in step c) is 36.5°C, the temperature in step d) is 36°C and the temperature in step d) is 35.5°C. The soluble rHuPH20 purification can be effected by column chromatography, such as beaded crosslinked agarose column chromatography, beaded crosslinked phenyl-substituted agarose column chromatography, amino phenyl boronate column chromatography and hydroxyapatite column chromatography.

[0009] In one example, the method for producing soluble rHuPH20, includes steps of a) inoculating cell medium in a bioreactor with an inoculum of cells that encode soluble rHuPH20 to produce a cell culture, wherein the cells contain between 150 and 300 copies of nucleic acid encoding soluble rHuPH20, the bioreactor contains at least 100 liters of cell culture, the inoculation cell density is at or about 4×10^5 cells/mL and the cells are cultured at 37°C; b) feeding the cells with a first feed medium containing at or about 33 g/L glucose, 32 mM L-alanyl-L-glutamine, 16.6 g/L yeast extract and 33 mg/L insulin, wherein the feed medium is added to the culture at a volume of 4% of the cell culture volume; c) feeding the cells with a second feed medium containing at or about 33 g/L glucose, 16 mM L-alanyl-L-glutamine, 33.4

g/L yeast extract and 0.92 g/L sodium butyrate, wherein the feed medium is added to the culture at a volume of 4% of the cell culture volume and the temperature is lowered to 36.5°C; d) feeding the cells with a third feed medium containing at or about 50 g/L glucose, 10 mM L-alanyl-L-glutamine, 50 g/L yeast extract and 1.8 g/L sodium butyrate, wherein the feed medium is added to the culture at a volume of 4% of the cell culture volume and the temperature is lowered to 36°C; e) feeding the cells with a fourth feed medium containing at or about 33 g/L glucose, 6.6 mM L-alanyl-L-glutamine, 50 g/L yeast extract and 0.92 g/L sodium butyrate, wherein the feed medium is added to the culture at a volume of 4% of the cell culture volume and the temperature is lowered to 36°C; f) continuing to culture the cells until viability drops below at least or about 50%; g) obtaining the harvest cell culture fluid; h) filtering the harvest cell culture fluid; and i) purifying the rHuPH20 from the harvest culture fluid using beaded crosslinked agarose column chromatography, beaded crosslinked phenyl-substituted agarose column chromatography, amino phenyl boronate column chromatography and hydroxyapatite column chromatography.

[0010] In another example, the method for producing soluble rHuPH20 includes the steps of a) inoculating cell medium in a bioreactor with an inoculum of cells that encode soluble rHuPH20 to produce a cell culture, wherein the cells comprise between 150 and 300 copies of nucleic acid encoding soluble rHuPH20; the bioreactor contains at least 100 liters of cell culture; the inoculation cell density is at or about 4×10^5 cells/mL; and the cells are cultured at or about 37°C; b) feeding the cells with a first feed medium containing or containing about 33 g/L glucose, 32 mM L-alanyl-L-glutamine, 83.3 g/L yeast extract and 33 mg/L insulin, wherein the feed medium is added to the culture at a volume of 4 % or about 4% of the cell culture volume; c) feeding the cells with a second feed medium containing or containing about 33 g/L glucose, 13 mM L-alanyl-L-glutamine, 166.7 g/L yeast extract and 0.92 g/L sodium butyrate, wherein the feed medium is added to the culture at a volume of at or about 4% of the cell culture volume and the temperature is lowered to 36.5°C; d) feeding the cells with a third feed medium containing or containing about 50 g/L glucose, 10 mM L-alanyl-L-glutamine, 250 g/L yeast extract and 1.8 g/L sodium butyrate, wherein the feed medium is added to the culture at a volume of 4 % or about 4% of the cell culture volume and the temperature is lowered to 36°C; e) feeding the cells with a fourth feed medium containing or containing about 33 g/L glucose, 6.7 mM L-alanyl-L-glutamine, 250 g/L yeast extract and 0.92 g/L sodium butyrate, wherein the feed medium is added to the culture at a volume of 4 % or about 4% of the cell culture volume and the temperature is lowered to 36°C; f) continuing to culture the cells until viability drops below at least or about 50%; g) obtaining the harvest cell culture fluid; h) filtering the harvest cell culture fluid; and i) purifying the rHuPH20 from the harvest culture fluid using beaded crosslinked agarose column chromatography, beaded crosslinked phenyl-substituted agarose column chromatography, amino phenyl boronate column chromatography and hydroxyapatite column chromatography.

[0011] In some examples, the volume of cell culture in the bioreactor is or is about 200, 300, 400, 500, 1000, 1500, 2000, 2500, 3000 or 3500 liters. In some examples, the amount of soluble rHuPH20 is produced per 100 L of cell culture using the methods provided herein at least or about 1, 5, 10, 15, 20, 25, 30, 35 or 40 grams of soluble rHuPH20. The specific activity of the soluble rHuPH20 can be at least or about 80000, 100000, 120000, 140000, 160000 or 180,000 units/mg. The cells that encode soluble rHuPH20 can, in some instances, be DG44 CHO cells. Further, the rHuPH20 can be encoded by nucleic acid set forth in SEQ ID NO:47.

[0012] Provided herein are cell culture media containing soluble rHuPH20 with an enzymatic activity of greater than 5000 units/mL, such as 10,000, 12,000, 14,000, 16,000, 18,000, 20,000, 22,000 or 24,000 units/mL. Also provided herein are harvested cell culture fluid containing soluble rHuPH20 with an enzymatic activity of greater than 5000 units/mL, such as 10,000, 12,000, 14,000, 16,000, 18,000, 20,000, 22,000 or 24,000 units/mL. The claimed invention however relates to the composition as defined in claims 1 to 16. Thus, also in the following detailed description section, methods, therapeutic uses, hyaluronidase-expressing cells, cell culture expansion etc are useful for understanding the invention but are not claimed as such.

DETAILED DESCRIPTION

Outline

[0013]

A. Definitions

B. Overview

C. Hyaluronidase

1. Structure and function

2. PH20

3. Therapeutic uses of hyaluronidases

- a. Use as a spreading agent
- b. Use in hypodermoclysis
- c. Use in vitrectomy and ophthalmic disorders and conditions
- d. Use in gene therapy
- e. Cosmetic uses
- f. Use in organ transplantation
- g. Use in cancer treatment
- h. Use in treatment of glycosaminoglycan accumulation in the brain
- i. Use in treatment of glycosaminoglycan accumulation in cardiovascular disease
- j. Use in pulmonary disease
- k. Other uses

D. Hyaluronidase-expressing, cells

- a. 3D35M cells
- b. 2B2 cells

E. Cell culture expansion

F. Protein production

G. Protein concentration and buffer exchange

H. Purification

- 1. Beaded crosslinked agarose column
- 2. Beaded crosslinked phenyl-substituted agarose column
- 3. Amino Phenyl Boronate column
- 4. Hydroxyapatite column
- 6. Virus removal, protein concentration and buffer exchange

I. Filling

J. Monitoring and assays

- 1. Monitoring conditions
- 2. Monitoring soluble rHuPH20 production

K. Examples

A. DEFINITIONS

[0014] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong.

[0015] In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

[0016] As used herein, hyaluronidase refers to an enzyme that degrades hyaluronic acid. Hyaluronidases include bacterial hyaluronidases (EC 4.2.99.1), hyaluronidases from leeches, other parasites, and crustaceans (EC 3.2.1.36), and mammalian-type hyaluronidases (EC 3.2.1.35). Hyaluronidases also include any of non-human origin including, but not limited to, murine, canine, feline, leporine, avian, bovine, ovine, porcine, equine, piscine, ranine, bacterial, and any from leeches, other parasites, and crustaceans. Exemplary non-human hyaluronidases include, hyaluronidases from cows (SEQ ID NO:10), yellow jacket wasp (SEQ ID NOS:11 and 12), honey bee (SEQ ID NO:13), white-face hornet (SEQ ID NO:14), paper wasp (SEQ ID NO:15), mouse (SEQ ID NOS:16-18, 29), pig (SEQ ID NOS: 19-20), rat (SEQ ID NOS:21-23, 28), rabbit (SEQ ID NO:24), sheep (SEQ ID NO:25), orangutan (SEQ ID NO:26), cynomolgus monkey (SEQ ID NO:27), guinea pig (SEQ ID NO:30), *Staphylococcus aureus* (SEQ ID NO:31), *Streptococcus pyogenes* (SEQ ID NO:32), and *Clostridium perfringens* (SEQ ID NO:33). Exemplary human hyaluronidases include HYAL1 (SEQ ID NO:34), HYAL2 (SEQ ID NO:35), HYAL3 (SEQ ID NO:36), HYAL4 (SEQ ID NO:37), and PH20 (SEQ ID NO:1). Also included amongst hyaluronidases are soluble human PH20 and soluble rHuPH20.

[0017] Reference to hyaluronidases includes precursor hyaluronidase polypeptides and mature hyaluronidase polypeptides (such as those in which a signal sequence has been removed), truncated forms thereof that have activity,

and includes allelic variants and species variants, variants encoded by splice variants, and other variants.

[0018] For example, reference to hyaluronidase also includes the human PH20 precursor polypeptide variants set forth in SEQ ID NOS:48-49. Hyaluronidases also include those that contain chemical or posttranslational modifications and those that do not contain chemical or posttranslational modifications. Such modifications include, but are not limited to, pegylation, albumination, glycosylation, farnesylation, carboxylation, hydroxylation, phosphorylation, and other polypeptide modifications known in the art.

[0019] As used herein, soluble human PH20 or sHuPH20 include mature polypeptides lacking all or a portion of the glycosylphosphatidyl inositol (GPI) attachment site at the C-terminus such that upon expression, the polypeptides are soluble. Exemplary sHuPH20 polypeptides include mature polypeptides having an amino acid sequence set forth in any one of SEQ ID NOS:4-9 and 45-46. The precursor polypeptides for such exemplary sHuPH20 polypeptides include a signal sequence. Exemplary of the precursors are those set forth in SEQ ID NOS:3 and 38-44, each of which contains a 35 amino acid signal sequence at amino acid positions 1-35. Soluble HuPH20 polypeptides also include those degraded during or after the production and purification methods described herein.

[0020] As used herein, soluble rHuPH20 refers to a soluble form of human PH20 that is recombinantly expressed in Chinese Hamster Ovary (CHO) cells. Soluble rHuPH20 is encoded by nucleic acid that includes the signal sequence and is set forth in SEQ ID NO:47. Also included are DNA molecules that are allelic variants thereof and other soluble variants. The nucleic acid encoding soluble rHuPH20 is expressed in CHO cells which secrete the mature polypeptide. As produced in the culture medium there is heterogeneity at the C-terminus so that the product includes a mixture of species that can include one or more of SEQ ID NOS. 4-8 in various abundance. Corresponding allelic variants are included.

[0021] As used herein, "soluble rHuPH20-expressing cells" refers to any CHO cell that expresses soluble rHuPH20. Exemplary soluble rHuPH20-expressing cells include 2B2 and 3D35M cells. Soluble rHuPH20-expressing cells are CHO cells into which nucleic acid that contains the sequence set forth in SEQ ID NO:55 has been introduced.

[0022] As used herein, hyaluronidase activity refers to any activity exhibited by a hyaluronidase polypeptide. Such activities can be tested *in vitro* and/or *in vivo* and include, but are not limited to, enzymatic activity, such as to effect cleavage of hyaluronic acid, ability to act as a dispersing or spreading agent and antigenicity. hyaluronidase activity refers to any activity exhibited by a hyaluronidase polypeptide.

[0023] As used herein, enzymatic activity refers to the activity of a hyaluronidase, as assessed in *in vitro* enzymatic assays, to cleave a substrate, such as hyaluronic acid. *In vitro* assays to determine the enzymatic activity of hyaluronidases, such as soluble rHuPH20, are known in the art and described herein. Exemplary assays include the microturbidity assay described below (see e.g. Example 9 and section I) that measures cleavage of hyaluronic acid by hyaluronidase indirectly by detecting the insoluble precipitate formed when the uncleaved hyaluronic acid binds with serum albumin.

[0024] As use herein, specific activity with reference to soluble rHuPH20 is the enzyme activity relative to the amount of soluble rHuPH20. Specific activity is calculated by dividing the enzymatic activity (units/mL) by the protein concentration (mg/mL).

[0025] As used herein, "exhibits at least one activity" or "retains at least one activity" refers to the activity exhibited by a variant soluble rHuPH20 as compared to any soluble rHuPH20 set forth in SEQ ID NOS:4-9 under the same conditions. Typically, a variant soluble rHuPH20 that retains or exhibits at least one activity of a soluble rHuPH20 set forth in SEQ ID NOS:4-9 retains at or about 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more of the activity of a soluble rHuPH20 set forth in SEQ ID NOS:4-9. Exemplary activities include, but are not limited to, hyaluronidase activity and enzymatic activity.

[0026] As used herein, beaded crosslinked agarose column chromatography refers to chromatography using a column packed with beaded crosslinked agarose. Exemplary of beaded crosslinked agarose is Q Sepharose™.

[0027] As used herein, beaded crosslinked phenyl-substituted agarose column chromatography refers to chromatography using a column packed with beaded phenyl-substituted crosslinked agarose. Exemplary of beaded phenyl-substituted crosslinked agarose is Phenyl Sepharose™.

[0028] As used herein, amino phenyl boronate column chromatography refers to chromatography using a column packed with amino phenyl boronate agarose.

[0029] As used herein, hydroxyapatite column chromatography refers to chromatography using a column packed with hydroxyapatite.

[0030] As used herein, harvested cell culture fluid or harvest cell culture fluid (HCCF) refers to the fluid obtained following harvest of the cells from the bioreactor and separation of the cell culture medium from the cells, cell debris and other aggregates. The cell culture that is harvested from the bioreactor can be filtered to clarify the culture, removing the cells, cell debris and other aggregates to leave the harvested cell culture fluid.

[0031] As used herein, cell density refers to the number of cells in a given volume of medium.

[0032] As used herein, cell culture or culture refers to a cell population that is suspended in a medium under conditions suitable to maintain viability of the cells or grow the cells.

[0033] As used herein, medium, cell medium or cell culture medium refers to a solution containing nutrients sufficient to promote the growth of cells in a culture. Typically, these solutions contain essential and non-essential amino acids, vitamins, energy sources, lipids and/or trace elements. The medium also can contain additional supplements, such as hormones, growth factors and growth inhibitors. Reference to cell culture medium included

[0034] As used herein, the residues of naturally occurring α -amino acids are the residues of those 20 α -amino acids found in nature which are incorporated into protein by the specific recognition of the charged tRNA molecule with its cognate mRNA codon in humans.

[0035] As used herein, "in amounts sufficient to increase" when referring to a substance increasing parameters such as cell growth rate, peak cell density, protein synthesis or cell cycle arrest refers to the amount of a substance that effects an increase in one of these parameters compared to that observed in the absence of the substance. The parameters can be assessed in the presence and absence of a substance, and the amount of substance that increases the parameter (such as cell growth rate, peak cell density, protein synthesis or cell cycle arrest) compared to in the absence of the substance can be determined. The growth rate, peak cell density, protein synthesis or cell cycle arrest in the presence of the substance can be increased by at or about 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500% or more compared to the growth rate, peak cell density, protein synthesis or cell cycle arrest in the absence of the substance.

[0036] As used herein, nucleic acids include DNA, RNA and analogs thereof, including peptide nucleic acids (PNA) and mixtures thereof. Nucleic acids can be single or double-stranded. When referring to probes or primers, which are optionally labeled, such as with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that their target is statistically unique or of low copy number (typically less than 5, generally less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous nucleotides of sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleic acids long.

[0037] As used herein, a peptide refers to a polypeptide that is from 2 to 40 amino acids in length.

[0038] As used herein, the amino acids which occur in the various sequences of amino acids provided herein are identified according to their known, three-letter or one-letter abbreviations (Table 1). The nucleotides which occur in the various nucleic acid fragments are designated with the standard single-letter designations used routinely in the art.

[0039] As used herein, an "amino acid" is an organic compound containing an amino group and a carboxylic acid group. A polypeptide contains two or more amino acids. For purposes herein, amino acids include the twenty naturally-occurring amino acids, non-natural amino acids and amino acid analogs (i.e., amino acids wherein the α -carbon has a side chain).

[0040] As used herein, "amino acid residue" refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are presumed to be in the "L" isomeric form. Residues in the "D" isomeric form, which are so designated, can be substituted for any L-amino acid residue as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in J. Biol. Chem., 243: 3552-3559 (1969), and adopted 37 C.F.R., §§ 1.821-1.822, abbreviations for amino acid residues are shown in Table 1:

Table 1- Table of Correspondence

SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	Tyrosine
G	Gly	Glycine
F	Phe	Phenylalanine
M	Met	Methionine
A	Ala	Alanine
S	Ser	Serine
I	Ile	Isoleucine
L	Leu	Leucine
T	Thr	Threonine
V	Val	Valine

(continued)

SYMBOL		
1-Letter	3-Letter	AMINO ACID
P	Pro	Proline
K	Lys	Lysine
H	His	Histidine
Q	Gln	Glutamine
E	Glu	Glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	Tryptophan
R	Arg	Arginine
D	Asp	Aspartic acid
N	Asn	Asparagine
B	Asx	Asn and/or Asp
C	Cys	Cysteine
X	Xaa	Unknown or other

[0041] It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence (Table 1) and modified and unusual amino acids, such as those referred to in 37 C.F.R. §§ 1.821-1.822. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues, to an amino-terminal group such as NH₂ or to a carboxyl-terminal group such as COOH.

[0042] As used herein, "naturally occurring amino acids" refer to the 20 L-amino acids that occur in polypeptides.

[0043] As used herein, "non-natural amino acid" refers to an organic compound that has a structure similar to a natural amino acid but has been modified structurally to mimic the structure and reactivity of a natural amino acid. Non-naturally occurring amino acids thus include, for example, amino acids or analogs of amino acids other than the 20 naturally-occurring amino acids and include, but are not limited to, the D-isostereomers of amino acids. Exemplary non-natural amino acids are described herein and are known to those of skill in the art.

[0044] As used herein, a DNA construct is a single or double stranded, linear or circular DNA molecule that contains segments of DNA combined and juxtaposed in a manner not found in nature. DNA constructs exist as a result of human manipulation, and include clones and other copies of manipulated molecules.

[0045] As used herein, "similarity" between two proteins or nucleic acids refers to the relatedness between the sequence of amino acids of the proteins or the nucleotide sequences of the nucleic acids. Similarity can be based on the degree of identity and/or homology of sequences of residues and the residues contained therein. Methods for assessing the degree of similarity between proteins or nucleic acids are known to those of skill in the art. For example, in one method of assessing sequence similarity, two amino acid or nucleotide sequences are aligned in a manner that yields a maximal level of identity between the sequences. "Identity" refers to the extent to which the amino acid or nucleotide sequences are invariant. Alignment of amino acid sequences, and to some extent nucleotide sequences, also can take into account conservative differences and/or frequent substitutions in amino acids (or nucleotides). Conservative differences are those that preserve the physico-chemical properties of the residues involved. Alignments can be global (alignment of the compared sequences over the entire length of the sequences and including all residues) or local (the alignment of a portion of the sequences that includes only the most similar region or regions).

[0046] "Identity" per se has an art-recognized meaning and can be calculated using published techniques. (See, e.g.: Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exists a number of methods to measure identity between two polynucleotide or polypeptides, the term "identity" is well known to skilled artisans (Carillo, H. & Lipton, D., SIAM J Applied Math 48:1073

(1988)).

[0047] As used herein, homologous (with respect to nucleic acid and/or amino acid sequences) means about greater than or equal to 25% sequence homology, typically greater than or equal to 25%, 40%, 50%, 60%, 70%, 80%, 85%, 90% or 95% sequence homology; the precise percentage can be specified if necessary. For purposes herein the terms "homology" and "identity" are often used interchangeably, unless otherwise indicated. In general, for determination of the percentage homology or identity, sequences are aligned so that the highest order match is obtained (see, e.g.: Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; Carillo et al. (1988) SIAM J Applied Math 48:1073). By sequence homology, the number of conserved amino acids is determined by standard alignment algorithms programs, and can be used with default gap penalties established by each supplier. Substantially homologous nucleic acid molecules would hybridize typically at moderate stringency or at high stringency all along the length of the nucleic acid of interest. Also contemplated are nucleic acid molecules that contain degenerate codons in place of codons in the hybridizing nucleic acid molecule.

[0048] Whether any two molecules have nucleotide sequences or amino acid sequences that are at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" or "homologous" can be determined using known computer algorithms such as the "FASTA" program, using for example, the default parameters as in Pearson et al. (1988) Proc. Natl. Acad. Sci. USA 85:2444 (other programs include the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1):387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., et al., J Molec Biol 275:403 (1990)); Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo et al. (1988) SIAM J Applied Math 48:1073). For example, the BLAST function of the National Center for Biotechnology Information database can be used to determine identity. Other commercially or publicly available programs include, DNASTar "MegAlign" program (Madison, WI) and the University of Wisconsin Genetics Computer Group (UWG) "Gap" program (Madison WI). Percent homology or identity of proteins and/or nucleic acid molecules can be determined, for example, by comparing sequence information using a GAP computer program (e.g., Needleman et al. (1970) J. Mol. Biol. 48:443, as revised by Smith and Waterman (1981) Adv. Appl. Math. 2:482). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids), which are similar, divided by the total number of symbols in the shorter of the two sequences. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov et al. (1986) Nucl. Acids Res. 14:6745, as described by Schwartz and Dayhoff, eds., ATLAS OF PROTEIN SEQUENCE AND STRUCTURE, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

[0049] Therefore, as used herein, the term "identity" or "homology" represents a comparison between a test and a reference polypeptide or polynucleotide. As used herein, the term at least "90% identical to" refers to percent identities from 90 to 99.99 relative to the reference nucleic acid or amino acid sequence of the polypeptide. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide length of 100 amino acids are compared. No more than 10% (i.e., 10 out of 100) of the amino acids in the test polypeptide differs from that of the reference polypeptide. Similar comparisons can be made between test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of a polypeptide or they can be clustered in one or more locations of varying length up to the maximum allowable, e.g. 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. At the level of homologies or identities above about 85-90%, the result should be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often by manual alignment without relying on software.

[0050] As used herein, an aligned sequence refers to the use of homology (similarity and/or identity) to align corresponding positions in a sequence of nucleotides or amino acids. Typically, two or more sequences that are related by 50% or more identity are aligned. An aligned set of sequences refers to 2 or more sequences that are aligned at corresponding positions and can include aligning sequences derived from RNAs, such as ESTs and other cDNAs, aligned with genomic DNA sequence.

[0051] As used herein, "primer" refers to a nucleic acid molecule that can act as a point of initiation of template-directed DNA synthesis under appropriate conditions (e.g., in the presence of four different nucleoside triphosphates and a polymerization agent, such as DNA polymerase, RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. It will be appreciated that a certain nucleic acid molecules can serve as a "probe" and as a "primer." A primer, however, has a 3' hydroxyl group for extension. A primer can be used in a variety of methods, including, for example, polymerase chain reaction (PCR), reverse-transcriptase (RT)-PCR, RNA PCR, LCR, multiplex PCR, pan-handle PCR, capture PCR, expression PCR, 3' and 5' RACE, in situ PCR, ligation-mediated PCR and other amplification protocols.

[0052] As used herein, an allelic variant or allelic variation references any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and can result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or can encode polypeptides having altered amino acid sequence. The term "allelic variant" also is used herein to denote a protein encoded by an allelic variant of a gene. Typically the reference form of the gene encodes a wildtype form and/or predominant form of a polypeptide from a population or single reference member of a species. Typically, allelic variants, which include variants between and among species typically have at least 80%, 90% or greater amino acid identity with a wildtype and/or predominant form from the same species; the degree of identity depends upon the gene and whether comparison is interspecies or intraspecies. Generally, intraspecies allelic variants have at least about 80%, 85%, 90% or 95% identity or greater with a wildtype and/or predominant form, including 96%, 97%, 98%, 99% or greater identity with a wildtype and/or predominant form of a polypeptide. Reference to an allelic variant herein generally refers to variations in proteins among members of the same species.

[0053] As used herein, "allele," which is used interchangeably herein with "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for that gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide or several nucleotides, and can include substitutions, deletions and insertions of nucleotides. An allele of a gene also can be a form of a gene containing a mutation.

[0054] As used herein, species variants refer to variants in polypeptides among different species, including different mammalian species, such as mouse and human.

[0055] As used herein, a splice variant refers to a variant produced by differential processing of a primary transcript of genomic DNA that results in more than one type of mRNA.

[0056] As used herein, modification is in reference to modification of a sequence of amino acids of a polypeptide or a sequence of nucleotides in a nucleic acid molecule and includes deletions, insertions, and replacements of amino acids and nucleotides, respectively. Methods of modifying a polypeptide are routine to those of skill in the art, such as by using recombinant DNA methodologies.

[0057] As used herein, the term promoter means a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding region of genes.

[0058] As used herein, isolated or purified polypeptide or protein or biologically-active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. Preparations can be determined to be substantially free if they appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound, however, can be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

[0059] The term substantially free of cellular material includes preparations of proteins in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly-produced. In one embodiment, the term substantially free of cellular material includes preparations of enzyme proteins having less than about 30% (by dry weight) of non-enzyme proteins (also referred to herein as a contaminating protein), generally less than about 20% of non-enzyme proteins or 10% of non-enzyme proteins or less than about 5% of non-enzyme proteins. When the enzyme protein is recombinantly produced, it also is substantially free of culture medium, *i.e.*, culture medium represents less than about or at 20%, 10% or 5% of the volume of the enzyme protein preparation.

[0060] As used herein, the term substantially free of chemical precursors or other chemicals includes preparations of enzyme proteins in which the protein is separated from chemical precursors or other chemicals that are involved in the synthesis of the protein. The term includes preparations of enzyme proteins having less than about 30% (by dry weight) 20%, 10%, 5% or less of chemical precursors or non-enzyme chemicals or components.

[0061] As used herein, synthetic, with reference to, for example, a synthetic nucleic acid molecule or a synthetic gene or a synthetic peptide refers to a nucleic acid molecule or polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods.

[0062] As used herein, an expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Such additional segments can include promoter and terminator sequences, and optionally can include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both. Thus, an expression vector

refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

[0063] As used herein, vector also includes "virus vectors" or "viral vectors." Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

[0064] As used herein the term assessing is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the activity of a protease, or a domain thereof, present in the sample, and also of obtaining an index, ratio, percentage, visual or other value indicative of the level of the activity. Assessment can be direct or indirect and the chemical species actually detected need not of course be the proteolysis product itself but can for example be a derivative thereof or some further substance. For example, detection of a cleavage product of a complement protein, such as by SDS-PAGE and protein staining with Coomassie blue.

[0065] As used herein, a composition refers to any mixture. It can be a solution, suspension, liquid, powder, paste, aqueous, non-aqueous or any combination thereof. As used herein, a kit is a packaged combination that optionally includes other elements, such as additional reagents and instructions for use of the combination or elements thereof.

[0066] As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from cause or condition including, but not limited to, infections, acquired conditions, genetic conditions, and characterized by identifiable symptoms.

[0067] As used herein, "treating" a subject with a disease or condition means that the subject's symptoms are partially or totally alleviated, or remain static following treatment. Hence treatment encompasses prophylaxis, therapy and/or cure. Prophylaxis refers to prevention of a potential disease and/or a prevention of worsening of symptoms or progression of a disease. Treatment also encompasses any pharmaceutical use of a modified interferon and compositions provided herein.

[0068] As used herein, a pharmaceutically effective agent, includes any therapeutic agent or bioactive agents, including, but not limited to, for example, anesthetics, vasoconstrictors, dispersing agents, conventional therapeutic drugs, including small molecule drugs and therapeutic proteins.

[0069] As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease or other indication, are ameliorated or otherwise beneficially altered.

[0070] As used herein, a patient refers to a human subject.

[0071] As used herein, an effective amount is the quantity of a therapeutic agent necessary for preventing, curing, ameliorating, arresting or partially arresting a symptom of a disease or disorder.

[0072] As used herein, animal includes any animal, such as, but are not limited to primates including humans, gorillas and monkeys; rodents, such as mice and rats; fowl, such as chickens; ruminants, such as goats, cows, deer, sheep; ovine, such as pigs and other animals. Non-human animals exclude humans as the contemplated animal. Most enzymes are of animal origin, including mammalian origin.

[0073] As used herein, a control refers to a sample that is substantially identical to the test sample, except that it is not treated with a test parameter, or, if it is a plasma sample, it can be from a normal volunteer not affected with the condition of interest. A control also can be an internal control.

[0074] As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a compound, comprising "an extracellular domain" includes compounds with one or a plurality of extracellular domains.

[0075] As used herein, ranges and amounts can be expressed as "about" a particular value or range. About also includes the exact amount. Hence "about 5 mM" means "about 5 mM" and also "5 mM."

[0076] As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) Biochem. 11:1726).

B. OVERVIEW

[0077] Provided herein are methods for the large scale production of soluble rHuPH20. The methods utilize bioreactors to culture cells that produce the soluble rHuPH20, specifically CHO cells (e.g. DG44 CHO cells). Exemplary of such cells are 2B2 cells, which produce soluble rHuPH20. The volume of cell culture in the bioreactor typically is or is about 200, 300, 400, 500, 1000, 1500, 2000, 2500, 3000 or 3500 liters. Prior to inoculation of the bioreactor, the cells are expanded through a series of increasing cell culture volumes to generate the required number of cells for seeding of the bioreactor. The cell culture in the bioreactor is inoculated with 10^{10} - 10^{11} per 100 liters cell culture.

[0078] The cells are then incubated in the bioreactor for 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or more days.

[0079] During this incubation, feed media is added to the cell culture to supply additional nutrients and supplements. Exemplary supplements or nutrients that can be included in the feed media include, but are not limited to, glucose,

glutamine or glutamine-substitute, such as L-Alanyl-L-Glutamine, insulin, and sodium butyrate. The type and amount of supplement added can influence cell growth and protein production. For example, insulin and glutamine-substitute are incorporated into the first feed media added to the cell culture to increase cell growth and peak cell density. Subsequent feed media are designed to promote protein production more than cell growth. Supplements such as insulin are excluded and supplements such as glutamine-substitute, such as L-Alanyl-L-Glutamine are reduced in amount. In contrast, supplements such as yeast extract that enhance protein synthesis are increased in amount. In addition, sodium butyrate that enhances cell cycle arrest and, therefore, increases protein production, also is included.

[0080] Following protein production in the bioreactor, the cells are harvested and the soluble rHuPH20 that has been secreted into the cell culture media is concentrated prior to initiation of the purification process. The soluble rHuPH20 is then purified from the concentrated protein solution using a series of purification steps. Exemplary of purification methods that are used for the methods herein is a combination of ion-exchange chromatography, hydrophobic interaction chromatography and affinity chromatography. The purified protein is then concentrated and diafiltered.

[0081] Utilizing the methods described herein, between about 0.5-50 grams of soluble rHuPH20 is produced per 100 L of cell culture. In some examples, the amount of soluble rHuPH20 produced per 100 L of culture is or is about 1, 2, 3, 4, 5, 10, 15, 20, 30, or 40 grams or more. In some examples, the yield of soluble rHuPH20 following purification can range from between or between about 10% to 50% of the amount produced before purification: For example, the yield following purification can be or be about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45 % or 50% of the amount produced before purification. Generally, the specific activity of soluble rHuPH20 produced using the methods herein is at least or about 80000, 100000, 120000, 140000, 160000 or 180,000 units/mg.

C. HYALURONIDASES

[0082] Hyaluronidases are a family of enzymes that degrade hyaluronic acid (also known as hyaluronan or hyaluronate), an essential component of the extracellular matrix and a major constituent of the interstitial barrier. By catalyzing the hydrolysis of hyaluronic acid, hyaluronidase lowers the viscosity of hyaluronic acid, thereby increasing tissue permeability. As such, hyaluronidases have been used, for example, as a spreading or dispersing agent in conjunction with other agents, drugs and proteins to enhance their dispersion and delivery.

1. Structure and function of hyaluronidases

[0083] There are three general classes of hyaluronidases; mammalian hyaluronidase, bacterial hyaluronidase and hyaluronidase from leeches, other parasites and crustaceans. Mammalian-type hyaluronidases (EC 3.2.1.35) are *endo-β-N*-acetyl-hexosaminidases that hydrolyze the $\beta 1 \rightarrow 4$ glycosidic bond of hyaluronic acid into various oligosaccharide lengths such as tetrasaccharides and hexasaccharides. They have both hydrolytic and transglycosidase activities and can degrade hyaluronic acid and chondroitin sulfates, such as C4-S and C6-S. Hyaluronidases of this type include, but are not limited to, hyaluronidases from cows (SEQ ID NO:10), yellow jacket wasp (SEQ ID NOS:11 and 12), honey bee (SEQ ID NO:13), white-face hornet (SEQ ID NO:14), paper wasp (SEQ ID NO:15), mouse (SEQ ID NOS:16-18, 29), pig (SEQ ID NOS:19-20), rat (SEQ ID NOS:21-23, 228), rabbit (SEQ ID NO:24), sheep (SEQ ID NO:25), orangutan (SEQ ID NO:26), cynomolgus monkey (SEQ ID NO:27), guinea pig (SEQ ID NO:30) and human hyaluronidases.

[0084] There are six hyaluronidase-like genes in the human genome: HYAL1, HYAL2, HYAL3, HYAL4, HYALP1 and PH20/SPAM1. HYALP1 is a pseudogene, and HYAL3 (SEQ ID NO:36) has not been shown to possess enzyme activity toward any known substrates. HYAL4 (precursor polypeptide set forth in SEQ ID NO:37) is a chondroitinase and exhibits little activity towards hyaluronic acid. HYAL1 (precursor polypeptide set forth in SEQ ID NO:34) is the prototypical acid-active enzyme and PH20 (precursor polypeptide set forth in SEQ ID NO:1) is the prototypical neutral-active enzyme. Acid-active hyaluronidases, such as HYAL1 and HYAL2 (precursor polypeptide set forth in SEQ ID NO:35) generally lack catalytic activity at neutral pH (i.e. pH 7). For example, HYAL1 has little catalytic activity *in vitro* over pH 4.5 (Frost et al. (1997) Anal Biochemistry 251:263-269). HYAL2 is an acid-active enzyme with a very low specific activity *in vitro*. The hyaluronidase-like enzymes also can be characterized by those which are generally locked to the plasma membrane via a glycosylphosphatidyl inositol anchor such as human HYAL2 and human PH20 (Daniilovitch-Miagkova, et al. (2003) Proc Natl Acad Sci USA. 100(8):4580-5), and those which are generally soluble such as human HYAL1 (Frost et al, (1997) Biochem Biophys Res Commun. 236(1):10-5). By catalyzing the hydrolysis of hyaluronic acid, a major constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronic acid, thereby increasing tissue permeability. It also has been shown to exhibit anti-cancer and anti-carcinogenic activities.

[0085] N-linked glycosylation of some hyaluronidases can be very important for their catalytic activity and stability. While altering the type of glycan modifying a glycoprotein can have dramatic effects on a protein's antigenicity, structural folding, solubility, and stability, many enzymes are not thought to require glycosylation for optimal enzyme activity. Hyaluronidases are, therefore, unique in this regard, in that removal of N-linked glycosylation can result in near complete inactivation of the hyaluronidase activity. For such hyaluronidases, the presence of N-linked glycans is critical for gen-

erating an active enzyme.

[0086] There are seven potential N-linked glycosylation sites at N82, N166, N235, N254, N368, N393, N490 of human PH20 exemplified in SEQ ID NO: 1. Disulfide bonds form between the cysteine residues C60 and C351 and between C224 and C238 to form the core hyaluronidase domain. However, additional cysteines are required in the carboxy terminus for neutral enzyme catalytic activity such that amino acids 36 to 464 of SEQ ID NO:1 contains the minimally active human PH20 hyaluronidase domain. Thus, N-linked glycosylation site N490 is not required for proper hyaluronidase activity.

[0087] N-linked oligosaccharides fall into several major types (oligomannose, complex, hybrid, sulfated), all of which have (Man)₃-GlcNAc-GlcNAc-cores attached via the amide nitrogen of Asn residues that fall within-Asn-Xaa-Thr/Ser-sequences (where Xaa is not Pro). Glycosylation at an-Asn-Xaa-Cys-site has been reported for coagulation protein C. In some instances, the hyaluronidase can contain both N-glycosidic and O-glycosidic linkages. For example, rHuPH20 (as produced in the methods described herein) has O-linked oligosaccharides as well as N-linked oligosaccharides.

[0088] The methods described herein provide a process for the production and purification of large quantities of a soluble preparation of human PH20 hyaluronidase preparation.

2. PH20

[0089] Human PH20 (also known as sperm surface protein PH20), as noted above, is the prototypical neutral-active enzyme that is generally locked to the plasma membrane via a glycosylphosphatidyl inositol (GPI) anchor. It is naturally involved in sperm-egg adhesion and aids penetration by sperm of the layer of cumulus cells by digesting hyaluronic acid. The PH20 mRNA transcript is normally translated to generate a 509 amino acid precursor protein containing a 35 amino acid signal sequence at the N-terminus (amino acid residue positions 1-35). The mature PH20 polypeptide is, therefore, a 474 amino acid polypeptide with an amino acid sequence set forth in SEQ ID NO:2.

[0090] Soluble forms of human PH20 (sHuPH20) can be produced and purified using the methods described herein. The generation of sHuPH20 are described in related U.S. Patent Application Nos. 10/795,095, 11/065,716 and 11/238,171 (also referred to in these applications as sHASEGP or rHuPH20), and in Examples 1 and 4, below. The soluble forms are produced by expressing nucleic acid encoding C-terminal truncations of the mature PH20 polypeptide that lack the GPI-attachment sites.

[0091] The soluble form of human PH20, rHuPH20, is produced and purified using the methods provided herein.

3. Therapeutic uses of hyaluronidases

[0092] Various forms of hyaluronidases have been prepared and approved for therapeutic use in humans. For example, animal-derived hyaluronidase preparations include Vitrase® (ISTA Pharmaceuticals), a purified ovine testicular hyaluronidase, and Amphadase® (Amphastar Pharmaceuticals), a bovine testicular hyaluronidase. Hylenex® (Halozyme Therapeutics) is a human recombinant hyaluronidase produced by genetically engineered Chinese Hamster Ovary (CHO) cells containing nucleic acid encoding for soluble rHuPH20. Approved therapeutic uses for hyaluronidase include use as an adjuvant to increase the absorption and dispersion of other injected drugs, for hypodermoclysis (subcutaneous fluid administration), and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents. In addition to these indications, hyaluronidases, including sHuPH20, can be used as a therapeutic or cosmetic agent for the treatment of additional diseases and conditions.

[0093] As noted above, hyaluronidase is a spreading or diffusing substance which modifies the permeability of connective tissue through the hydrolysis of hyaluronic acid, a polysaccharide found in the intercellular ground substance of connective tissue, and of certain specialized tissues, such as the umbilical cord and vitreous humor. When no spreading factor is present, materials injected subcutaneously, such as drugs, proteins, peptides and nucleic acid, spread very slowly. Co-injection with hyaluronidase, however, can cause rapid spreading. The rate of diffusion is proportional to the amount of enzyme, and the extent of diffusion is proportional to the volume of solution. Absorption and dispersion of injected drugs and agents can be enhanced by adding 10-1000 units hyaluronidase to the injection solution. In some examples, 150 U hyaluronidase is added. Hyaluronidases have multiple uses, including and in addition to their use as a spreading agent. Hyaluronidase is commonly used, for example, for peribulbar block in local anesthesia prior ophthalmic surgery. The presence of the enzyme prevents the need for additional blocks and speeds the time to the onset of akinesia (loss of eye movement). Peribulbar and sub-Tenon's block are the most common applications of hyaluronidase for ophthalmic procedures. Hyaluronidase also can promote akinesia in cosmetic surgery, such as blepharoplasties and face lifts. Exemplary therapeutic and cosmetic uses for hyaluronidase are described below.

a. Use as a spreading agent

[0094] Soluble rHuPH20 produced using the methods described herein, can be used to promote or enhance the

delivery agents and molecules to any of a variety of mammalian tissues *in vivo*. It can be used to facilitate the diffusion and, therefore, promote the delivery, of small molecule pharmacologic agents as well as larger molecule pharmacologic agents, such as proteins, nucleic acids and ribonucleic acids, and macromolecular compositions that can contain a combination of components including, but not limited to, nucleic acids, proteins, carbohydrates, lipids, lipid-based molecules and drugs. For example, molecules and macromolecular complexes ranging from about 10 nm to about 500 nm in diameter, can exhibit dramatic improvements in delivery through interstitial spaces when the interstitial space has been previously, or is coincidentally, exposed to hyaluronidase (see e.g. U.S. Patent Application Nos. 10/795,095, 11/065,716 and 11/238,171).

[0095] Examples of pharmaceutical, therapeutic and cosmetic agents and molecules that can be administered with rHuPH20 include, but are not limited to, anesthetics; anti-metabolites, anti-neoplastics and other anti-cancer agents; anti-virals; anti-infectives, including anti-bacterials and other antibiotics, anti-fungals and other anti-infectives; immunomodulatory agents; steroidal and non-steroidal antiinflammatories; beta blockers; sympathomimetics; ducosanoids, prostaglandins and prostaglandin analogs; mitotics, cholinergics and anti-cholinesterases; anti-allergens and decongestants; hormonal agents; growth factors; immunosuppressants; vaccines and toxoids; immune sera; antibodies; and any combination thereof. In one example, soluble rHuPH20 is administered with a cathepsin, such as cathepsin L.

b. Use in hypodermoclysis

[0096] Hypodermoclysis, the infusion of fluids and electrolytes into the hypodermis of the skin, is a useful and simple hydration technique suitable for mildly to moderately dehydrated adult patients, especially the elderly. Although considered safe and effective, the most frequent adverse effect is mild subcutaneous edema that can be treated by local massage or systemic diuretics. Approximately 3 L can be given in a 24-hour period at two separate sites. Common infusion sites include the chest, abdomen, thighs and upper arms. Solutions used in hypodermoclysis include, for example, normal saline, half-normal saline, glucose with saline and 5% glucose. Potassium chloride also can be added to the solution. The addition of hyaluronidase to the solution can enhance fluid absorption and increase the overall rate of administration.

c. Use in vitrectomy and ophthalmic disorders and conditions

[0097] Hyaluronidase can be used to minimize the detachment or tearing of the retina during vitrectomy. This could cause, for example, the vitreous body to become uncoupled or "disinserted" from the retina, prior to removal of the vitreous body. Such disinsertion or uncoupling of the vitreous body can minimize the likelihood that further tearing or detachment of the retina will occur as the vitreous body is removed.

[0098] Hyaluronidase can be used for various ophthalmic applications, including the vitrectomy adjunct application described in U.S. Pat. No. 5,292,509. The use of a highly purified hyaluronidase, such as soluble rHuPH20 produced and purified by the methods described herein, is preferable for intraocular procedures to minimize immunogenicity and toxicity. In some examples, a pegylated hyaluronidase can be used to prolong residence within the vitreous and prevent localized uptake.

[0099] Hyaluronidases can be used to treat and/or prevent ophthalmic disorders by, for example, preventing neovascularization and increasing the rate of clearance from the vitreous of materials toxic to the retina. Hyaluronidase can be administered in an amount effective to liquefy the vitreous humor of the eye without causing toxic damage to the eye. Liquefaction of the vitreous humor increases the rate of liquid exchange from the vitreal chamber. This increase in exchange removes the contaminating materials whose presence can cause ophthalmologic and retinal damage.

[0100] Hyaluronidase also can be used to reduce postoperative pressure. Hyaluronic acid has been used in eye procedures such as glaucoma, vitreous and retina surgery and in corneal transplantation. A common side effect occurring in postoperative cataract patients is a significant early, and occasionally prolonged, rise in intraocular pressure. Such a condition is sometimes serious, especially in patients with glaucomatous optic disc changes. Hyaluronidase can be co-administered with hyaluronic acid to the eye prior to surgery to reduce postoperative pressure in the eye. The hyaluronidase is administered in an amount effective to reduce the intraocular pressure to pre-operative levels by breaking down the hyaluronic acid without decreasing its effectiveness during surgery nor causing side effects in the patient (U.S. Patent No. 6,745,776).

[0101] Hyaluronidase also can be administered to patients with glaucoma to remove glycosaminoglycans from the trabecular meshwork and reduce intraocular pressure, and can be applied to the vitreous to promote the resolution of vitreous hemorrhages (i.e. extravasation of blood into the vitreous), which can occur in connection with conditions such as diabetic retinopathy, retinal neovascularization, retinal vein occlusion, posterior vitreous detachment, retinal tears, ocular traumas and the like. The presence of vitreous hemorrhages, which are typically slow to resolve, can delay, complicate or prevent procedures that require the retina to be visualized through the vitreous for diagnosis and/or for

treatment procedures such as laser photocoagulation and the like which are often primary treatments for conditions such as proliferative diabetic retinopathy.

d. Use in gene therapy

[0102] The efficacy of most gene delivery vehicles *in vivo* does not correspond to the efficacy found observed *in vitro*. Glycosaminoglycans can hinder the transfer and diffusion of DNA and viral vectors into many cell types. The levels such extracellular matrix material can hinder the process considerably. Administration of hyaluronidase can open channels in the extracellular matrix, thus enhancing delivery of gene therapy. For example, hyaluronidase can be administered with collagenase to facilitate transduction of DNA *in vivo* (Dubensky et al. (1984) Proc Natl Acad Sci USA 81(23):7529-33). Hyaluronidase also can enhance gene therapy using adeno-associated virus (Favre et al, (2000) Gene Therapy 7(16):1417-20). The channels opened following administration of hyaluronidase are of a size that typically enhance diffusion of smaller molecules such as retroviruses, adenoviruses, adeno-associated viruses and DNA complexes (as well as other therapeutic and pharmacological agents of interest). The pores are not so large, however, as to promote the dislocation and movement of cells.

[0103] In some examples, viruses can be engineered to express hyaluronidase to facilitate their replication and spread within a target tissue. The target tissue can be, for example, a cancerous tissue whereby the virus is capable of selective replication within the tumor. The virus also can be a non-lytic virus wherein the virus selectively replicates under a tissue specific promoter. As the viruses replicate, the co-expression of hyaluronidase with viral genes can facilitate the spread of the virus *in vivo*.

e. Cosmetic uses

[0104] Hyaluronidases can be by administered to remove glycosaminoglycans involved in the accumulation of cellulite and to promote lymphatic flow. In some examples, human hyaluronidases, such as for example, soluble rHuPH20, are used for the treatment of cellulite. The hyaluronidase can be administered through repeated subcutaneous injections, through transdermal delivery in the form of ointments or creams or through the use of injectable slow release formulations to promote the continual degradation of glycosaminoglycans and prevent their return.

[0105] Hyaluronidase also can be used to treat conditions such as "pigskin" edema or "orange peel" edema. Hyaluronidases can effect depolymerization of the long mucopolysaccharide chains that can accumulate in the dermis and which are responsible for the retention of bound water and of the slowing, by capillary compression, of the diffusion of organic liquids, which eliminate metabolic wastes. Such retention of water and wastes associated with fat overloading of the lipocytes, constitutes classical "pigskin" edema or "orange peel" edema. Depolymerization can cut the long chains of mucopolysaccharides into shorter chains, resulting in the elimination of the bound water and wastes and restoration of the venous and lymphatic circulation, culminating in the disappearance of local edema.

f. Use in organ transplantation

[0106] The content of hyaluronic acid in an organ can increase with inflammation. An increased concentration of hyaluronic acid has been observed in tissue from different organs characterized by inflammatory-immunological injury such as alveolitis (Nettelbladt et al. (1991) Am. Rev. Resp. Dis. 139: 759-762) and myocardial infarction (Waldenstrom et al. (1991) J. Clin. Invest. 88(5): 1622-1628). Other examples include allograft rejection after a renal (Ha'llgren et al. (1990) J. Exp. Med. 171: 2063-2076; Wells et al. (1990) Transplantation 50: 240-243), small bowel (Wallander et al. (1993) Transplant. Int. 6: 133-137) or cardiac (Haellgren et al. (1990) J Clin Invest 185:668-673) transplantation; or a myocardial inflammation of viral origin (Waldenstrdm et al. (1993) Eur. J. Clin. Invest. 23: 277-282). The occurrence of interstitial edemas in connection with the grafting of an organ constitutes a severe problem in the field of transplantation surgery. Grafts with interstitial edemas can swell to such a degree that the function is temporarily be lost. In some instances, the swelling can cause disruption of the kidney, resulting in a massive hemorrhage. Hyaluronidases can be used to degrade accumulated glycosaminoglycans in an organ transplant. Removal of such glycosaminoglycans promotes removal of water from the graft and thus enhances organ function.

g. Use in cancer treatment

[0107] Hyaluronidase has direct anti carcinogenic effects. Hyaluronidase prevents growth of tumors transplanted into mice (De Maeyer et al., (1992) Int. J. Cancer 51:657-660) and inhibits tumor formation upon exposure to carcinogens (Pawlowski et al. (1979) Int. J. Cancer 23:105-109) Hyaluronidase is effective as the sole therapeutic agent in the treatment of brain cancer (gliomas) (WO 198802261). In addition to these effects, hyaluronidases also can be used to enhance penetration of chemotherapeutic agents into solid tumors. They can be injected intratumorally with anti-cancer

agents or intravenously for disseminated cancers or hard to reach tumors. The anticancer agent can be a chemotherapeutic, an antibody, a peptide, or a gene therapy vector, virus or DNA. Additionally, hyaluronidase can be used to recruit tumor cells into the cycling pool for sensitization in previously chemorefractory tumors that have acquired multiple drug resistance (St Croix et al., (1998) *Cancer Lett* September 131(1): 35-44). Hyaluronidases, such as, for example soluble rHuPH20, also can enhance delivery of biologics such as monoclonal antibodies, cytokines and other drugs to tumors that accumulate glycosaminoglycans.

[0108] Hyaluronidases can also be used to increase the sensitivity of tumors that are resistant to conventional chemotherapy. For example, hyaluronidase, such as soluble rHuPH20, can be administered to a patient having a tumor associated with a HYAL1 defect in an amount effective to increase diffusion around the tumor site (e.g., to facilitate circulation and/or concentrations of chemotherapeutic agents in and around the tumor site), inhibit tumor cell motility, such as by hyaluronic acid degradation, and/or to lower the tumor cell apoptosis threshold. This can bring the tumor cell(s) to a state of anoikis, which renders the tumor cell more susceptible to the action of chemotherapeutic agents. Administration of hyaluronidase can induce responsiveness of previously chemotherapy-resistant tumors of the pancreas, stomach, colon, ovaries, and breast (Baumgartner et al. (1988) *Reg. Cancer Treat.* 1 :55-58; Zanker et al. (1986) *Proc. Amer. Assoc. Cancer Res.* 27:390).

[0109] In one example, hyaluronidases are used in the treatment of metastatic and non-metastatic cancers, including those that have decreased endogenous hyaluronidase activity relative to non-cancerous cells. Hyaluronidases can be used as a chemotherapeutic agent alone or in combination with other chemotherapeutics. Exemplary cancers include, but are not limited to, small lung cell carcinoma, squamous lung cell carcinoma, and cancers of the breast, ovaries, head and neck, or any other cancer associated with depressed levels of hyaluronidase activity or decreased hyaluronic acid catabolism.

h. Use in treatment of glycosaminoglycan accumulation in the brain

[0110] Hyaluronic acid levels are elevated in a number of cerebrospinal pathologic conditions. Levels of cerebrospinal hyaluronic acid are normally less than 200 $\mu\text{g/L}$ in adults (Laurent et al. (1996) *Acta Neurol Scand* September 94(3):194-206), but can elevate to levels of over 8000 $\mu\text{g/L}$ in diseases such as meningitis, spinal stenosis, head injury and cerebral infarction. Hyaluronidases, such as, for example, soluble rHuPH20, can be utilized to degrade critically elevated levels of substrate.

[0111] The lack of effective lymphatics in the brain also can lead to life threatening edema following head trauma. Hyaluronic acid accumulation is a result of increased synthesis by hyaluronic acid synthases and decreased degradation. Accumulation of hyaluronic acid can initially serve the beneficial purpose of increasing water content in the damaged tissue to facilitate leukocyte extravasation, but continued accumulation can be lethal. Administration of hyaluronidase, such as intrathecally or intravenously, to a patient suffering from head trauma can serve to remove tissue hyaluronic acid accumulation and the water associated with it.

[0112] Hyaluronidases also can be used in the treatment of edema associated with brain tumors, particularly that associated with glioblastoma multiform. The edema associated with brain tumors results from the accumulation of hyaluronic acid in the non-cancerous portions of the brain adjacent the tumor. Administration of hyaluronidase to the sites of hyaluronic acid accumulation (e.g., by intravenous injection or via a shunt) can relieve the edema associated with such malignancies by degrading the excess hyaluronic acid at these sites.

i. Use in treatment of glycosaminoglycan accumulation in cardiovascular disease

[0113] Hyaluronidase can be used in the treatment of some cardiovascular disease. Administration of hyaluronidase in animal models following experimental myocardial infarct can reduce infarct size (Maclean, et al (1976) *Science* 194(4261): 199-200). One proposed mechanism by which this can occur is by reducing hyaluronic acid accumulation that occurs following ischemia reperfusion. Reduction of infarct size is believed to occur from increased lymph drainage and increased tissue oxygenation and reduction of myocardial water content.

[0114] Hyaluronidases also can be used to limit coronary plaques from arteriosclerosis. Such plaques accumulate glycosaminoglycans and mediate macrophage and foam cell adhesion (Kolodgie et al. (2002) *Arterioscler Thromb Vase Biol.* 22(10): 1642-8).

j. Use in pulmonary disease

[0115] Levels of hyaluronic acid in bronchoalveolar lavages (BAL) from normal individuals are generally below 15 ng/ml. However, hyaluronic acid levels in BAL rise dramatically in conditions of respiratory distress (Bjerner et al. (1987) *Br Med J (Clin Res Ed)* 295(6602):803-6). The increased hyaluronic acid in the lung can prevent oxygen diffusion and gas exchange as well as activating neutrophil and macrophage responses. Purified preparations of soluble rHuPH20,

such as those produced using the methods provided herein, can be delivered by either pulmonary or intravenous delivery to patients presenting with such conditions to reduce hyaluronan levels. Hyaluronidases also can be administered to patients suffering from other pulmonary complications that are associated with elevated glycosaminoglycans or to enhance the delivery of other co delivered molecules to the lung.

k. Other uses

[0116] In further examples of its therapeutic use, hyaluronidase can be used for such purposes as an antidote to local necrosis from paravenous injection of necrotic substances such as vinka alkaloids (Few et al. (1987) Amer. J. Matern. Child Nurs. 12, 23-26), treatment of ganglion cysts (Paul et al. (1997) J Hand Surg. 22 (2): 219-21) and treatment of tissue necrosis due to venous insufficiency (Elder et al. (1980) Lancet 648-649). Hyaluronidases also can be used to treat ganglion cysts (also known as a wrist cyst, Bible cyst, or dorsal tendon cyst), which are the most common soft tissue mass of the hand and are fluid filled sacs that can be felt below the skin.

[0117] Hyaluronidases can be used in the treatment of spinal cord injury by degrading chondroitin sulfate proteoglycans (CSPGs). Following spinal cord injury, glial scars containing CSPGs are produced by astrocytes. CSPGs play a crucial role in the inhibition of axon growth. In addition, the expression of CSPG has been shown to increase following injury of the central nervous system (CNS). Hyaluronidases also can be utilized for the treatment of herniated disks in a process known as chemonucleolysis. Chondroitinase ABC, an enzyme cleaving similar substrates as hyaluronidase, can induce the reduction of intradiscal pressure in the lumbar spine. There are three types of disk injuries. A protruded disk is one that is intact but bulging. In an extruded disk, the fibrous wrapper has torn and the NP has oozed out, but is still connected to the disk. In a sequestered disk, a fragment of the NP has broken loose from the disk and is free in the spinal canal. Chemonucleolysis is typically effective on protruded and extruded disks, but not on sequestered disk injuries.

D. Soluble rHuPH20-expressing cells

[0118] The methods described herein can be used to generate and purify large quantities of soluble rHuPH20. Soluble rHuPH20 is expressed in CHO cells that are grown in large-scale cell culture. Expression is effected using an expression vector that contains the nucleotide sequence encoding the sequence of amino acids set forth in SEQ ID NO:3 (corresponding to amino acids 1 to 482 of the precursor human PH20 polypeptide set forth in SEQ ID NO:1). Following translation, the 35 amino acid signal sequence is cleaved and soluble rHuPH20 is secreted into the medium. The vector also contains an IRES downstream of the soluble rHuPH20 encoding region, a mouse dihydrofolate reductase gene and the SV40 pA sequence. The expression vector was introduced into DG44 cells, which are dihydrofolate reductase deficient (dhfr-) that have been adapted to grow in suspension culture in a chemically defined, animal product-free medium. The resulting soluble rHuPH20-expressing cells include those described in Examples 1 and 4, below, and include cells designated 3D35M, 2B2, 3E10B, 1B3, 5C1, 1G11 and 2G10 cells.

[0119] Other cells can be used to produce hyaluronidases similar to rHuPH20. Generally, protein expression systems suitable for the introduction of critical N-linked glycosylation residues on hyaluronidases are used. Such cells include, for example, yeast cells, fungal cells, plant cells, insect cells and mammalian cells. Many cell lines are available for mammalian expression including mouse, rat human, monkey, chicken and hamster cells. Exemplary cell lines include but are not limited to CHO (including DG44 cells and CHO-S cells), Balb/3T3, HeLa, MT2, mouse NS0 (nonsecreting) and other myeloma cell lines, hybridoma and heterohybridoma cell lines, lymphocytes, fibroblasts, Sp2/0, COS, NIH3T3, HEK293, 293S, 2B8, and HKB cells. Cell lines also are available adapted to serum-free media, which facilitates purification of secreted proteins from the cell culture media.

a. 3D35M cells

[0120] Exemplary of soluble rHuPH20-expressing cells are 3D35M cells, described in Example 1, below, and U.S. Patent Publication Nos. 20040268425, 20050260186 and 20060104968. 3D35M cells are dihydrofolate reductase deficient (dhfr-) DG44 CHO cells that express soluble rHuPH20. The cells were transformed with an HZ24 expression vector having the nucleotide sequence set forth in SEQ ID NO:50. This vector contains a CMV promoter driving expression of nucleic acid encoding a 482 amino acid (SEQ ID NO:3) polypeptide that corresponds to amino acid positions 1 to 482 of the full length human PH20 set forth in SEQ ID NO:1. This includes a 35 amino acid N-terminal signal sequence. The vector also contains an internal ribosome entry site (IRES) after the PH20-encoding sequence, followed by a mouse dihydrofolate reductase gene and the SV40 polyadenylation sequence. Following translation, the 482 amino acid polypeptide is processed to remove the 35 amino acid signal sequence, resulting in the secretion of soluble rHuPH20.

[0121] Characterization of 3D35M cells demonstrated that the nucleic acid region encoding soluble rHuPH20 is present in the cells at a copy number of approximately 318 copies/cells. Soluble rHuPH20 produced from 3D35M cells by the methods herein is a mixture of species that can include one or more of the polypeptides having sequences set forth in

SEQ ID NOS:4-8. In an exemplary characterization of these species (described in Example 11), the species set forth in SEQ ID NO:4 was present at an abundance of 0.2%, the species set forth in SEQ ID NO:5 (corresponding to amino acids 1 to 446 of SEQ ID NO:4) was present at an abundance of 18.4%, the species set forth in SEQ ID NO:6 (corresponding to amino acids 1 to 445 of SEQ ID NO:4) was present at an abundance of 11.8%, the species set forth in SEQ ID NO:7 (corresponding to amino acids 1 to 444 of SEQ ID NO:4) was present at an abundance of 56.1%; and the species set forth in SEQ ID NO:8 (corresponding to amino acids 1 to 443 of SEQ ID NO:4) was present at an abundance of 13.6%. Such heterogeneity in the soluble rHuPH20 preparation is likely a result of C-terminal cleavage by peptidases present during the production and purification methods provided herein.

[0122] The 3D35M cells can be grown in cell culture medium with or without methotrexate. Additional supplements, such as glutamine, also can be added. In some examples, the cells are grown in cell culture medium containing, for example, 50 nM, 100 nM, 500 nM, 1 μ M, or 2 μ M methotrexate and lacking hypoxanthine and thymidine. In one example, 3D35M cells are cultured at 37°C in 5-7% CO₂ in culture medium (such as CD CHO Medium, Invitrogen) without hypoxanthine and thymidine and with 100 nM methotrexate and glutamine or a glutamine substitute, such as L-Alanyl-L-Glutamine, a stabilized, dipeptide form of L-glutamine. Other cell culture media appropriate for CHO cells can be used to culture 3D35M cells including, but not limited to, Dulbecco's modified Eagle's medium (DMEM), Eagle's Minimum essential medium (EMEM), Iscove's modified Eagle's medium (IMEM), F12 and RPMI. 3D35M cells grown under such conditions in shaking flasks can produce in excess of 1000 units/mL hyaluronidase activity. When cultured in a bioreactor, such as described in Comparative Example 3, below, 3D35M cells can produce soluble rHuPH20 with enzymatic activity in excess of 2000 units/mL.

b. 2B2 cells

[0123] Exemplary of soluble rHuPH20-expressing cells for production of rHuPH20 in the methods provided herein are described in Example 4 and designated 2B2 cells. 2B2 cells were generated by adapting 3D35M cells to higher methotrexate levels (i.e. 20 μ M) and selecting clones that grew in the higher methotrexate concentration. This adaptation increased the hyaluronidase activity produced by the cells. DG44 cells are dihydrofolate reductase-deficient (dhfr-) and, therefore, cannot make nucleosides. The expression vector present in 3D35M and 2B2 cells contains, in addition to the PH20 gene, the coding sequence for mouse dihydrofolate reductase. Methotrexate is a strong competitive dihydrofolate reductase inhibitor. Therefore, by increasing the concentration of methotrexate in the culture media, the hyaluronidase-expressing cells are forced to produce more mouse dihydrofolate reductase to remain viable. This can be effected by, for example, gene amplification or rearrangement of the integrated DNA to a more stable and productive arrangement. Thus, forcing an increase in the production of mouse dihydrofolate reductase also can result in an increase in the production of sHuPH20. A comparison of enzymatic activity of soluble rHuPH20 produced by 2B2 cells and 3D35M cells demonstrated that activity was typically between 80% and 100% higher in 2B2 cells (see e.g. Example 5, below) compared to 3D35M cells.

[0124] 2B2 cells were selected from amongst the cell clones that were isolated following selection with 20 μ M methotrexate as the cell line that produced soluble rHuPH20 having the greatest enzymatic activity (see, e.g. Example 4). When characterized, it was observed that the nucleic acid region encoding soluble rHuPH20 was present in 2B2 cells at a copy number of approximately 206 copies/cell. Southern blot analysis of Spe I-, Xba I- and BamH I/Hind III-digested genomic 2B2 cell DNA using a probe specific for the nucleic acid region encoding soluble rHuPH20 revealed the following restriction digest profile: one major hybridizing band of ~7.7 kb and four minor hybridizing bands (~13.9, ~6.6, ~5.7 and ~4.6 kb) with DNA digested with Spe I; one major hybridizing band of ~5.0 kb and two minor hybridizing bands (~13.9 and ~6.5 kb) with DNA digested with Xba I; and one single hybridizing band of ~1.4 kb observed using 2B2 DNA digested with BamH I/Hind III.

[0125] 2B2 cells can be grown in cell culture medium with or without methotrexate. Additional supplements, such as glutamine, insulin and yeast extract also can be added. In some examples, the cells are grown in cell culture medium containing, for example, 50 nM, 100 nM, 500 nM, 1 μ M, 2 μ M, 5 μ M, 10 μ M, 20 μ M or more methotrexate and lacking hypoxanthine and thymidine. In one example, 2B2 cells are cultured at 37°C in 5-7% CO₂ in culture medium (such as CD CHO Medium, Invitrogen) without hypoxanthine and thymidine and with 20 μ M methotrexate and glutamine or L-alanyl-L-glutamine, a stabilized, dipeptide form of L-glutamine. Other cell culture media appropriate for CHO cells can be used to culture 2B2 cells, including, but not limited to, Dulbecco's modified Eagle's medium (DMEM), Eagle's Minimum essential medium (EMEM), Iscove's modified Eagle's medium (IMEM), F12 and RPMI. 2B2 cells grown under such conditions in shaking flasks can produce in excess of 3000 units/mL hyaluronidase activity. When cultured in a bioreactor, such as described in Example 8, below, 2B2 cells can produce soluble rHuPH20 having enzymatic activity in excess of 17000 units/mL hyaluronidase activity.

[0126] Soluble rHuPH20 produced from 2B2 cells by the methods herein is a mixture of species of polypeptides having sequences set forth in SEQ ID NOS: 4-8. In an exemplary characterization the soluble rHuPH20 product produced by 2B2 cells (described in Example 11), the species set forth in SEQ ID NO:4 was present at an abundance of 1.9%, the

species set forth in SEQ ID NO:5 (corresponding to amino acids 1 to 446 of SEQ ID NO:4) was present at an abundance of 46.7%, the species set forth in SEQ ID NO:6 (corresponding to amino acids 1 to 445 of SEQ ID NO:4) was present at an abundance of 16.7%, the species set forth in SEQ ID NO:7 (corresponding to amino acids 1 to 444 of SEQ ID NO:4) was present at an abundance of 27.8%; and the species set forth in SEQ ID NO:8 (corresponding to amino acids 1 to 443 of SEQ ID NO:4) was present at an abundance of 6.9%. As noted for soluble rHuPH20 produced from 3D35M cells, the heterogeneity in the soluble rHuPH20 preparation from 2B2 cells is likely a result of C-terminal cleavage by peptidases present during the production and purification methods provided herein.

E. Cell culture expansion

[0127] The methods described herein employ bioreactors to grow large volumes of cell culture to produce large quantities of soluble rHuPH20. As described in detail below, these methods include a cell expansion phase, a protein production phase, a protein concentration and buffer exchange phase, and a purification phase. The soluble rHuPH20-expressing cells, such as 2B2 cells, are initially expanded from an original inoculum, such as an aliquot of cells from a working cell bank (WCB) or master cell bank (MCB), to a larger volume prior to culture in the bioreactor for the production phase. The final culture volume in the expansion phase is directly proportional to the volume of the bioreactor used in the following production phase. Typically, a larger bioreactor is inoculated using a larger final culture volume from the expansion phase than is a smaller bioreactor.

[0128] The soluble rHuPH20-expressing cells are expanded through a series of cultures, each increasing in volume from the previous one, and each being used as the inoculum for the subsequent culture. Exemplary of such cells are 2B2 cells. The original inoculum is typically one in which the purity and identity of the cells and the cell number are defined. These cells can be stored frozen, such as at -20°C, -70°C or -80°C, or can be maintained in liquid media at, for example, 4 °C, or maintained in culture at, for example, 37°C. In some instances, the original inoculum is a master cell bank or working cell bank aliquot that has been stored frozen. In such cases, the inoculum is thawed, such as in a 37°C waterbath. The original cell inoculum is typically centrifuged and the cells are resuspended in an appropriate cell culture media. For example, 2B2 cells can be resuspended in, and subsequently cultured in, basal media, such as CD CHO media (Invitrogen), or reconstituted powdered CD CGO AGT™ media (Invitrogen), supplemented with 8mM glutamine or L-alanyl-L-glutamine and 20 μM methotrexate. In another example, cells can be grown in basal media supplemented with 8 mM glutamine or L-alanyl-L-glutamine and 100 mM methotrexate. Any other suitable cell culture media also can be used to expand hyaluronidase-expressing cells. For example, cells can be cultured in Dulbecco's modified Eagle's medium (DMEM), Eagle's Minimum essential medium (EMEM), Iscove's modified Eagle's medium (IMEM), F12, RPMI, or other chemically-defined or undefined media, with or without additional supplements. Typically, the cells are grown in serum-free media, but also can be grown in media containing serum. One of skill in the art could prepare cell culture media using other basal cell culture media to which various nutrients can be supplemented to make the cell culture media in which soluble rHuPH20-expressing cells are cultured.

[0129] The cell inoculum is added to the first of a series of increasing volumes of cell culture media, thus expanding the cell culture. Following the initial inoculation, the cells are expanded in a humidified incubator or bioreactor at an appropriate temperature with an appropriate amount of CO₂. Typically, the amount of CO₂ is between 4% and 9%, typically between 6.0% and 8.0%, such as 7.0% and the temperature is between 35 °C and 39 °C, typically between 36 °C and 38 °C, such as 37 °C. For example, 2B2 and 3D35M cells can be grown in a 37°C humidified incubator with 7% CO₂. The culture can be agitated, such as at 90-130 rpm, during this process. When the cells reach the desired density, such as, for example, greater than 1.0×10^6 cells/mL (e.g. between 1.5×10^6 cells/mL and 2.5×10^6 cells/mL), the cell culture is used to inoculate a larger volume of fresh cell culture media. For example, cells can be inoculated into the next culture at a density of 4×10^4 to 4×10^6 cells/mL, typically 2×10^5 to 6×10^5 cells/mL, such as 4×10^5 cells/mL. The process is repeated until the cells have been expanded to the desired volume and cell density for seeding of, for example, 4×10^4 to 4×10^6 cells/mL into the bioreactor.

[0130] In one example, soluble rHuPH20-expressing cells, such as 2B2 cells, are initially added to approximately 20 mL of fresh cell culture media in a 125 mL shaker flask, resulting in a culture volume of 20-30 mL, typically 25 mL. Following incubation at 37°C, 7% CO₂ and expansion of the cells to a density of greater than 1.5×10^6 cells/mL, fresh media is added to the flask to expand the cell culture to 40 mL. The cells are incubated again until a density of greater than 1.5×10^6 cells/mL is attained, after which the entire cell culture (approximately 40 mL) is added to fresh media to make 100 mL culture volume in a 125 mL spinner flask. This process is repeated by transferring the entire cell culture (approximately 100 mL) to a 250 mL spinner flask containing sufficient fresh media to make a final culture volume of 200 mL, then a 1 L spinner flask containing sufficient fresh media to make a final culture volume of 800 mL, then a 6 L spinner flask containing sufficient fresh media to make a final culture volume a final volume of 5 L, and finally to a 36 L spinner flask containing sufficient fresh media to make a final culture volume of 32 L. Between each transfer, the cells are incubated until the culture reached a density of greater than 1.5×10^6 cells/mL. In some examples, a higher cell density is attained following incubation of the final 36 L spinner flask. For example, cells in the 36 L spinner flask can

be expanded to a cell density of 3.55×10^6 cells/mL to 6.05×10^6 cells/mL. This process can be used to expand soluble rHuPH20-expressing cells before introduction to a 400 L bioreactor (300 L culture volume) for the protein production phase (see, e.g. Example 8).

[0131] This process, like any of the processes described herein, also can be scaled-up by one of skill in the art for introduction of the cells into a bioreactor with a culture volume larger than 300 L. For example, the process can be scaled up for introduction of the cells into a bioreactor with a 2500 L culture volume, such as described in Example 12. Thus, in one example of the methods provided herein, following thawing, the cells are serially expanded through a 125 mL shaker flask (working volume of 20-30 mL, such as 25 mL), a 250 mL shaker flask (working volume of 45-55 mL, such as 50 mL), a 1 L shaker flask (working volume of 190-210 mL, such as 200 mL), two 2 L shaker flasks (working volume of 350-450 mL per flask, such as 400 mL per flask), six 2 L shaker flasks (working volume of 350-450 mL per flask, such as 400 mL per flask), a 25 L wave bioreactor (working volume of 14-16 L, such as 15 L), a 100 L wave bioreactor (working volume of 75-85 L, such as 80 L), and a 600 L seed bioreactor (working volume of 440-520 L, such as 480 L).

F. Protein production

[0132] Following cell expansion, the soluble rHuPH20-expressing cells are transferred to a bioreactor for the production phase, during which large quantities of soluble rHuPH20 are secreted into the cell media. This phase typically is designed such that the cells growth is maximized in the first half of the bioreactor run, and soluble rHuPH20 production is maximized in the second half of the bioreactor run. The cells are provided with a series of feed media at particular time points throughout the production to regulate this process. The bioreactor conditions also are typically monitored to ensure optimal conditions are maintained throughout the process. The methods described herein for protein can be scaled up or down by one of skill in the art. Further, modifications to, for example, cell media, incubation times, feeding protocols. One of skill in the art can empirically determine the appropriate conditions for protein production for any given bioreactor and cell type.

[0133] Bioreactors of different sizes and designs can be utilized in the methods herein. In some examples, a 125 L, 400 L or 3500 L bioreactor is used in the methods herein to culture cells in volumes of approximately 100L, 300L and 2500 L, respectively. Typically, the bioreactor is sterilized prior to the addition of cell culture media or cells. Sterilization can be effected by autoclaving or otherwise treating with steam for some bioreactors, or by treatment with a sterilizing solution, such as dilute sodium hydroxide, dilute nitric acid or sodium hypochlorite. In some examples, the bioreactor is sterilized by steam at 121°C, 20 PSI for 30 minutes. Following sterilization, cell culture media can be added to the bioreactor and then assessed for contamination, such by microbial contamination, after a period of time to ensure that the sterilization process was effective.

[0134] The cell culture from the expansion phase, described above, is added to the sterilized bioreactor containing fresh cell culture media. The soluble rHuPH20-expressing cells are inoculated into the fresh cell culture media at a cell density of 10^5 to 10^6 cells/mL.

[0135] In one example, soluble rHuPH20-expressing cells are inoculated at a cell density of 4×10^5 cells/mL. The total cell count following inoculation depends on the size of the bioreactor and the cell density. For example, a cell culture volume of 100 L can have a cell density following inoculation of approximately 10^{10} or 10^{11} cells.

[0136] The volumes of inoculating cell culture and fresh culture media used are dependent upon the size of the bioreactor and the cell density of the inoculum. For example, approximately 30 L of soluble rHuPH20-expressing cells, such as 2B2 cells, can be added to a 400 L bioreactor containing 230 L fresh cell culture media, for a total volume of approximately 260 L and an inoculation cell density of 4×10^5 cells/mL (total cell count of approximately 10^{11} cells). This can be scaled up or down as necessary, depending on the bioreactor.

[0137] In one example, for production in a 3500 L bioreactor, 2B2 cells are added to fresh cell culture media for a total cell culture volume of 1900-2300 L, such as 2100 L.

[0138] The fresh cell culture media contains the appropriate supplements to provide the necessary nutrients to the cells to promote cell growth. Supplements that can be added to the basal cell medium include, but are not limited to, glucose, insulin, sodium butyrate, yeast extract and glutamine or a glutamine substitute, such as L-alanyl-L-glutamine. In some instances, the basal medium contains sufficient glucose that no further glucose needs to be added. In other instances, glucose is added to the media later in the production process, such as in a subsequent feed media. The addition of insulin to the medium can promote cell growth and increase peak cell density. Glutamine or glutamine-substitutes, such as or L-alanyl-L-glutamine, can support cell cycle progression and also enhance cell growth. One of skill in the art can empirically determine the amount and quality of the nutrients that can be supplemented to the basal medium. In some examples, glutamine or glutamine-substitute is added to the basal cell culture medium at 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 15 mM or 20 mM. Insulin can be added to the cell culture medium at, for example, 0.5 mg/L to 50 mg/L, such as 1 mg/L to 40 mg/L, 2 mg to 30 mg/L, or 5 mg/L to 20 mg/L. For example, basal cell culture medium supplemented with 5mg/L insulin and 8 mM L-alanyl-L-glutamine can be used as the fresh cell culture media into which the soluble rHuPH20-expressing cells are inoculated. Additional supplements,

such as antibiotics, anti-fungals, indicators, salts, vitamins, amino acids and growth factors also can be added.

[0139] The individual parameters of the bioreactor can be set to maintain optimal conditions throughout the protein production process. The specific parameters that can be set depend on the bioreactor used, and can include, but are not limited to, temperature, pH, dissolved oxygen, impeller speed, vessel pressure, air sparge and air overlay. In one example, the conditions of a 125 L bioreactor containing 100 L cell culture of 3D35M cells are set to; temperature: 37°C; dissolved oxygen: 25% $\pm$ 10%; impeller speed: 50 RPM; vessel pressure: 3 psi; air sparge: 1 L/minute; air overlay: 1 L/minute, pH: 7.2 In another example, the conditions of a 400L bioreactor containing an initial cell culture volume of 260 L are set to; temperature: 37°C; impeller speed 40-55 RPM; vessel pressure: 3 psi; air sparge: 0.5-1.5 L/minute; air overlay: 1 L/minute. In a further example, the conditions of a 3000L bioreactor containing an initial culture volume of 2100 L are set to; temperature: 37°C (or between 36.5°C and 37.5 °C; impeller speed: 35 RPM (or 70-80 RPM); vessel pressure: 5 psi (or 3-7 psi); air sparge: 12 L/minute (or 11-13 L/minute); dissolved oxygen: 25%, or > 25%; pH pre inoculation: 7.2 (or pH 7.1-7.3); pH post inoculation: $\leq$ 7.2 (or $\leq$ 7.3). One of skill in the art can empirically determine the appropriate conditions for growth of a particular soluble rHuPH20-expressing cell in a particular bioreactor.

[0140] The soluble rHuPH20-expressing cells are typically cultured in the bioreactor for between 10 and 25 days. In some examples, the soluble rHuPH20-expressing cells are cultured in the bioreactor for 12, 13, 14, 15 or 16 days before harvesting. In other examples, the cells are harvested when the viable cell count (VCC) falls to a particular level, such as, for example, 25 %, 30 %, 35 %, 40 %, 45 %, 50 %, 55 %, 60% or 70 %. In one example, the cells are harvested when the VCC is between 30 % and 35 %. In another example, the cells are harvested within 24 hours of the VCC dropping below 50 %.

[0141] During the bioreactor culture, the cells are grown as fed-batch cultures and provided with a series of feed media at particular time points to supplement nutrients and glucose throughout. In some instances, the nutrients provided in the cell culture medium into which the cells were inoculated have depleted by 3, 4, 5, 6, 7 days or more post-inoculation. Thus, providing additional nutrients or supplements can produce higher yields of protein than batch cultures. In one example, cells are provided with feed media on days 6, 9 and 11 post-inoculation. In another example, cells are provided with feed media on days 7, 9 and 11 post-inoculation. In a further example, cells are provided with feed media on days 5, 7, 9 and 11 post-inoculation. The volume of feed media added to the bioreactor culture can range between, for example, 0.5 % and 20%, such as 1-20%, 2-15%, 3-10% or 4-5% of the cell culture volume. In some instances, the feed media is added at a volume equivalent to 4% of the cell culture volume.

[0142] The addition of various supplements to the feed media is used to regulate the growth and/or cell cycle of the cells. Nutrients and supplements that are included in the feed media include, but are not limited to, glutamine or glutamine-substitute, such as L-Alanyl-L-Glutamine, insulin, yeast extract, glucose and sodium butyrate or sodium butyrate. Furthermore, the basal media used in the feed media also can be concentrated, thus providing additional nutrients, such as essential amino acids, that may have been depleted during cell culture. The basal media in the feed media can be 2 $\times$, 3 $\times$, 4 $\times$, 5 $\times$, 6 $\times$ or more concentrated. In other examples, the basal media is less concentrated, or the same concentration as the cell culture media in the bioreactor.

[0143] The supplements included in the feed media can be used to regulate cell growth and protein production. The first feed media added to the cell culture can include nutrients that enhance cell cycle progression, cell growth and peak cell density. Subsequent feed medias promote cell growth arrest and/or protein synthesis. The amount of each supplement in each feed media can vary, such as by increasing or decreasing from one feed media to the next, or can be the same from one feed media to the next. In some examples, the amount of a supplement is increased from one feed media to the next, such as by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 400% or more. In other examples, the amount of a supplement is decreased from one feed media to the next, such as by 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more. In one example, a supplement is omitted from a feed media. In other examples, the amount of a supplement in the feed media stays the same. One of skill in the art can empirically determine the optimum amount of each supplement for each feed media to promote the desired amount of cell growth and protein production.

[0144] Supplements or nutrients that are included in the feed media include, but are not limited to, glucose, glutamine or glutamine-substitute, such as L-Alanyl-L-Glutamine, insulin, and sodium butyrate. The type and amount of supplement added can influence cell growth and protein production. Insulin and glutamine-substitute L-alanyl-L-glutamine are incorporated into the first feed media added to the cell culture to increase cell growth and peak cell density. Subsequent feed media are designed to promote protein production more than cell growth. The supplement insulin is excluded and L-Alanyl-L-Glutamine is reduced in amount. In contrast, the supplement yeast extract that enhances protein synthesis is increased in amount. Sodium butyrate that enhances cell cycle arrest and, therefore, increases protein production, is also included.

[0145] The addition of insulin can increase peak cell density by, for example, 2%, 5%, 10%, 15%, 20%, 25%, 30% or more.

Insulin is added to early feed media, i.e., the first feed media, to promote maximal cell growth in the initial phase of the

bioreactor run. For example, the first feed media, can contain amount of insulin at or about 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, 25 mg/L, 30 mg/L, 35 mg/L, 40 mg/L, 45 mg/L, 50 mg/L, 55 mg/L, 60 mg/L or more.

[0146] Glutamine-substitute L-Alanyl-L-Glutamine is also added to the feed media.

[0147] The amount of glutamine-substitute added to the first feed media is more than the amount of glutamine-substitute added to subsequent feed media. The amount of glutamine-substitute added to each subsequent feed media is reduced compared to the amount added in the prior feed media. The optimal amount added to each feed media can be determined empirically by one of skill in the art, and can include, for example, concentrations of glutamine-substitute at or about 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM or more.

[0148] Typically, the basal media used in the feed media also is supplemented with glucose. The amount of glucose added to each feed media can be increased or decreased relative to the previous feed media, or can stay approximately constant. In some examples, the amount of glucose added to the feed media is or is about 10 g/L, 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 55 g/L, 60 g/L, 75 g/L, 80 g/L or more.

[0149] In addition, supplements that promote protein synthesis are also included. Such nutrients include yeast extract. In some instances, the amount of yeast extract included in the feed media is increased during the bioreactor run. For example, the amount of yeast extract in the third feed media can be increased compared to the amount in the second feed media, which can be increased compared to the amount in the second feed media. In some examples, the amount of yeast extract added to the feed media is between 5 and 1000 g/L, such as or as about 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 75 g/L, 100 g/L, 125 g/L, 150 g/L, 175 g/L, 200 g/L, 250 g/L, 300 g/L, 350 g/L, 400 g/L or more.

[0150] Supplements that enhance cell cycle arrest and, therefore, increase protein production, are also included, i.e., sodium butyrate. Typically, such supplement is included in feed media that are added to the bioreactor later in the run and not included in the first feed media. Sodium butyrate that enhances cell cycle arrest is added to the second feed media and subsequent feed media.

[0151] In some examples, the amount of sodium butyrate added to the feed media is between 0.1 g/L and 10 g/L, such as or as about 0.2 g/L, 0.3 g/L, 0.4 g/L, 0.5 g/L, 0.6 g/L, 0.7 g/L, 0.8 g/L, 0.9 g/L, 1.0 g/L, 1.1 g/L, 1.2 g/L, 1.3 g/L, 1.4 g/L, 1.5 g/L, 1.6 g/L, 1.7 g/L, 1.8 g/L, 1.9 g/L, 2.0 g/L, 2.5 g/L, 3.0 g/L, 3.5 g/L, or more.

[0152] Further, any one or more of the bioreactor conditions can be altered during the production phase to optimize protein production. The temperature is lowered. This can serve to promote cell cycle arrest, prolong cell viability (thereby increasing total protein production), and help stabilize the hyaluronidase that has been secreted. For example, the temperature of the bioreactor can be reduced at each feeding, such as from 37°C to 36.5°C on the second feeding, to 36°C on the third feeding and to 35.5°C on the fourth feeding. One of skill in the art can empirically determine the appropriate feed media and the time at which to provide the feed, as well as the appropriate conditions in the bioreactor.

[0153] In one example, cells are provided with feed media on days 6, 9 and 11 post-inoculation. In another example, cells are provided with feed media on days 7, 9 and 11 post-inoculation. In a further example, cells are provided with feed media on days 5, 7, 9 and 11 post-inoculation. The feed media provided at each time-point are different, and can include supplements such as, but not limited to, glucose, sodium butyrate, insulin, glutamine or a glutamine substitute and yeast extract as defined in the claims. For example, 2B2 cells growing in a 260 L culture in a 400 L bioreactor can be provided with a first feed at day 5 containing 10.4 L of 4 × basal media (e.g. CD CHO media) with 33 g/L Glucose, 32 mM L-alanyl-L-glutamine, 16.6 g/L Yeast extract and 33 mg/L Insulin, a second feed at day 7 containing 10.8 L of 2× basal media (e.g. CD CHO media), 33 g/L Glucose, 16 mM L-alanyl-L-glutamine, 33.4 g/L Yeast extract and 0.92 g/L Sodium Butyrate, a third feed at day 9 containing 10.8 L 1× basal media (e.g. CD CHO media), 50 g/L Glucose, 10 mM L-alanyl-L-glutamine, 50 g/L Yeast extract and 1.80 g/L Sodium Butyrate, and a fourth feed at day 11 containing 1× basal media (e.g. CD CHO media), 33 g/L Glucose, 6.6 mM L-alanyl-L-glutamine, 50 g/L Yeast extract and 0.92 g/L Sodium Butyrate. This can be scaled up or down by one of skill in the art for production of rHuPH20 in larger or smaller bioreactors, respectively. Further, one of skill in the art can alter the amount of type of one more supplements added to the media to enhance cell growth and/or protein production.

[0154] In another example, the following feed media are provided to cells on days 5, 7, 9 and 11: Feed #1 Medium: basal media + 33 g/L Glucose + 26.6 mM L-alanyl-L-glutamine + 83.3 g/L Yeastolate + 33 mg/L rHuInsulin; Feed #2: basal media + 33 g/L Glucose + 13.4 mM L-alanyl-L-glutamine + 166.7 g/L Yeastolate + 0.92 g/L Sodium Butyrate; Feed #3: basal media + 50 g/L Glucose + 10 mM L-alanyl-L-glutamine + 250 g/L Yeastolate + 1.8 g/L Sodium Butyrate; Feed #4: basal media + 33.3 g/L Glucose + 6.7 mM L-alanyl-L-glutamine + 250 g/L Yeastolate + 0.92 g/L Sodium butyrate.

G. Cell culture harvest, protein concentration and buffer exchange

[0155] Following the protein production phase, the cells are harvested and the soluble rHuPH20 that has been secreted into the cell culture media is concentrated prior to initiation of the purification process. In addition to concentrating the protein, the cell culture media can be exchanged with an appropriate buffer at this time. Multiple systems and processes to effect protein concentration and buffer exchange are known in the art and can be used in the methods herein. Described below are exemplary methods of such, and one of skill in the art will recognize that these methods can be modified or

substituted with other effective methods to achieve a satisfactory level of protein concentration and buffer exchange.

[0156] The cells are harvested from the bioreactor and processed through a cell removal and clarification system to separate the cell culture fluid containing the hyaluronidase from cells and cell debris. An example of such a system is one that contains a series of filters that allow only the protein to pass through and be collected. Any filter or series of filters capable of separating the hyaluronidase from cells and cell debris can be used. For example, the cell culture harvest can be passed through a series of capsule filters, such as polyethersulfone filters. These can have decreasing pore sizes to incrementally remove, for example, cells, cell debris and smaller particles, such as viruses. In some examples, a series of four filters with pore sizes of 8.0 μm , 0.65 μm , 0.22 μm and 0.22 μm is used to clarify the cell culture to obtain the harvested cell culture fluid (HCCF). Another example of a cell removal and clarification system that can be used in the methods herein is a series of filters that in the first stage contains four modules in parallel, each containing a layer of diatomaceous earth graded to 4-8 μm and a layer of diatomaceous earth graded to 1.4-1.1 μm , followed by a cellulose membrane. The second stage contains a single module containing a layer of diatomaceous earth graded to 0.1-0.11 μm and a layer of diatomaceous earth graded to <0.1 μm followed by a cellulose membrane, and the third stage is a 0.22 μm polyethersulfone capsule filter.

[0157] Once the cells and debris have been separated from the HCCF, the protein in the HCCF typically is concentrated and the cell culture media exchanged with an appropriate buffer. The protein can be concentrated by 2 $\times$, 3 $\times$, 4 $\times$, 5 $\times$, 6 $\times$, 7 $\times$, 8 $\times$, 9 $\times$, 10 $\times$, 11 $\times$, 12 $\times$, 13 $\times$ or more. In some examples, the protein is concentrated 10 $\times$. In other examples, the protein is concentrated 6 $\times$. Any method of protein concentration known in the art can be utilized. Exemplary of such methods include concentration using tangential flow filtration (TFF) systems with molecular weight cut off (MWCO) filters. For example, the clarified HCCF can be passed through a series of two 30 kDa MWCO spiral polyethersulfone filters to concentrate the protein 10 $\times$. In another example, the HCCF is passed through a series of four 30 kDa MWCO filters. For large-scale production of hyaluronidase, such as, for example, 100 L and 300 L cultures, filters with surface areas of between 0.5 and 5 square meters are typically employed for this purpose. In some examples, filters with a surface area of 1.2 square meters or 2.8 square meters are used.

[0158] A buffer exchange is performed following protein concentration. One of skill in the art can empirically determine an appropriate buffer. Exemplary of suitable buffers is a 10 mM Hepes, 25 mM NaCl, pH 7.0 buffer, or a 10 mM Tris, 20 mM Na₂SO₄, pH 7.5 buffer. Following harvesting, concentration and buffer exchange, the concentrated protein solution is typically passed through another filter, such as a 0.22 μm capsule filter, before being stored in a sterile storage bag.

[0159] In some examples, the concentrated protein solution is treated to inactivate any residual virus contamination. Virus inactivation can be effected by any method known in the art. For example, the concentrated protein solution can be mixed with a 10% Triton X-100, 3% tri (n-butyl) phosphate (TNBP), to a final concentration of 1% Triton X-100, 0.3% TNBP, at room temperature for between 15 and 75 minutes. In some examples, the protein is exposed to the viral inactivation solution for 30-45 minutes.

H. Purification

[0160] The soluble rHuPH20 is purified from the concentrated protein solution using a series of purification steps. Many purification techniques are known in the art and can be utilized in the methods herein. Such methods can include, but are not limited to, chromatographic methods such as ion-exchange chromatography, size-exclusion chromatography, affinity chromatography (AC), high performance liquid chromatography (HPLC), reversed phase chromatography (RPC) and hydrophobic interaction chromatography (HIC), and gel filtration methods, or any combination thereof.

[0161] Exemplary of purification methods that are used for the methods herein is a combination of ion-exchange chromatography, hydrophobic interaction chromatography and affinity chromatography. In ion-exchange chromatography, the proteins can be separated from a complex solution or mixture based on electrostatic forces between charged functional groups of the proteins and charged functional groups of the chromatography-column matrix. Cation-exchange resins have negatively charged functional groups that attract positively charged functional groups of proteins, and anion-exchange resins have positively charged functional groups that attract negatively charged functional groups of proteins. Proteins bound through electrostatic forces to the matrix can be eluted by increasing the ionic strength of the buffer solution within the chromatography column over time. In hydrophobic interaction chromatography, a protein can be separated from a complex solution or mixture based on its hydrophobicity. A complex solution containing the protein is applied to a chromatography column equilibrated with a high salt buffer that facilitates binding of the protein to the resin. A salt-gradient mobile phase with decreasing ionic strength is then introduced into the chromatography column to release bound proteins from the matrix. Alternatively, hydrophobic interaction chromatography may separate a monomeric protein from a complex solution or mixture by binding hydrophobic impurities, including inactive dimers and aggregates of the protein, while permitting the monomeric protein to flow through the chromatography column relatively unimpeded. In affinity chromatography, a proteins can be separated from a complex solution based on the affinity of the protein for a ligand or ligand-binding entity that is covalently bound to the matrix. Other proteins in the complex solution or mixture

with weak affinity, or lacking affinity, for the ligand or ligand-binding entity flow through the chromatography column unimpeded, leaving the protein of interest bound to the matrix. The protein can then be eluted from the chromatography column by altering buffer conditions to decrease the affinity for the ligand or ligand-binding entity.

[0162] In one example, the soluble rHuPH20 is purified from the concentrated protein solution by sequential purification through a beaded crosslinked agarose column, such as a Q Sepharose™ column (ion-exchange chromatography), beaded crosslinked phenyl-substituted agarose column, such as a Phenyl Sepharose™ column (hydrophobic interaction chromatography), an Amino Phenyl Boronate column (affinity chromatography) and finally through a Hydroxyapatite column (ion-exchange chromatography). Each of these columns exhibit different binding properties with regards to hyaluronidase, such that the beaded crosslinked agarose column (e.g. Q Sepharose™ column) is a capture step (i.e. soluble rHuPH20 is bound to the resin while some other proteins flow through), the beaded crosslinked phenyl-substituted agarose (e.g. Phenyl Sepharose™ column) is a flow through step (i.e. soluble rHuPH20 flows through the column while some other proteins are captured), the Amino Phenyl Boronate column is another capture step, and the Hydroxyapatite column is a polishing step to further purify the soluble rHuPH20.

[0163] Prior to use, the columns are typically sterilized and the equilibrated. Sterilization can be effected by any method known in the art, including, but not limited to, sterilization with 1.0 M NaOH. Equilibration can be effected by the addition of an appropriate buffer to the column, such as a buffer similar to or the same as the buffer used to subsequently wash the column or the buffer in which the protein is contained in prior to loading. One of skill in the art can readily determine buffers suitable for use in equilibrating each column. Exemplary buffers are provided below. Between each chromatography step, the eluted protein can be filtered, such as through a 0.22 µm filter, to remove any contaminating microorganism or large aggregates. In some examples, the filtered eluate is stored, such as in sterile storage bags, prior to use in the next step. Following column chromatography, the purified hyaluronidase can subsequently be subjected to a virus removal step, followed by protein concentration and buffer exchange for final formulation. Exemplary purification methods are described in more detail below.

1. Beaded crosslinked agarose column

[0164] The concentrated protein obtained from the harvested cell culture fluid (HCCF) can be loaded onto a beaded crosslinked agarose column, such as, for example, a Q Sepharose™ column, which is a strong anion exchanger and captures soluble rHuPH20 while allowing other proteins to flow through. The bound soluble rHuPH20 can then be eluted using an appropriate buffer. The dimensions of the column used is typically dependent on the volume of concentrated protein obtained from the HCCF. For example, concentrated protein obtained from culture of hyaluronidase-expressing cells in a 100 L bioreactor culture can be loaded onto a column that is 20 cm high, 14 cm in diameter and contains 3 L resin. In another example, concentrated protein obtained from culture of soluble rHuPH20-expressing cells in a 300 L bioreactor culture can be loaded onto a column that is 29 cm high, 20 cm in diameter and contains 9 L resin. This can be scaled up or down as necessary, depending on the volume of the concentrated protein solution and the expected amount of protein. For example, concentrated protein obtained from culture of soluble rHuPH20-expressing cells in a 20 L bioreactor culture can be loaded onto a Q Sepharose™ column that is 28 cm high, 7 cm in diameter and contains 1.1 L resin, and concentrated protein obtained from culture of soluble rHuPH20-expressing cells in a 2500 L bioreactor culture can be loaded onto a Q Sepharose™ column that is 26 cm high, 63 cm in diameter and contains 81 L resin.

[0165] Prior to loading with the protein, the column is typically equilibrated. Equilibration can be effected by passing through 1, 2, 3, 4, 5, 6, 7, 8, 9 or more column volumes of buffer. In some examples, 5 column volumes of buffer is passed through the column for equilibration. Buffers suitable for equilibration include those similar to the buffers that will be used to wash the column after the protein had been loaded. For example, a beaded crosslinked agarose column, such as a Q Sepharose™ column can be equilibrated with 10 mM Hepes, 25 mM NaCl, pH 7.5. Other neutral pH buffers can be used, as will be recognized by one of skill in the art.

[0166] After loading the protein concentrate, the column is washed and the protein eluted. Suitable buffers for washing such columns containing bound soluble rHuPH20 include, for example, 10 mM Hepes, 25 mM NaCl, pH 7.0; 10 mM Hepes, 50 mM NaCl, pH 7.0; and 10 mM Tris, 20 mM Na₂SO₄, pH 7.5. The column can be washed with one or more types of buffer. For example, the column can be washed with 20 mM Na₂SO₄, pH 7.5 and 10 mM Hepes, 50 mM NaCl, pH 7.0. Typically, washing is effected by passing through 1, 2, 3, 4, 5, 6, 7, 8, 9 or more column volumes of buffer. In some examples, 5 column volumes of buffer is used to wash the column. The soluble rHuPH20 is then eluted using a buffer with a higher salt concentration, such as for example, 10 mM Hepes, 400 mM NaCl, pH 7.0. In some examples, the absorbance at A₂₈₀ is monitored to determine when to collect the eluate, as any absorbance during this process generally indicates the presence of soluble rHuPH20. Thus, in one example, the eluate is collected when the absorbance begins reading is 0.025. Typically, the eluate is filtered through an appropriate filter, such as a 0.22 µm filter, before being stored, such as in a sterile storage bag.

2. Beaded crosslinked phenyl-substituted agarose column

[0167] Following purification through a beaded crosslinked agarose column, the protein solution can be subjected to hydrophobic interaction chromatography using a beaded crosslinked phenyl-substituted agarose column, such as a Phenyl Sepharose™ column, in which the soluble rHuPH20 flows through the column while other contaminating proteins are captured. The column used in the methods herein can range in size, depending on the volume and amount of protein being purified through it. Exemplary sizes include columns that are 29 cm high, 20 cm in diameter with 9 L resin for use in the purification of soluble rHuPH20 from cells grown in a 100 L bioreactor culture, columns that are 29 cm high, 30 cm in diameter with 19-21 L resin for use in the purification of hyaluronidase from cells grown in a 300 L bioreactor culture, and columns that are 35 cm high, 80 cm in diameter with 176 L resin for use in the purification of soluble rHuPH20 from cells grown in a 2500 L bioreactor culture. One of skill in the art can scale up or down as appropriate.

[0168] The sterilized beaded crosslinked phenyl-substituted agarose column, such as a Phenyl Sepharose™ column, can be equilibrated prior to loading of the protein with an appropriate buffer, such as, for example, 5 mM potassium phosphate, 0.5 M ammonium sulfate, 0.1 mM CaCl_2 , pH 7.0. The protein eluate from the Q Sepharose column purification also is supplemented with ammonium sulfate, potassium phosphate and CaCl_2 . These can be supplemented to the protein to final concentrations of, for example, about 5 mM potassium phosphate, 0.5 M ammonium sulfate and 0.1 mM CaCl_2 , pH 7.0. Following loading of the protein, 5 mM potassium phosphate, 0.5 M ammonium sulfate and 0.1 mM CaCl_2 , pH 7.0 also is added to the column and the flow through filtered is collected, such as in a sterile bag.

3. Amino Phenyl Boronate column

[0169] Following hydrophobic interaction chromatography, the column-purified protein can be loaded onto an Amino Phenyl Boronate column for further purification. Amino Phenyl boronate ligand-mediated chromatography differs from many other ligands used for affinity chromatography. Whereas most ligands bind to a particular binding site on a protein by a mixture of noncovalent interactions, phenyl boronate interacts predominantly by forming a temporary covalent bond with 1,2-cis-diol groups. The boronate ligand will bind to any molecule containing the appropriate group, including soluble rHuPH20, which is highly glycosylated.

[0170] The Amino Phenyl Boronate column used in the methods herein can range in size, depending on the volume and amount of protein being purified through it. Exemplary sizes include columns that are 29 cm high, 20 cm in diameter with 6.3 L resin for use in the purification of hyaluronidase from cells grown in a 100 L bioreactor culture, columns that are 29 cm high, 30 cm in diameter with 19-21 L resin for use in the purification of hyaluronidase from cells grown in a 300 L bioreactor culture, and, and columns that are 35 cm high, 80 cm in diameter with 176 L resin for use in the purification of hyaluronidase from cells grown in a 2500 L bioreactor culture. One of skill in the art can scale up or down as appropriate. Buffers suitable for equilibrating the Amino Phenyl Boronate column include, for example, buffers containing 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0.

[0171] Following loading of the Phenyl Sepharose column-purified protein onto the Amino Phenyl Boronate column, the column is washed with suitable wash buffers. Exemplary wash buffers include, but are not limited to, 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0, and 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0 and 20 mM bicine, 100 mM NaCl, pH 9.0. In one example, the Amino Phenyl Boronate column with the bound hyaluronidase is washed first with 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0, then with 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0 and finally with 20 mM bicine, 100 mM NaCl, pH 9.0. The bound hyaluronidase can then be eluted, such as with 50 mM Hepes, 100 mM NaCl, pH 7.0. One of skill in the art can modify one or more of the buffers to similarly effect purification. Typically, the eluted soluble rHuPH20 also is filtered to remove any microbial contamination or large aggregates.

4. Hydroxyapatite column

[0172] Following Phenyl Boronate column chromatography, the protein solution containing the soluble rHuPH20 can be loaded onto a Hydroxyapatite column in a final polishing step. Hydroxyapatite is a crystalline form of calcium phosphate with the molecular formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. It can be used as a polishing step to separate closely copurifying proteins, operating by mixed-mode ion exchange due to its inclusion of both positively and negatively charged moieties. Various hydroxyapatite chromatographic media are available commercially, and any available form of the material can be used in the methods herein. Examples of hydroxyapatites include, but are not limited to, those that are agglomerated to form particles and sintered at high temperatures into a stable porous ceramic mass. The particle size can vary, such as ranging from about 1 μm to about 1,000 μm in diameter. The porosity also can also vary, such as from about 100 Å to about 10,000 Å.

[0173] The Hydroxyapatite column used in the methods herein can range in size, depending on the volume and amount of protein being purified through it. Exemplary sizes include columns that are 20 cm high, 30 cm in diameter with 13 L

resin for use in the purification of hyaluronidase from cells grown in a 300 L bioreactor culture, and columns that are 23 cm high, 80 cm in diameter with 116 L resin for use in the purification of hyaluronidase from cells grown in a 2500 L bioreactor culture. One of skill in the art can scale up or down as appropriate.

[0174] For the methods described herein, the Hydroxyapatite column can be equilibrated with 5 mM potassium phosphate, 200 mM NaCl or 5 mM potassium phosphate, 200 mM NaCl, 0.1 mM CaCl_2 , pH 7.0. Equilibration using solutions such as these make the column compatible with the partially-purified hyaluronidase, which itself is supplemented with potassium phosphate and CaCl_2 to final concentrations of 5 mM and 0.1 mM, respectively. Following loading of the protein onto the column, the column can be washed with, for example, 10 mM potassium phosphate, 100 mM NaCl, 0.1 mM CaCl_2 , pH 7.0, to remove any unbound contaminating proteins. The bound soluble rHuPH20 can then be eluted with an appropriate elution buffer. For example, elution can be effected by the addition of 70 mM potassium phosphate, pH 7.0. In some examples, the eluate is filtered, such as through a 0.22 μm filter.

6. Virus removal, protein concentration and buffer exchange

[0175] The soluble rHuPH20 obtained flowing column chromatography can be subjected to post-purification steps that serve to formulate the protein in the desired buffer at the desired concentration. The protein also can be subjected to a viral removal step to ensure it is free from contamination and suitable for use as a therapeutic. Viral removal is typically effected with the use of a filter that allows only the soluble protein to pass through while trapping any viruses (and other contaminants that are equal to in size or larger than viruses). Such filters are available commercially, and any can be used in the methods herein. Pores sizes of filters useful for viral removal include, but are not limited to, 10 nm, 15 nm, 20 nm, 25 nm, 30 nm, 40 nm, 50 nm, 60 nm, 75 nm and 100 nm. In one example, the purified hyaluronidase is filtered through a filter containing 20 nm pores. The protein can be pumped into the filter by, for example, peristaltic pump or by use of a pressure tank.

[0176] Following viral removal, the soluble rHuPH20 can be concentrated and subjected to buffer exchange. The soluble rHuPH20 can be concentrated by 2 $\times$, 3 $\times$, 4 $\times$, 5 $\times$, 6 $\times$, 7 $\times$, 8 $\times$, 9 $\times$, 10 $\times$, 11 $\times$, 12 $\times$, 13 $\times$ or more. In some examples, the protein is concentrated approximately 6 $\times$. This can result in, for example, a concentration of between 0.1 mg/mL and 50 mg/mL. In some examples, the purified hyaluronidase is concentrated to approximately 1 mg/mL. In other examples, the purified hyaluronidase is concentrated to approximately 10 mg/mL. Any method of protein concentration known in the art can be utilized. Exemplary of such methods include concentration using tangential flow filtration (TFF) systems with molecular weight cut off (MWCO) filters. For example, the purified hyaluronidase can be passed through a 10 kDa MWCO spiral polyethersulfone filters to concentrate the protein 10 $\times$. In another example, the protein is passed through a series of four 30 kDa MWCO filters. For large-scale production of hyaluronidase, such as, for example, 100 L and 300 L cultures, filters with surface areas of between 0.5 and 5 square meters are typically employed for this purpose. In some examples, filters with a surface area of 1.2 square meters or 2.8 square meters are used.

[0177] A buffer exchange is generally performed following protein concentration to formulate the protein in the desired buffer for subsequent use, for example, as a therapeutic. One of skill in the art can empirically determine an appropriate buffer. Exemplary of suitable buffers are saline buffers, including, but not limited to, 10 mM Hepes, 130 mM NaCl, pH 7.0, and 10 mM Histidine, 130 mM NaCl, pH 6.0. The purified hyaluronidase can, in some example, be passed through another filter, such as a 0.22 μm capsule filter, before being stored in a sterile environment.

I. Filling

[0178] The methods described herein for the production and purification of soluble rHuPH20 also can include a filling step, in which the purified protein is aseptically filled into smaller containers for long-term storage and use. The soluble rHuPH20 can be filled into the containers as a liquid formulation, or as a powder, such as following lyophilization. For large-scale production, automated filling systems that include, for example, pumps to transfer the protein to the containers and weighing stations to measure the fill volume are typically used and are widely available. Manual or a combination of automated and manual filling of containers also can be performed, however. Suitable containers include, but are not limited to, glass or plastic vials, blister packs, bottles, tubes, inhalers, pumps, bags, syringes, bottles, or any other suitable container. Suitable closures or caps also can be used to seal the container. The filling process can include first passing the soluble rHuPH20 through a filter prior to filling to remove microbial contaminant and larger aggregates or sediment. For example, the protein can be filtered through a 0.22 μm filter before being aliquoted into suitable containers. One of skill in the art can determine the appropriate fill volume and can include, for example, volumes ranging from 0.1 mL to 100 mL. In some examples, vials are aseptically filled with 1 mL, 5 mL or 20 mL soluble rHuPH20. Following capping or closure of the containers, the containers can be stored at an appropriate temperature. In some examples, the containers are flash frozen and stored at between -15°C and -35°C. In other examples, the containers are refrigerated, such as at between 3°C and 15°C. Typically, long-term storage of liquids is at lower temperatures to minimize degradation. Soluble rHuPH20 in powder form can be stored for long periods at room temperature without significant degradation.

J. Monitoring and Assays

[0179] The methods described herein can be monitored at one or more steps, measuring one or more conditions, parameters or products at each point. This can ensure that optimal conditions are maintained throughout, and also can be used to assess efficiency and productivity of the process. Monitoring can occur, for example, one or more times during the cell expansion phase, protein production phase (i.e. in the bioreactor), and/or the protein purification stage, as well as any time between, before or after, such as during concentration/buffer exchange procedures or filling. Monitoring can include, but is not limited to, measuring pH, temperature, volumes, contamination, purity, protein concentration, enzyme activity, cell viability and cell number. In addition to monitoring conditions, parameters or products throughout the process, the purified soluble rHuPH20 produced as the end product also can be assessed and characterized with respect to, for example, protein concentration, enzyme activity, impurities, contamination, osmolarity, degradation, post-translational modifications and monosaccharide content.

1. Monitoring the conditions

[0180] The conditions during one or more of the steps in the methods provided herein can be monitored to ensure optimal conditions are maintained throughout the process. If the monitoring demonstrates that the conditions are not within an optimal range, then the conditions can be altered. Conditions that can be monitored vary for each process. For example, during the cell culture phases (i.e. cell expansion and protein production in the bioreactor), conditions to be monitored include, but are not limited to, temperature, cell culture pH, cell culture nutrients (e.g. glucose), CO₂ levels and O₂ levels. Typically, the conditions are monitored automatically using in-built systems in, for example the incubator or bioreactor.

[0181] During the protein purification stage, conditions that can be monitored include, but are not limited to, pH, conductivity and flow rate. These conditions can be monitored before, during and/or after one or more column chromatography steps. For example, the buffers used to equilibrate, wash or elute the column can be monitored. This can be performed before the buffer is loaded or after the buffer has run through the column.

2. Monitoring soluble rHuPH20 production

[0182] Soluble rHuPH20 production, and parameters associated with soluble rHuPH20 production, also can be monitored throughout the process. These include, but are not limited to, cell number, cell viability, contamination, protein concentration, enzyme activity, purity, osmolarity, post-translational modifications,. Any method to assess these parameters can be used. For example, mammalian cell viability can be assessed by taking a small aliquot of the cell culture and staining with trypan blue, which permeates only damaged cell membranes, thus staining only dead cells. The cells can be visualized under microscope and counted using, for example, a hemocytometer. Other methods include assessing cell viability by measuring metabolic activity. For example, an aliquot of the cell culture can be incubated with a tetrazolium salt (e.g. MTT, XTT or WST-1) that is cleaved into a colored formazan product by metabolically active cells.

[0183] Soluble rHuPH20 concentration in a particular sample can be assessed by methods well-known in the art, including but not limited to, enzyme-linked immunosorbant assays (ELISA); SDS-PAGE; Bradford, Lowry, and/or BCA methods; UV absorbance, and other quantifiable protein labeling methods, such as, but not limited to, immunological, radioactive and fluorescent methods and related methods. Additionally, the presence and extent of degradation can be measured by standard techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting of electrophoresed hyaluronidase-containing samples and chromatography, such as, for example, RP-HPLC. The purity of a hyaluronidase-containing sample can be assessed by, for example, SDS-PAGE, RP-HPLC, size-exclusion chromatography, anion-exchange chromatography and isoelectric focusing (IEF). Soluble rHuPH20-containing samples, such as samples containing purified hyaluronidase, can be further characterized by assessing the sialic acid and monosaccharide content. This can be accomplished by, for example, hydrolyzing the sample with 40% trifluoroacetic acid, fluorescently labeling the released monosaccharides and separating them using RP-HPLC (see Example 10).

[0184] Soluble rHuPH20 produced and purified using the methods provided herein also can be assessed for the presence of post-translational modifications. Such assays are known in the art and include assays to measure glycosylation, hydroxylation, and carboxylation. In an exemplary assay for glycosylation, carbohydrate analysis can be performed, for example, with SDS page analysis of soluble rHuPH20 exposed to hydrazinolysis or endoglycosidase treatment. Hydrazinolysis releases N- and O-linked glycans from glycoproteins by incubation with anhydrous hydrazine, while endoglycosidase release involves PNGase F, which releases most N-glycans from glycoproteins. Hydrazinolysis or endoglycosidase treatment of soluble rHuPH20 polypeptides generates a reducing terminus that can be tagged with a fluorophore or chromophore label. Labeled soluble rHuPH20 polypeptides can be analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE). The fluorescent tag for glycans also can be used for monosaccharide analysis, profiling or fingerprinting of complex glycosylation patterns by HPLC. Exemplary HPLC methods include hydrophilic

interaction chromatography, electronic interaction, ion-exchange, hydrophobic interaction, and size-exclusion chromatography. Exemplary glycan probes include, but are not limited to, 3-(acetylamino)-6-aminoacridine (AA-Ac) and 2-aminobenzoic acid (2-AA). Carbohydrate moieties can also be detected through use of specific antibodies that recognize the glycosylated hyaluronidase polypeptide.

[0185] An exemplary assay to measure β -hydroxylation comprises reverse phase HPLC analysis of soluble rHuPH20 polypeptides that have been subjected to alkaline hydrolysis (Przywiecki et al. (1987) PNAS 84:7856-7860). Carboxylation and γ -carboxylation of hyaluronidase polypeptides can be assessed using mass spectrometry with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis, as described in the art (see, e.g. Harvey et al. J Biol Chem 278:8363-8369, Maum et al. Prot Sci 14:1171-1180).

[0186] The enzymatic activity of soluble rHuPH20 in a sample can be assessed at any point during the methods described herein. In one example, activity is measured using a microturbidity assay (see e.g. Example 10). This is based on the formation of an insoluble precipitate when hyaluronic acid binds with serum albumin. The activity is measured by incubating soluble rHuPH20 with sodium hyaluronate (hyaluronic acid) for a set period of time (e.g. 10 minutes) and then precipitating the undigested sodium hyaluronate with the addition of acidified serum albumin. The turbidity of the resulting sample is measured at 640 nm after an additional development period. The decrease in turbidity resulting from enzyme activity on the sodium hyaluronate substrate is a measure of the soluble rHuPH20 enzymatic activity. In another example, enzymatic activity is measured using a microtiter assay in which residual biotinylated hyaluronic acid is measured following incubation with the sample containing soluble rHuPH20 (see e.g. Frost and Stem (1997) Anal. Biochem. 251:263-269, U.S. Patent Publication No. 20050260186). The free carboxyl groups on the glucuronic acid residues of hyaluronic acid are biotinylated, and the biotinylated hyaluronic acid substrate is covalently couple to a microtiter plate. Following incubation with the sample containing soluble rHuPH20, the residual biotinylated hyaluronic acid substrate is detected using an avidin-peroxidase reaction, and compared to that obtained following reaction with hyaluronidase standards of known activity. Other assays to measure enzymatic activity also are known in the art and can be used in the methods herein (see e.g. Delpech et al., (1995) Anal. Biochem. 229:35-41; Takahashi et al., (2003) Anal. Biochem. 322:257-263).

[0187] The presence of any contamination also can be monitored. Contamination can include, but is not limited to, microbial contamination (e.g. viruses, bacteria and mycoplasma), microbial products contamination (e.g. endotoxin), or other process-related impurities. Any suitable method or assay can be used. For example, viruses and bacteria can be cultured using methods well known in the art to determine whether they are present or not in a sample, and if so, in what quantities. Microscopy also can be used to detect microbial contamination. For example, a sample can be assessed for the presence of viruses or bacteria using transmission electron microscopy (TEM). Mycoplasma detection can be effected using, for example, biochemical or molecular techniques, including, but not limited to, PCR to amplify mycoplasma-specific nucleic acid, biochemical tests to detect mycoplasmal enzymes and cell-based fluorescence to detect mycoplasmal antigens or nucleic acid.

[0188] The presence of microbial products, such as bacterial endotoxins, also can be monitored. An example of a suitable assay for detecting the presence of endotoxin is the Limulus Amebocyte Lysate (LAL) assay. Two types of LAL assays can be used: gel clot and photometric (chromogenic and turbometric). LAL is an aqueous extract of blood cells (amebocytes) from the horseshoe crab. Endotoxin triggers a cascade of enzymatic reactions, which result in activated clotting enzyme. In the presence of bacterial endotoxins, at an elevated temperature, the LAL reagent will clot after addition of reagent. The formation of the gel clot is proportional to the concentration of the endotoxin. In the kinetic assay, the proenzyme in the LAL is activated when in contact with endotoxins produced by gram negative bacteria. The rate of activation is directly proportional with the concentration of the endotoxin present. The level of activation can be measured through a subsequent substrate reaction which is measured spectrophotometrically.

K. EXAMPLES

EXAMPLE 1

Generation of a soluble rHuPH20 -expressing cell line

[0189] The HZ24 plasmid (set forth in SEQ ID NO:50) was used to transfect Chinese Hamster Ovary (CHO cells) (see e.g. related application Nos. 10,795,095, 11/065,716 and 11/238,171). The HZ24 plasmid vector for expression of soluble rHuPH20 contains a pCI vector backbone (Promega), DNA encoding amino acids 1-482 of human PH20 hyaluronidase (SEQ ID NO:47), an internal ribosomal entry site (IRES) from the EMCV virus (Clontech), and the mouse dihydrofolate reductase (DHFR) gene. The pCI vector backbone also includes DNA encoding the Beta-lactamase resistance gene (AmpR), an *ori* origin of replication, a Cytomegalovirus immediate-early enhancer/promoter region (CMV), a chimeric intron, and an SV40 late polyadenylation signal (SV40). The DNA encoding the soluble rHuPH20 construct contains an NheI site and a Kozak consensus sequence prior to the DNA encoding the methionine at amino acid position 1 of the

native 35 amino acid signal sequence of human PH20, and a stop codon following the DNA encoding the tyrosine corresponding to amino acid position 482 of the human PH20 hyaluronidase set forth in SEQ ID NO:1), followed by a BamHI restriction site. The construct pCI-PH20-IRES-DHFR-SV40pa (HZ24), therefore, results in a single mRNA species driven by the CMV promoter that encodes amino acids 1-482 of human PH20 (set forth in SEQ ID NO:3) and amino acids 1-186 of mouse dihydrofolate reductase (set forth in SEQ ID NO:51), separated by the internal ribosomal entry site (IRES).

[0190] Non-transfected DG44 CHO cells growing in GIBCO Modified CD-CHO media for DHFR(-) cells, supplemented with 4 mM Glutamine and 18 ml/L Plurionic F68/L (Gibco), were seeded at 0.5×10^6 cells/ml in a shaker flask in preparation for transfection. Cells were grown at 37° C in 5% CO₂ in a humidified incubator, shaking at 120 rpm. Exponentially growing non-transfected DG44 CHO cells were tested for viability prior to transfection.

[0191] Sixty million viable cells of the non-transfected DG44 CHO cell culture were pelleted and resuspended to a density of 2×10^7 cells in 0.7 mL of 2x transfection buffer (2x HeBS: 40 mM Hepes, pH 7.0, 274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 12 mM dextrose). To each aliquot of resuspended cells, 0.09 mL (250 µg) of the linear HZ24 plasmid (linearized by overnight digestion with Cla I (New England Biolabs) was added, and the cell/DNA solutions were transferred into 0.4 cm gap BTX (Centronics) electroporation cuvettes at room temperature. A negative control electroporation was performed with no plasmid DNA mixed with the cells. The cell/plasmid mixes were electroporated with a capacitor discharge of 330 V and 960 µF or at 350 V and 960 µF.

[0192] The cells were removed from the cuvettes after electroporation and transferred into 5 mL of Modified CD-CHO media for DHFR(-) cells, supplemented with 4 mM Glutamine and 18 ml/L Plurionic F68/L (Gibco), and allowed to grow in a well of a 6-well tissue culture plate without selection for 2 days at 37° C in 5% CO₂ in a humidified incubator.

[0193] Two days post-electroporation, 0.5 mL of tissue culture media was removed from each well and tested for the presence of hyaluronidase activity, using the microturbidity assay described in Example 9.

Table 1: Initial Hyaluronidase Activity of HZ24 Transfected DG44 CHO cells at 40 hours post-transfection

	Dilution	Activity Units/ml
Transfection 1 330V	1 to 10	0.25
Transfection 2 350V	1 to 10	0.52
Negative Control	1 to 10	0.015

[0194] Cells from Transfection 2 (350V) were collected from the tissue culture well, counted and diluted to 1×10^4 to 2×10^4 viable cells per mL. A 0.1 mL aliquot of the cell suspension was transferred to each well of five, 96 well round bottom tissue culture plates. One hundred microliters of CD-CHO media (GIBCO) containing 4 mM GlutaMAX™-I supplement (GIBCO™, Invitrogen Corporation) and without hypoxanthine and thymidine supplements were added to the wells containing cells (final volume 0.2 mL).

[0195] Ten clones were identified from the 5 plates grown without methotrexate.

Table 2. Hyaluronidase activity of identified clones

Plate/Well ID	Relative Hyaluronidase
1C3	261
2C2	261
3D3	261
3E5	243
3C6	174
2G8	103
1B9	304
2D9	273
4D10	302

[0196] Six HZ24 clones were expanded in culture and transferred into shaker flasks as single cell suspensions. Clones 3D3, 3E5, 2G8, 2D9, 1E11, and 4D10 were plated into 96-well round bottom tissue culture plates using a two-dimensional

infinite dilution strategy in which cells were diluted 1:2 down the plate, and 1:3 across the plate, starting at 5000 cells in the top left hand well. Diluted clones were grown in a background of 500 non-transfected DG44 CHO cells per well, to provide necessary growth factors for the initial days in culture. Ten plates were made per subclone, with 5 plates containing 50 nM methotrexate and 5 plates without methotrexate.

[0197] Clone 3D3 produced 24 visual subclones (13 from the no methotrexate treatment, and 11 from the 50 nM methotrexate treatment. Significant hyaluronidase activity was measured in the supernatants from 8 of the 24 subclones (>50 Units/mL), and these 8 subclones were expanded into T-25 tissue culture flasks. Clones isolated from the methotrexate treatment protocol were expanded in the presence of 50 nM methotrexate. Clone 3D35M was further expanded in 500 nM methotrexate giving rise to clones producing in excess of 1,000 Units/ml in shaker flasks (clone 3D35M; or Gen1 3D35M). A master cell bank (MCB) of the 3D35M cells was then prepared.

EXAMPLE 2

Determination of the copy number of the nucleic acid region encoding soluble rHuPH20 in 3D35M cells

[0198] The copy number of the nucleic acid region encoding soluble rHuPH20 in 3D35M cells was determined by quantitative PCR. Total genomic DNA was extracted from 3D35M cells from the MCB. Six independent dilutions of the DNA were prepared for analysis in duplicate, each of which contained approximately 6.6 ng DNA (equivalent to approximately 100 cells). Negative controls containing no template also were prepared, as were positive controls containing the plasmid and the DNA equivalent of 1000 CHO cells (6.6 ng). The reactions were assembled according to the TaqMan Universal PCR Master Mix Protocol (Applied Biosystems) and run in duplicate. A standard curve was generated using eight dilutions of the HZ24 plasmid, representing a range of approximately 5×10^6 to 49 copies of plasmid DNA. The standard was diluted in CHO control genomic DNA (equivalent of 100 cells). The reactions were assembled according to the TaqMan™ Universal PCR Master Mix Protocol (Applied Biosystems) using the HZM3.P 1 forward primer and the HZM3.P2 reverse primer (set forth in SEQ ID NOS:52 and 53, respectively) and the HZM3 probe (SEQ ID NO:54), which contained the fluorescent dyes 6FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethylrhodamine). The reactions were run in duplicate using the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A standard quantitative PCR reaction to assay GAPDH copies for each DNA sample also was performed. Data was collected by the ABI Prism 7700™ Sequence Detection System software version 1.9 (Applied Biosystems).

[0199] The target gene copy numbers per cell were calculated as the ratio of target copies (soluble rHuPH20) to normalized copies (GAPDH) for the six dilutions of 3D35M genomic DNA. The Dixon Q Outlier statistics test was applied to the data set. The copy number of the sHuPH20 region in 3D35M cells was found to be 317.87 ± 11.64 .

EXAMPLE 3 (COMPARATIVE)

Production and Purification of Gent soluble rHuPH20

A. 5 L Bioreactor Process

[0200] A vial of 3D35M was thawed and expanded from T-25 flasks through 1 L spinner flasks in CD CHO (Invitrogen, Carlsbad Calif.) supplemented with 100 nM Methotrexate and 40 mL/L GlutaMAX™-I (Invitrogen; 200 mM stock solution). Cells were transferred from spinner flasks to a 5 L bioreactor (Braun) at an inoculation density of 4×10^5 viable cells per ml in 5 L media. Parameters were temperature Setpoint 37°C, pH 7.2 (starting Setpoint), with Dissolved Oxygen Setpoint 25% and an air overlay of 0-100 cc/min. At 168 hrs, 250 ml of Feed #1 Medium (CD CHO with 50 g/L Glucose) was added. At 216 hours, 250 ml of Feed #2 Medium (CD CHO with 50 g/L Glucose and 10 mM Sodium Butyrate) was added, and at 264 hours 250 ml of Feed #2 Medium was added. This process resulted in a final productivity of 1600 Units per ml with a maximal cell density of 6×10^6 cells/ml. The addition of sodium butyrate was to enhance the production of soluble rHuPH20 in the final stages of production.

[0201] Conditioned media from the 3D35M clone was clarified by depth filtration and tangential flow diafiltration into 10 mM Hepes pH 7.0. Soluble rHuPH20 was then purified by sequential chromatography on Q Sepharose (Pharmacia) ion exchange, Phenyl Sepharose (Pharmacia) hydrophobic interaction chromatography, Amino phenyl boronate (Pro-Medics) and Hydroxyapatite Chromatography (Biorad, Richmond, CA).

[0202] Soluble rHuPH20 bound to Q Sepharose and eluted at 400 mM NaCl in the same buffer. The eluate was diluted with 2M ammonium sulfate to a final concentration of 500 mM ammonium sulfate and passed through a Phenyl Sepharose (low sub) column, followed by binding under the same conditions to a phenyl boronate resin. The soluble rHuPH20 was eluted from the Phenyl Sepharose resin in Hepes pH 6.9 after washing at pH 9.0 in 50 mM bicine without ammonium sulfate. The eluate was loaded onto a ceramic hydroxyapatite resin at pH 6.9 in 5 mM potassium phosphate and 1 mM

CaCl₂ and eluted with 80 mM potassium phosphate, pH 7.4 with 0.1 mM CaCl₂.

[0203] The resultant soluble rHuPH20 possessed a specific activity in excess of 65,000 Units/mg protein by way of the microturbidity assay (see Example 9). Purified soluble rHuPH20 eluted as a single peak from 24 to 26 minutes from a Pharmacia 5RPC styrene divinylbenzene column with a gradient between 0.1% TFA/H₂O and 0.1% TFA/90% acetonitrile/10% H₂O and resolved as a single broad 61 kDa band by SDS electrophoresis that reduced to a sharp 51 kDa band upon treatment with PNGase-F. N-terminal amino acid sequencing revealed that the leader peptide had been efficiently removed.

B. Upstream Cell Culture Expansion Process into 100 L Bioreactor Cell Culture

[0204] A scaled-up process was used to separately purify soluble rHuPH20 from four different vials of 3D35M cell to produce 4 separate batches of sHuPH20; HUA0406C, HUA0410C, HUA0415C and HUA0420C. Each vial was separately expanded and cultured through a 125 L bioreactor, then purified using column chromatography. Samples were taken throughout the process to assess such parameters as enzyme yield. The description of the process provided below sets forth representative specifications for such things as bioreactor starting and feed media volumes, transfer cell densities, and wash and elution volumes. The exact numbers vary slightly with each batch, and are detailed in Tables 3 to 10.

[0205] Four vials of 3D35M cells were thawed in a 37°C water bath, CD CHO containing 100 nM methotrexate and 40 mL/L GlutaMAX™-I was added and the cells were centrifuged. The cells were re-suspended in a 125 mL shake flask with 20 mL of fresh media and placed in a 37°C, 7% CO₂ incubator. The cells were expanded up to 40 mL in the 125 mL shake flask. When the cell density reached $1.5 - 2.5 \times 10^6$ cells/mL, the culture was expanded into a 125 mL spinner flask in a 100 mL culture volume. The flask was incubated at 37°C, 7% CO₂. When the cell density reached $1.5 - 2.5 \times 10^6$ cells/mL, the culture was expanded into a 250 mL spinner flask in 200 mL culture volume, and the flask was incubated at 37°C, 7% CO₂. When the cell density reached $1.5 - 2.5 \times 10^6$ cells/mL, the culture was expanded into a 1 L spinner flask in 800 mL culture volume and incubated at 37°C, 7% CO₂. When the cell density reached $1.5 - 2.5 \times 10^6$ cells/mL, the culture was expanded into a 6 L spinner flask in 5 L culture volume and incubated at 37°C, 7% CO₂. When the cell density reached $1.5 - 2.5 \times 10^6$ cells/mL, the culture was expanded into a 36 L spinner flask in 20 L culture volume and incubated at 37°C, 7% CO₂.

[0206] A 125 L reactor was sterilized with steam at 121°C, 20 PSI and 65 L of CD CHO media was added. Before use, the reactor was checked for contamination. When the cell density in the 36 L spinner flasks reached $1.8 - 2.5 \times 10^6$ cells/mL, 20 L cell culture were transferred from the 36L spinner flasks to the 125 L bioreactor (Braun), resulting a final volume of 85 L and a seeding density of approximately 4×10^5 cells/mL. Parameters were temperature setpoint, 37°C; pH: 7.2; Dissolved oxygen: 25% $\pm$ 10%; Impeller Speed 50 rpm; Vessel Pressure 3 psi; Air Sparge 1 L/min.; Air Overlay: 1 L/min. The reactor was sampled daily for cell counts, pH verification, media analysis, protein production and retention. Nutrient feeds were added during the run. At Day 6, 3.4 L of Feed #1 Medium (CD CHO + 50 g/L Glucose + 40 mL/L GlutaMAX™-I) was added, and culture temperature was changed to 36.5°C. At day 9, 3.5 L of Feed #2 (CD CHO + 50 g/L Glucose + 40 mL/L GlutaMAX™-I + 1.1 g/L Sodium Butyrate) was added, and culture temperature was changed to 36°C. At day 11, 3.7 L of Feed #3 (CD CHO + 50 g/L Glucose + 40 mL/L GlutaMAX™-I + 1.1 g/L Sodium Butyrate) was added, and the culture temperature was changed to 35.5°C. The reactor was harvested at 14 days or when the viability of the cells dropped below 50%. The process resulted in production of soluble rHuPH20 with an enzymatic activity of 1600 Units/ml with a maximal cell density of 8 million cells/mL. At harvest, the culture was sampled for mycoplasma, bioburden, endotoxin, and virus *in vitro* and *in vivo*, transmission electron microscopy (TEM) for viral particles, and enzyme activity.

[0207] The one hundred liter bioreactor cell culture harvest was filtered through a series of disposable capsule filters having a polyethersulfone medium (Sartorius): first through a 8.0 μ m depth capsule, a 0.65 μ m depth capsule, a 0.22 μ m capsule, and finally through a 0.22 μ m Sartopore 2000 cm² filter and into a 100 L sterile storage bag. The culture was concentrated 10 $\times$ using two TFF with Spiral Polyethersulfone 30 kDa MWCO filters (Millipore), followed by a 6 $\times$ buffer exchange with 10 mM HEPES, 25 mM Na₂SO₄, pH 7.0 into a 0.22 μ m final filter into a 20 L sterile storage bag. Table 3 provides monitoring data related to the cell culture, harvest, concentration and buffer exchange steps.

Table 3. Monitoring data for cell culture, harvest, concentration and buffer exchange steps.

Parameter	HUA0406C	HUA04010C	HUA0415C	HUA0420C
Time from thaw to inoculate 100 L bioreactor (days)	21	19	17	18
100 L inoculation density ($\times 10^6$ cells/mL)	0.45	0.33	0.44	0.46
Doubling time in logarithmic growth (hr)	29.8	27.3	29.2	23.5

(continued)

Parameter	HUA0406C	HUA04010C	HUA0415C	HUA0420C
Max. cell density ($\times 10^6$ cells/mL)	5.65	8.70	6.07	9.70
Harvest viability (%)	41	48	41	41
Harvest titer (U/ml)	1964	1670	991	1319
Time in 100-L bioreactor (days)	13	13	12	13
Clarified harvest volume (mL)	81800	93300	91800	89100
Clarified harvest enzyme assay (U/mL)	2385	1768	1039	1425
Concentrate enzyme assay (U/mL)	22954	17091	8561	17785
Buffer exchanged concentrate enzyme assay (U/mL)	15829	11649	9915	8679
Filtered buffer exchanged concentrate enzyme assay (U/mL)	21550	10882	9471	8527
Buffer exchanged concentrate volume (mL)	10699	13578	12727	20500
Ratio enzyme units concentration/harvest	0.87	0.96	1.32	1.4

[0208] A Q Sepharose (Pharmacia) ion exchange column (3 L resin, Height = 20 cm, Diameter = 14 cm) was prepared. Wash samples were collected for a determination of pH, conductivity and endotoxin (LAL) assay. The column was equilibrated with 5 column volumes of 10 mM Tris, 20 mM Na₂SO₄, pH 7.5. The concentrated, diafiltered harvest was loaded onto the Q column at a flow rate of 100 cm/hr. The column was washed with 5 column volumes of 10 mM Tris, 20 mM Na₂SO₄, pH 7.5 and 10 mM Hepes, 50 mM NaCl, pH 7.0. The protein was eluted with 10 mM Hepes, 400 mM NaCl, pH 7.0 and filtered through a 0.22 μ m final filter into a sterile bag.

[0209] Phenyl-Sepharose (Pharmacia) hydrophobic interaction chromatography was next performed. A Phenyl-Sepharose (PS) column (9.1 L resin, Height = 29 cm, Diameter = 20cm) was prepared. The column was equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate, 0.1 mM CaCl₂, pH 7.0. The protein eluate from above was supplemented with 2M ammonium sulfate, 1 M potassium phosphate and 1 M CaCl₂ stock solutions to final concentrations of 5 mM, 0.5 M and 0.1 mM, respectively. The protein was loaded onto the PS column at a flow rate of 100 cm/hr. 5 mM potassium phosphate, 0.5 M ammonium sulfate and 0.1 mM CaCl₂ pH 7.0 was added at 100 cm/hr. The flow through was passed through a 0.22 μ m final filter into a sterile bag.

[0210] The PS-purified protein was loaded onto an aminophenyl boronate column (ProMedics) (6.3 L resin, Height = 20 cm, Diameter = 20cm) that had been equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The protein was passed through the column at a flow rate of 100 cm/hr, and the column was washed with 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0. The column was then washed with 20 mM bicine, 100 mM NaCl, pH 9.0 and the protein eluted with 50 mM Hepes, 100 mM NaCl pH 6.9 through a sterile filter and into a 20 L sterile bag. The eluate was tested for bioburden, protein concentration and enzyme activity.

[0211] A hydroxyapatite (HAP) column (BioRad) (1.6 L resin, Height = 10 cm, Diameter = 14 cm) was equilibrated with 5 mM potassium phosphate, 100 mM NaCl, 0.1 mM CaCl₂ pH 7.0. Wash samples were collected and tested for pH, conductivity and endotoxin (LAL assay). The aminophenyl boronate purified protein was supplemented with potassium phosphate and CaCl₂ to yield final concentrations of 5 mM potassium phosphate and 0.1 mM CaCl₂ and loaded onto the HAP column at a flow rate of 100 cm/hr. The column was washed with 5 mM potassium phosphate pH 7.0, 100 mM NaCl, 0.1 mM CaCl₂, then 10 mM potassium phosphate pH 7.0, 100 mM NaCl, 0.1 mM CaCl₂ pH. The protein was eluted with 70 mM potassium phosphate pH 7.0 and filtered through a 0.22 μ m filter into a 5 L sterile storage bag. The eluate was tested for bioburden, protein concentration and enzyme activity.

[0212] The HAP-purified protein was then pumped through a 20 nm viral removal filter via a pressure tank. The protein was added to the DV20 pressure tank and filter (Pall Corporation), passing through an Ultipor DV20 Filter with 20 nm pores (Pall Corporation) into a sterile 20 L storage bag. The filtrate was tested for protein concentration, enzyme activity, oligosaccharide, monosaccharide and sialic acid profiling, and process-related impurities. The protein in the filtrate was then concentrated to 1 mg/mL using a 10 kD molecular weight cut off (MWCO) Sartocore Slice tangential flow filtration (TFF) system (Sartorius). The filter was first prepared by washing with a Hepes/saline solution (10 mM Hepes, 130 mM

NaCl, pH 7.0) and the permeate was sampled for pH and conductivity. Following concentration, the concentrated protein was sampled and tested for protein concentration and enzyme activity. A 6× buffer exchange was performed on the concentrated protein into the final buffer: 10 mM Hepes, 130 mM NaCl, pH 7.0. The concentrated protein was passed through a 0.22 μm filter into a 20 L sterile storage bag. The protein was sampled and tested for protein concentration, enzyme activity, free sulfhydryl groups, oligosaccharide profiling and osmolarity.

[0213] Tables 4 to 10 provide monitoring data related to each of the purification steps described above, for each 3D35M cell lot.

Table 4. Q sepharose column data

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Load volume (mL)	10647	13524	12852	20418
Load Volume/Resin Volume ratio	3.1	4.9	4.5	7.3
Column Volume (mL)	2770	3840	2850	2880
Eluate volume (mL)	6108	5923	5759	6284
Protein Conc. of Eluate (mg/mL)	2.8	3.05	2.80	2.86
Eluate Enzyme Assay (U/mL)	24493	26683	18321	21052
Enzyme Yield (%)	65	107	87	76

Table 5. Phenyl Sepharose column data

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Volume Before Stock Solution Addition (mL)	5670	5015	5694	6251
Load Volume (mL)	7599	6693	7631	8360
Column Volume (mL)	9106	9420	9340	9420
Load Volume/Resin Volume ratio	0.8	0.71	0.82	0.89
Eluate volume (mL)	16144	18010	16960	17328
Protein Conc. of Eluate (mg/mL)	0.4	0.33	0.33	0.38
Eluate Enzyme Assay (U/mL)	8806	6585	4472	7509
Protein Yield (%)	41	40	36	37
Enzyme Yield (%)	102	88	82	96

Table 6. Amino Phenyl Boronate column data

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Load Volume (mL)	16136	17958	16931	17884
Load Volume/Resin Volume ratio	2.99	3.15	3.08	2.98
Column Volume (mL)	5400	5700	5500	5300
Eluate volume (mL)	17595	22084	20686	19145
Protein Conc. of Eluate (mg/mL)	0.0	0.03	0.03	0.04
Protein Conc. of Filtered Eluate (mg/mL)	not tested	0.03	0.00	0.04
Eluate Enzyme Assay (U/mL)	4050	2410	1523	4721

(continued)

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Protein Yield (%)	0	11	11	12
Enzyme Yield (%)	not determined	41	40	69

Table 7. Hydroxyapatite column data

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Volume Before Stock Solution Addition (mL)	16345	20799	20640	19103
Load Volume/Resin Volume ratio	10.95	13.58	14.19	12.81
Column Volume (mL)	1500	1540	1462	1500
Load volume (mL)	16429	20917	20746	19213
Eluate volume (mL)	4100	2415	1936	2419
Protein Conc. of Eluate (mg/mL)	not tested	0.24	0.17	0.23
Protein Conc. of Filtered Eluate (mg/mL)	NA	NA	0.17	NA
Eluate Enzyme Assay (U/mL)	14051	29089	20424	29826
Protein Yield (%)	Not tested	93		73
Enzyme Yield (%)	87	118	140	104

Table 8. DV20 filtration data

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Start volume (mL)	4077	2233	1917	2419
Filtrate Volume (mL)	4602	3334	2963	3504
Protein Conc. of Filtrate (mg/mL)	0.1	NA	0.09	NA
Protein Conc. of Filtered Eluate (mg/mL)	NA	0.15	0.09	0.16
Protein Yield (%)	not tested	93	82	101

Table 9. Final concentration data

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Start volume (mL)	4575	3298	2963	3492
Concentrate Volume (mL)	562	407	237	316
Protein Conc. of Concentrate (mg/mL)	0.9	1.24	1.16	1.73
Protein Yield (%)	111	102	103	98

Table 10. Buffer Exchange into Final Formulation data

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Start Volume (mL)	562	407	237	316
Final Volume Buffer Exchanged Concentrate (mL)	594	516	310	554

(continued)

Parameter	HUA0406C	HUA0410C	HUA0415C	HUA0420C
Protein Conc. of Concentrate (mg/mL)	1.00	0.97	0.98	1.00
Protein Conc. of Filtered Concentrate (mg/mL)	0.95	0.92	0.95	1.02
Protein Yield (%)	118	99	110	101

[0214] The purified and concentrated soluble rHuPH20 protein was aseptically filled into sterile vials with 5 mL and 1 mL fill volumes. The protein was passed through a 0.22 μ m filter to an operator controlled pump that was used to fill the vials using a gravimetric readout. The vials were closed with stoppers and secured with crimped caps. The closed vials were visually inspected for foreign particles and then labeled. Following labeling, the vials were flash-frozen by submersion in liquid nitrogen for no longer than 1 minute and stored at $\leq 15^{\circ}\text{C}$ ($-20 \pm 5^{\circ}\text{C}$). Production and purification of soluble rHuPH20 using this method yielded approximately 400-700 mg soluble rHuPH20 with a specific activity of 96,000 units/mg to 144,000 units/mg.

EXAMPLE 4

Production of Gen2 soluble rHuPH20

[0215] The Gen1 3D35M cell line described above was adapted to higher methotrexate levels to produce Gen2 clones. 3D35M cells were seeded from established methotrexate-containing cultures into CD CHO medium containing 8 mM GlutaMAXTM-I and 1.0 μ M methotrexate. The cells were adapted to a higher methotrexate level by growing and passaging them 9 times over a period of 46 days in a 37°C, 7% CO₂ humidified incubator. The amplified population of cells was cloned out by limiting dilution in 96-well tissue culture plates containing medium with 2.0 μ M methotrexate. After approximately 4 weeks, clones were identified and clone 3E10B was selected for expansion. 3E10B cells were grown in CD CHO medium containing 8 mM GlutaMAXTM-I and 2.0 μ M methotrexate for 20 passages, with testing for cell viability by trypan blue staining and counting with a hemocytometer, and enzyme activity by the microturbidity assay (described below in Example 9). A master cell bank (MCB) of the 3E10B cell line was created and frozen and used for subsequent studies.

[0216] Amplification of the cell line continued by culturing 3E10B cells in CD CHO medium containing 8 mM GlutaMAXTM-I and 4.0 μ M methotrexate. After the 12th passage, cells were frozen in vials as a research cell bank (RCB). One vial of the RCB was thawed and cultured in medium containing 8.0 μ M methotrexate. After 5 days, the methotrexate concentration in the medium was increased to 16.0 μ M, then 20.0 μ M 18 days later. Cells from the 8th passage in medium containing 20.0 μ M methotrexate were cloned out by limiting dilution in 96-well tissue culture plates containing CD CHO medium containing 4 mM GlutaMAXTM-I and 20.0 μ M methotrexate. Clones 1B3, 2B2 and 5C1 were identified 5-6 weeks later. Cells from the 9th passage of 3D35M also were cloned out by limiting dilution in 96-well tissue culture plates with CD CHO medium containing 8 mM GlutaMAXTM-I and 20.0 μ M methotrexate, and clones 1G11, 2E10 and 2G10 were identified.

[0217] Cells cultures of each of 1B3, 2B2, 5C1, 1G11, 2E10 and 2G10 were seeded at a density of 4×10^5 cells/mL in a volume of 50 mL in 250 mL shaker flasks. The cultures were allowed to grow and decline without additional feeds for 10-14 days to determine the growth rate and productivity of the cells. Samples were taken periodically and assayed for hyaluronidase activity (Tables 11 and 12).

Table 11. Hyaluronidase activity of 1B3, 2B2 and 5C1 cells

Hours post inoculation	Soluble rHuPH20 Enzyme activity in cell culture (units)		
	1B3	2B2	5C1
74			382
95		942	
101			582
142		.2287	
144	955		
169	1200		

(continued)

Hours post inoculation	Soluble rHuPH20 Enzyme activity in cell culture (units)		
	1B3	2B2	5C1
195			
238	1611		
242			2139
265		3070	
336	2252		

Table 12. Hyaluronidase activity of 1B3, 2B2 and 2E10 cells

Hours post inoculation	Soluble rHuPH20 Enzyme activity in cell culture (units)		
	1B3	2B2	2E10
98	470		
123			1179
143		2228	
216			2814
290	2860		
291			2542
337		2992	

[0218] Four cell lines (2B2, 2G10, 1G11 and 2E10) were compared in a study in which all were seeded at a density of 4×10^5 cells/mL in a volume of 50 mL in 250 mL shaker flask. All received 10% (v/v) feeds on day 8 and 5% feeds with feed media containing CD CHO medium supplemented with 50 g/L glucose, 40 g/L yeast extract and 1.1 g/L sodium butyrate. The cells were harvested on day 15. Samples were taken periodically and assayed for soluble rHuPH20 enzymatic activity (Table 13).

Table 13. Hyaluronidase activity of 2E10, 1G11, 2G10 and 2B2 cells

Hours post inoculation	Soluble rHuPH20 Enzyme activity in cell culture (units)			
	2E10	1G11	2G10	2B2
122	991	87	1688	
124				878
194	2151	1387	2430	
196				2642
285	6231	3831	7952	
287				8822
364	5880	2955	11064	
366				15684

[0219] In both the batch and fed-batch conditions, culture of 2B2 cells exhibited higher enzymatic activity, although other cells (e.g. 2G10 cells) also exhibited good enzyme productivity the 2B2 cell line was, therefore, selected for expansion in medium containing 20.0 μ M methotrexate. After the 11th passage, 2B2 cells were frozen in vials as a research cell bank (RCB).

EXAMPLE 5**Enzymatic activity of soluble rHuPH20 produced in 3E10B and 2B2 cells**

[0220] Soluble rHuPH20 produced by 3E10B and 2B2 cells was assayed for enzymatic activity using the Microturbidity assay (Example 9). Frozen vials of 3E10B and 2B2 cells banks were thawed and the cells were cultured separately for two passages in growth medium (CD CHO medium with 8 mM GlutaMAX™-I and either 2.0 μM methotrexate for 3E10B cells, or 20.0 μM methotrexate for 2B2 cells) in 37°C, 6% CO₂ in a humidified incubator. Cells were inoculated into 20 mL growth medium in 125 mL Erlenmeyer flasks (Corning) at 5×10^5 cells/mL, and grown for 8 days in 37°C, 6% CO₂ in a humidified incubator with a shaker platform rotating at approximately 100 rpm. On days 8 and 10, the cultures received 5% v/v of feed medium containing CD CHO medium supplemented with 50 g/L glucose, 50 g/L Yeast extract, and 2.2 g/L (20 mM) sodium butyrate to initiate the production phase. The cultures were sampled during the production phase on day 8 (190 hours), day 10 (240 hours), day 14 (332 hours), day 15 (258 hours), day 16 (382 hours) and day 18 (427 hours), and the viability was allowed to decline to zero. The samples were then analyzed for hyaluronidase activity.

[0221] Tables 14 and 15 set forth the viability (viable cell density (VCD) and percentage viability) and activity (units/flask) of the 3E10B and 2B2 cells at each time point. The enzyme activity of soluble rHuPH20 produced by 2B2 cells was consistently higher than that produced by 3E10B cells. For example, on day 8, the enzyme activity of soluble rHuPH20 produced by 2B2 cells was 69% higher than that of produced by 3E10B cells (2484 units/mL compared to 1469 units/mL). A similar trend was observed throughout the production phase. The viability of the cell cultures declined at a similar rate. When the production rate of the cells was calculated, it was observed that 3E10B cells produced 0.23 picograms soluble rHuPH20 per cell per day (pcd) on day 8 and 0.38 pcd on day 15. In comparison, 2B2 cells produced 0.46 picograms soluble rHuPH20 pcd on day 8 and 0.69 pcd on day 15, which was 100% and 82% higher than production by 3E10B on days 8 and 15, respectively. A master cell bank (MCB) of 2B2 cells was then prepared for subsequent studies.

Table 14. Viability and activity of Clone 3E10B

Hours post inoculation	VCD	% viability	Activity (Units/mL)	Volume (mL)
0	5	99	0	20
190	79.8	96	1469	20
240	61.6	76	2388	20
332	16.8	22	5396	20
358	16.4	17	5628	20
382	8.4	10	6772	20
427	0	0	6476	20
Total Activity (units) per flask (U/mL times volume (mL)): 129520				

Table 15. Viability and activity of Clone 2B2

Hours post inoculation	VCD	% viability	Activity (Units/flask)	Volume (mL)
0	5	99	0	20
190	68	94	2484	20
240	77.6	89	3532	20
332	32	38	8196	20
358	15.8	17	9680	20
382	9.8	13	10788	20
427	0	0	10044	20
Total Activity (units) per flask (U/mL times volume (mL)): 200880				

EXAMPLE 6**Genetic stability testing of 2B2 cells****A. Determination of copy number of the nucleic acid region encoding soluble rHuPH20 in 2B2 cells**

[0222] The copy number of the nucleic acid region encoding soluble rHuPH20 in 2B2 cells was determined by PCR. Total genomic DNA was extracted from 2×10^7 2B2 cells from the MCB using a QIAamp DNeasy kit (Qiagen). Genomic DNA also was extracted from DG-44 CHO cells as a negative control. The purity of the extracted DNA was verified by agarose gel electrophoresis and UV spectrophotometry. To generate fragments of DNA (versus high molecular weight DNA), the genomic DNA was sheared by sonication. This ensured more accurate pipetting and template accessibility. Six independent dilutions of the genomic DNA (to amounts dilutions equivalent to approximately 1000 cells/ μ l) from 2B2 and DG-44 cells were prepared and analyzed in duplicate in two assays; a target assay, which targeted and amplified a sequence specific to the nucleic acid region encoding soluble rHuPH20 plasmid DNA, and an endogenous control, which targeted and amplified GAPDH sequence. The endogenous control was used as a normalizing the results. The target assay included a standard curve generated from a serial dilution of known amounts of the HZ24 plasmid mixed into DG-44 CHO genomic DNA. The endogenous control included a standard curve generated from serial dilutions of DG-44 CHO genomic DNA mixed with HZ24 plasmid DNA. The mammalian genome size was assumed to be 3×10^9 base pairs. Each assay included a negative control (no template) and a positive control (HZ24 plasmid DNA for the target assay and host cell DNA for the endogenous control normalizing assay). The reactions were prepared using the HZM3.PI forward primer and the HZM3.P2 reverse primer (set forth in SEQ ID NOS:52 and 53, respectively) and the HZM3 probe (SEQ ID NO:54), which contained the fluorescent dyes 6FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethylrhodamine). The samples were amplified using the Applied Biosystems Prism[®] 7900 Sequence Detection System with standard cycling conditions (50 °C for 2 minutes, 95°C 10 min, 95 °C 15 seconds and 60°C for 1 min for 40 cycles).

[0223] The target gene copy numbers per cell were calculated as the ratio of target copies to normalized copies (GAPDH) for the six dilutions of 2B2 genomic DNA. The Dixon Q Outlier statistics test was applied to the data set. The copy number of the nucleic acid region encoding soluble rHuPH20 plasmid in 2B2 cells was found to be 206.61 ± 8.35 .

B. mRNA sequence analysis

[0224] The sequence of the PH20 mRNA generated from the HZ24 plasmid in 2B2 cells was determined. RNA was extracted from 2×10^7 2B2 cells from the MCB using a RNeasy Mini Kit (Qiagen). The sample was treated with DNase I to remove contaminating DNA, and the purity of the RNA was verified by agarose gel electrophoresis and UV spectrophotometry. A reverse transcription reaction using SuperScript[™] Reverse Transcriptase (Invitrogen) and a control reaction lacking reverse transcriptase was performed using the extracted RNA and oligo d(t) and random primers. The resulting cDNA products were then used as templates in PCR amplifications. Two different sets of primer pairs were used; AP01/AP03 and AP10/AP12. AP01/AP03 was designed to amplify 1719 base pair region, while primer pair AP10/AP12 was designed to amplify a larger region (1811 base pairs) to obtain the reverse strand sequence of the 3' end. Table 5 sets forth the sequences of the primers. Each PCR reaction included single primer controls, a negative control using the no reverse transcriptase control (described above) as template, and a positive control with control primers and control template. The amplification products were visualized by agarose gel electrophoresis and confirmed to be of the expected size, then purified to remove excess primers and dNTPs by gel extraction or EXOSAP (USB).

[0225] The purified products were sequenced using the BigDye[®] Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and the primers set forth in Table 16. The sequence data were assembled and the derived consensus sequence (SEQ ID NO:55) compared to the reference sequence using Sequencher software version 4.1.2 (Gene Code Corporation). A total of 1449 base pairs of sequence data were generated. The sequence was found to be identical to the reference sequence (SEQ ID NO:47) except for one base pair difference at position 1131, which was observed to be a thymidine (T) instead of the expected cytosine I. This is a silent mutation, with no effect on the amino acid sequence.

Table 16. Primers for PCR amplification and sequencing

Primer name	Sequence	SEQ ID NO.
AP01	TTCTCTCCACAGGTGTC	56
AP02	AAGATTTCTTACAAGAC	57
AP03	TGGCGAGAGGGGAAAGAC	58

(continued)

Primer name	Sequence	SEQ ID NO.
AP04	CCATTTATTTGAACACTC	59
AP06	CCGAACTCGATTGCGCAC	60
AP07	AGCCATTCCCAAATTGTC	61
AP08	CTCCCAGTTCAATTACAG	62
AP09	CGTTAGCTATGGATCCTC	63
AP10	CGAGACAGAGAAGACTCTTGCG	64
AP12	CATTCAACAGACCTTGCATTCC	65

C. Southern blot analysis of 2B2 cells

[0226] A Southern Blot analysis was performed on 2B2 cells to obtain a structure map. Total DNA was extracted from 1×10^7 2B2 cells and 1×10^7 control DG-44 cells using a Maxwell 16® system (Promega). The extracted DNA and a HZ24 plasmid control construct were evaluated for purity by agarose gel electrophoresis. DNA from 2B2 cells, DG-44 cells and the HZ24 plasmid control were digested with Spe I, Xba I, and a double digest using BamH I/Hind III. Another BamH I/Hind digest was performed on the HZ24 plasmid control and the approximately 1.4 kb was purified by gel extraction and radioactively labeled with α -³²P to generate a labeled probe. Approximately 10 µg each of digested 2B2 DNA and DG-44 DNA, and 10 µg DG-44 DNA with 250 pg HZ24 plasmid DNA, was electrophoresed on an agarose gel. An image was taken on the gel following electrophoresis, then a Southern blot transfer was performed. The nylon membrane was hybridized with the labeled probe then washed at room temperature for 30 minutes then twice at 55°C for 30 minutes. An initial autoradiograph was exposed for 24 hours and visually inspected. It was determined that a longer exposure was needed, so a second autoradiograph was exposed for 3 days for a darker exposure of the hybridized bands. After developing the film, the bands were sized using an Alphamager® (Alpha Innotech).

[0227] No hybridizing bands were observed for the DG-44 negative control digest and single hybridizing bands of expected sizes were observed in the HZ24 digests (BamH I/Hind III digest: ~1.4 kb; Spe I digest: ~6.6 kb; Xba I digest: ~6.5 kb). There was one major hybridizing band of ~7.7 kb and four minor hybridizing bands (~13.9, ~6.6, ~5.7 and ~4.6 kb) observed using 2B2 DNA digested with Spe I, one major hybridizing band of ~5.0 kb and two minor hybridizing bands (~13.9 and ~6.5 kb) observed using 2B2 DNA digested with Xba I, and one single hybridizing band of ~1.4 kb observed using 2B2 DNA digested with BamH I/Hind III. The presence of the single ~1.4 kb hybridizing band in the BamH I/Hind III indicated that there were no large sequence insertions or deletions within the probed region. The results from the single Xba I and Spe I digests indicate that there are multiple integration sites of the HZ24 plasmid in the genome of the 2B2 cells.

EXAMPLE 7

Production of Gen2 soluble rHuPH20 in 30 L Bioreactor Cell Culture

[0228] Soluble rHuPH20 was produced and purified from 2B2 cells using a 36 L bioreactor (30 L culture volume) to determine optimal processes for scale-up to a 400 L bioreactor (300 L culture volume). Four separate 36 L bioreactor runs are detailed below in sections A to D.

A. Production and characterization of soluble rHuPH20 Lots 056-099 and 056-100

[0229] A vial of 2B2 (1×10^7 cells) was thawed and cultured at 37°C, 7% CO₂ for 8 passages in CD CHO (Invitrogen, Carlsbad, CA) supplemented with 20 µM methotrexate and 40 mL/L GlutaMAX™-I (Invitrogen), after which it was expanded to 600 mL. One week later, the culture was expanded to 5L in CD CHO medium supplemented with 40 mL/L GlutaMAX™-I and no methotrexate. A 36 L bioreactor containing 25 L CD CHO medium supplemented with 1 L GlutaMAX™-I and 30 mL gentamicin sulfate was inoculated with the 5 L culture at an initial seeding density of 3.6×10^5 cell/mL. The agitation set point of the bioreactor was set to 75 RPM; temperature: 37°C; pH: 7.15; dissolved oxygen: 30%. The bioreactor received filtered air overlay and an air/oxygen/CO₂ sparge, as controlled by an Applikon controller.

[0230] The culture was fed 7 times throughout the bioreactor run, at 161, 184, 237, 256, 280, 304 and 328 hours post inoculation. The feed media were filtered into the bioreactor via peristaltic pump. The content of each feed media and

the bioreactor feed parameters throughout the run are provided in Tables 17 and 18, respectively.

Table 17. Feed Media formulations

Component	Feed #1	Feed #2	Feed #3	Feed #4	Feed #5	Feed #6	Feed #7
CD CHO liquid medium	1 L	1 L	1 L	1 L	1 L	1 L	1 L
GlutaMAX™ -I	120 mL	80 mL	40 mL	40 mL	40 mL	30 mL	30 mL
CD CHO AGT powder	48.6 g	24.3 g	0	0	0	0	0
Yeastolate Ultrafiltrate (200g/L)	150 mL	300 mL	300 mL	150 mL	150 ml	0	0
Sodium butyrate	1.65 g	2.35 g	1.65 g	1.65 g	1.65 g	1.65 g	1.65 g

Table 18. Bioreactor Parameters

Hours	VCD	% viability	pH	Hyaluronidase Units	Vol (L)	Glucose	Feed
0	3.6	97	7.28	0	31	6000	-
15	6.2	94	7.45	117	31	5780	-
44	11.3	97	7.15	290	31	5320	-
88	25.6	97	6.85	517	31	3430	-
115	42.6	95	6.75	1132	31	2920	-
139	56.4	96	6.74	1320	31	2220	-
161	71.9	97	6.82	2296	31	520	Feed #1
184	83.9	96	6.81	2748	32	610	Feed #2
213	82.7	96	6.87	3396	33	1190	-
237	80.5	89	7.21	4450	33	200	Feed #3
256	62.3	71	7.03	4750	34	240	Feed #4
280	52.7	66	7.01	5030	35	600	Feed #5
304	44.6	59	7.00	5970	36	560	Feed #6
328	33.3	47	7.00	7240	37	570	Feed #7
351	26.1	34	7.00	7360	37	250	-

[0231] The bioreactor was harvested and filtered through a system that contained a series of capsule filters (Sartorius) with pore sizes of 8 μm , 0.65 μm , 0.45 μm and 0.22 μm , respectively. The harvest was performed using a peristaltic pump and completed in approximately 5 hours, yielding approximately 32 L of harvested cell culture fluid (HCCF). The HCCF was supplemented with EDTA and Tris to final concentrations of 10 mM each, pH 7.6. The HCCF was then stored at 2-8°C before being concentrated and subjected to a buffer exchange.

[0232] To concentrate the protein, a 2.5 ft² Millipore spiral wound cartridge with a 30 kDa MWCO was first equilibrated in 150 mM NaCl, 10 mM Hepes, 10 mM EDTA, pH 7.5. Fifteen L of HCCF was concentrated 15 $\times$ to 1 L. The concentrate was 10 $\times$ buffer exchanged with the 150 mM NaCl, 10 mM Hepes, 10 mM EDTA, pH 7.5 buffer, and the retentate was filtered through a 0.2 μm capsule into a 2 L storage bag, for a final volume of 1.1 L. The retentate was then stored at 2-8°C.

[0233] The concentrated and buffer exchanged protein solution was then purified using column chromatography through a Q Sepharose column, a phenyl Sepharose column, an Amino Phenyl column and a Hydroxyapatite column. The hyaluronidase units in the protein solution before and after each chromatography step were assessed and used to determine the yield for each step.

[0234] Briefly, a Q sepharose column with a 1.1 L column bed was sanitized with 2.8 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then cleaned with 2.5 L of 10 mM Hepes, 400 mM NaCl, pH 7.0, rinsed in 4.1 L sterile water for injection (SWFI) and equilibrated with 2.5 L of 10 mM Hepes, 25 mM NaCl, pH 7.0. The buffer exchanged protein (1 L at 170,160 units/mL) was loaded onto the column. The flowthrough was 1.0 L at 479 units/mL, indicating that nearly all of the product bound the resin. The column was washed with 4 L of 10 mM Hepes, 25 mM NaCl, pH 7.0, and 4.2 L

of 10 mM Hepes, 50 mM NaCl, pH 7.0. The product was then eluted in 3.0 L of 10 mM Hepes, 400 mM NaCl, pH 7.0, yielding 3 L at 49,940 units/mL, and filtered through a 0.2 μ m filter.

[0235] A Phenyl Sepharose column with a 2.1 L column bed was sanitized with 4.8 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then rinsed with 5.0 L SWFI and cleaned with 4.6 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate and rinsed again with 6.8 L SWFI. The column was then equilibrated in 5.5 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate. To the eluate from the Q Sepharose column, 10.3 mL of 1 M potassium phosphate monobasic, 10.3 mL 1 M potassium phosphate dibasic and 0.42 mL 1 mL CaCl_2 was added. This was then loaded onto the column and the flow through and chase (1 L of 5 mM potassium phosphate, 0.5 mM ammonium sulfate) were collected, yielding 7.4 L at 20,030 units/mL. The product was filtered through a 0.2 μ m filter.

[0236] An Amino Phenyl Boronate column with a 1.8 L column bed was sanitized with 4.5 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then rinsed with 3.9 L SWFI, cleaned with 4.2 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate and rinsed again with 4.0 L SWFI. The column was then equilibrated with 7.5 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The flow through material from the Phenyl Sepharose column was loaded onto the Amino Phenyl Boronate column after being supplemented with ammonium sulfate to a final concentration of 0.5 M. The column was washed with 6.5 L of 5 mM potassium phosphate pH 7.0, then with 7.8 L of 20 mM bicine, pH 9.0 then with 9.0 L of 20 mM bicine, 100 mM NaCl, pH 9.0. The product was eluted with 4.8 L 50 mM Hepes, 100 mM NaCl, pH 7.0, resulting in 4.8 L at 22,940 units/mL, and filtered through a 0.2 μ m filter.

[0237] An Hydroxyapatite column with a 0.8 L column bed was sanitized with 2.7 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. The column was neutralized with 2.1 L of 200 mM potassium phosphate, pH 7.0 then equilibrated in 2.2 L of 5 mM potassium phosphate, 100 mM NaCl. To the eluate from the Amino Phenyl Boronate column, 9.1 mL of 1 M potassium phosphate monobasic, 9.1 mL 1 M potassium phosphate dibasic and 0.452 mL 1 mL CaCl_2 was added. This was then loaded onto the column and the flow through was 4.5 L at 10 units/mL, indicating good binding of the soluble HuPH20. The column was washed with 3.3 L of 5 mM potassium phosphate, 100 mM NaCl, 0.5 M ammonium sulfate, pH 7.0, then with 2.9 L of 10 mM potassium phosphate, 100 mM NaCl, 0.5 M ammonium sulfate, pH 7.0. The product was eluted with 1.0 L of 70 mM potassium phosphate, pH 7.0, resulting in 1 L at 130,000 units/mL, and filtered through a 0.2 μ m filter.

[0238] The purified product was concentrated using a 2.5 ft² Millipore 30 kDa MWCO cartridge that had been equilibrated in 130 mM NaCl, 10 mM Hepes, pH 7.0. The product was concentrated 74 mL, and buffer exchanged 10 $\times$ with the 130 mM NaCl, 10 mM Hepes, pH 7.0 buffer then filtered through a 0.2 μ m filter. A_{280} measurements were performed and indicated that the protein concentration was 11.3 mg/mL. An additional 9.6 mL of 130 mM NaCl, 10 mM Hepes, pH 7.0 buffer was added to bring the protein concentration to 10 mg/mL (Lot 056-99). Ten mL of the 10 mg/mL protein solution was diluted in the buffer to yield a 1 mg/mL solution (Lot 056-100). Both solutions were filtered through a 0.2 μ m filter.

[0239] The formulated product was filled into 10 mL and 1 mL glass vials, the combined total of which yielded 761 mg soluble rHuPH20. The vials were frozen at -80°C then transferred to -20°C for storage. Lot 056-99 and 056-100 were then characterized with respect to activity and purity. Lots 056-99 and 056-100 exhibited 1,350,786 units/mL and 129,982 units/mL enzyme activity, and 130,00 units/mg and 124,00 units/mg specific activity (calculated from enzyme activity and protein concentration). The purity of the soluble rHuPH20 samples was determined by SDS-PAGE, IsoElectric Focusing (IEF), reverse phase high pressure liquid chromatography (RP-HPLC), size-exclusion chromatography (SEC) and anion-exchange chromatography. As determined by RP-HPLC, purity of the two Lots was observed to be approximately 95%. As determined by SEC, purity of the two Lots was observed to be approximately 99%. Endotoxin levels were shown to be ≤ 0.5 EU/mL and 0.1 EU/mL for lots 056-99 and 056-100, respectively. Osmolarity was measured to be 271 mOsm/kg and 291 mOsm/kg for lots 056-99 and 056-100, respectively.

B. Modifications to increase soluble rHuPH20 production: Bioreactor batch 2B2-20K.5

[0240] Modifications were made to the method described above in section A. These modifications were intended to increase the product yield and improve the efficiency and scalability of manufacturing. The manufacturing steps described below include thaw of frozen cells from the research cell bank, expansion of cells in continuous culture, operation of fed-batch bioreactor system, harvest and clarification of cell culture fluid, and concentration and buffer exchange of bulk product. The modifications include, for example, the addition of recombinant human insulin to the bioreactor medium to increase the growth rate and product expression levels of the cells. Also, the number of feeds has been reduced from 7 to 5. Other modifications of the methods described above also were made.

[0241] A vial of 2B2 (1×10^7 cells) was thawed and cultured in CD CHO (Invitrogen, Carlsbad, CA) supplemented with 20 μ M methotrexate and 40 mL/L GlutaMAXTM-I (Invitrogen), after which it was expanded to 100 mL, 450 mL then to 4.5L in CD CHO medium supplemented with 40 mL/L GlutaMAXTM-I and no methotrexate. A 36 L bioreactor containing 20 L CD CHO medium supplemented with 800 L GlutaMAXTM-I, 30 mL gentamicin sulfate and 100 mg recombinant human insulin was inoculated with 3.6 L 2B2 culture at an initial seeding density of 4.3×10^5 cell/mL. The agitation set

point of the bioreactor was set to 80 RPM; temperature: 37°C; pH: 7.15; dissolved oxygen: 25%. The bioreactor received filtered air overlay and an air/oxygen/CO₂ sparge, as controlled by an Applikon controller.

[0242] The culture was fed 5 times throughout the 13 day bioreactor run, at 117, 143, 196, 235, and 283 hours post inoculation. The feed media were filtered into the bioreactor via peristaltic pump. The content of each feed media and the bioreactor feed parameters throughout the run are provided in Tables 19 and 20, respectively.

Table 19. Feed Media formulations

Component	Initial bioreactor medium	Feed #1	Feed #2	Feed #3	Feed #4	Feed #5
CD CHO liquid medium	12 L	0	0	0	0	0
GlutaMAX™ -I	800 mL	120 mL	80 mL	40 mL	40 mL	40 mL
CD CHO AGT powder	194.4 g	97.2 g	48.6 g	24.3 g	24.3 g	24.3 g
SWFI	8L	800 mL	900 mL	700 mL	700 mL	700 mL
Yeastolate Ultrafiltrate (200g/L)	0	0	0	200 mL	200 mL	200 mL
Dextrose	0	30 g	60 g	40 g	40 g	40 g
Gentamicin	30 mL	0	0	0	0	0
rHuInsulin	25 mL	0	0	0	0	0
Sodium butyrate	0	0	0	2.2 g	1.1 g	1.1 g

Table 20. Bioreactor Parameters

Hours	VCD	% viability	pH	Hyaluronidase Units	Vol (L)	Glucose	Feed
0	4.3	98	7.28	0	25	8820	-
55	17.1	99	7.07	580	25	4950	-
94	40.6	99	6.77	1059	25	3800	-
117	57.5	99	6.76	1720	25	2310	Feed #1
143	88.8	99	6.75	3168	26	2770	Feed #2
167	93.7	99	6.80	6982	27	3830	-
196	96.2	97	6.89	4560	27	2060	Feed #3
222	78.9	85	6.83	4920	28	2720	-
235	80	76	6.81	5670	28	1870	Feed #4
260	54.3	65	6.76	5865	29	2930	
283	38.7	44	6.73	6540	29	1880	Feed #5
308	37.3	39	6.78	8460	29	2400	-
313	33.7	34	6.78	8190	29	2300	-

[0243] The bioreactor was harvested and filtered through a system that contained a series of Millipore Pod filters D0HC (0.5 m²) and A1HC stacks, which contain layers of graded-pore-size diatomaceous earth, followed by final filtration through capsule filter (Sartorius Sartobran 300) into a 50 L storage bag. The harvest was performed using a peristaltic pump and completed in approximately 2 hours, yielding approximately 30 L of harvested cell culture fluid (HCCF). Twenty-eight L HCCF was supplemented with EDTA and Tris to final concentrations of 10 mM each, and a pH 7.5. The remaining 2 L HCCF was left without Tris/EDTA, to assess the effect of adding Tris/EDTA on the concentration/buffer exchange step. The HCCF was then stored at 2-8°C before being concentrated and subjected to a buffer exchange.

[0244] To concentrate the protein, a 0.1 m² Millipore Pellicon 2 biomax A screen cassette with a 30 kDa MWCO was

first equilibrated in 20 mM Na₂SO₄, 10 mM Tris, pH 7.5. 2L of HCCF with and without Tris/EDTA was concentrated 10× and buffer exchanged 10× with the 20 mM Na₂SO₄, 10 mM Tris, pH 7.5 buffer. The protein levels were measured by absorbance at A₂₆₀. The remaining HCCF (approximately 26.5 L) was then concentrated and subjected to buffer exchange. Two 0.1 m² Millipore Pellicon 2 biomax A screen cassettes with a 30 kDa MWCO were assembled in the TFF system and equilibrated in 20 mM Na₂SO₄, 10 mM Tris, pH 7.5. The HCCF was concentrated approximately 10× to 2.5 L, and buffer exchanged 10× with 20 mM Na₂SO₄, 10 mM Tris, pH 7.5. The retentate was filtered through a 0.2 μm vacuum filter into 1 L and 500 mL storage bags, for a final volume of 2.6 L. The retentate was then stored at 2-8°C. Samples taken during the concentration and buffer exchange process were analyzed by RP-HPLC to determine the effect of the addition of Tris/EDTA to the sample. It was observed that the addition of Tris/EDTA facilitated a more efficient processing step.

C. Production and characterization of soluble rHuPH20 Lots 056-122 and 056-123 (bioreactor batch 2B2-20K.6).

[0245] Modifications described above in section C were incorporated into the manufacturing steps to produce and purify two lots of soluble rHuPH20; Lots 056-122 and 056-123. The process described below include thaw of frozen cells from the research cell bank HZ24-2B2; expansion of cells in continuous culture; operation of 36 L fed-batch bioreactor system at the 30L scale; cell removal, clarification, and buffer exchange of bulk product; 4-step column chromatography; and formulation, fill, and finish operations.

[0246] A vial of 2B2 (1×10^7 cells) was thawed and cultured at 37°C, 7% CO₂ in CD CHO (Invitrogen, Carlsbad, CA) supplemented with 20 μM methotrexate and 40 mL/L GlutaMAX™-I (Invitrogen), after which it was expanded to 400 mL then 4.4 L in CD CHO medium supplemented with 40 mL/L GlutaMAX™-I and no methotrexate. A 36 L bioreactor (Bellco 1964 series) containing 20 L CD CHO medium supplemented with 800 L GlutaMAX™-I, 100 mg recombinant human insulin and 30 mL gentamicin sulfate was inoculated with the 4 L culture at an initial seeding density of 4.9×10^5 cell/mL. The agitation set point of the bioreactor was set to 80 RPM; temperature: 37°C; pH: 7.15; dissolved oxygen: 25%. The bioreactor received filtered air overlay and an air/oxygen/CO₂ sparge, as controlled by an Applikon ADI 1030 controller.

[0247] The culture was fed 4 times throughout the 13 day bioreactor run, at 127, 163, 208 and hours post inoculation. The feed media were filtered into the bioreactor via peristaltic pump. The temperature setpoint of the bioreactor was reduced from 37°C to 36.5 °C on day 7, to 36.0 °C on day 9 and finally to 35.5 °C on day 11. The content of each feed media and the bioreactor feed parameters throughout the run are provided in Tables 21 and 22, respectively.

Table 21. Feed Media formulations

Component	Initial bioreactor medium	Feed #1	Feed #2	Feed #3	Feed #4
CD CHO AGT powder (Invitrogen; part #: 10743-029; lot # 1366333)	0	97.2 g	48.6 g	24.3 g	24.3 g
CD CHO AGT powder (Invitrogen; part #: 10743-029; lot # 1320613)	267.3 g	0	0	0	0
CD CHO AGT powder (Invitrogen; part #: 12490-017; lot # 1300803)	218.7 g	0	0	0	0
SWFI	20 L	700 mL	700 mL	600 mL	600 mL
GlutaMAX™-I (Invitrogen)	800 mL	160 mL	80 mL	60 mL	40 mL
Yeastolate Ultrafiltrate (Invitrogen; 200g/L)	0	100 mL	200 mL	300 mL	300 mL
Dextrose (D-glucose)	0	40 g	40 g	60 g	40 g
Gentamicin	30 mL	0	0	0	0
rHuInsulin	100 mg	40 mg	0	0	0
Sodium butyrate	0	0	1.1 g	2.2 g	1.1 g

Table 22. Bioreactor Parameters

Hours	Viable cell density ($\times 10^5$ cells/mL)	% viability	pH	Hyaluronidase Units	Vol (L)	Glucose	Feed
0	4.9	92	7.26	79	25	7780	-
24	9.2	95	7.21	141	25	6060	-
48	17.3	97	7.13	243	25	5280	-
72	33	99	6.82	407	25	3910	-
98	49.3	99	6.77	658	25	3200	-
127	67	98	6.83	1296	25	1610	Feed #1
144	88.1	98	6.78	1886	26	2860	-
163	92.4	99	6.89	2439	26	1680	Feed #2
192	91	97	6.85	3140	27	1480	-
208	92.7	96	6.91	3188	27	230	Feed #3
235	70	76	6.86	5118	28	1940	-
261	63	61	6.84	5862	28	280	Feed #4
291	36.4	45	6.76	7072	29	1570	-
307	29.3	32	6.81	8160	29	1250	Harvest

[0248] The bioreactor was harvested and filtered through a system that contained a series of Millipore Pod filters D0HC (0.5 m²) and A1HC stacks (0.1 m²), which contain layers of graded-pore-size diatomaceous earth, followed by final filtration through capsule filter (Sartorius Sartobran 300) into 20 L storage bags. The harvest was performed using a peristaltic pump and completed in approximately 1 hour, yielding approximately 34 L of harvested cell culture fluid (HCCF). This includes the 29 L bioreactor volume plus approximately 5 L PBS chase. The HCCF was supplemented with EDTA and Tris to final concentrations of 10 mM each, and a final pH of 7.5. The HCCF was then stored at 2-8°C before being concentrated and subjected to a buffer exchange.

[0249] To concentrate the protein, a 7.0 ft² Sartorius Sartocn 2 crossflow cassette with a 30 kDa MWCO was first equilibrated in 20 mM Na₂SO₄, 10 mM Tris, pH 7.5. 34 L of HCCF was concentrated 10 $\times$ to 3 L and buffer exchanged 10 $\times$ with the 20 mM Na₂SO₄, 10 mM Tris, pH 7.5 buffer. The retentate was filtered through a 0.2 μ m capsule filter into a 5 L storage bags for a final volume of 3.0 L. The retentate was then stored at 2-8°C.

[0250] The concentrated and buffer exchanged protein solution was then purified using column chromatography through a Q Sepharose column, a phenyl Sepharose column, an Amino Phenyl column and a Hydroxyapatite column. The hyaluronidase units in the protein solution before and after each chromatography step were assessed and used to determine the yield for each step.

[0251] Briefly, a Q sepharose column with a 1.1 L column bed, diameter 7 cm, height 28 cm was sanitized with 2.1 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then cleaned with 2.5 L of 10 mM Hepes, 400 mM NaCl, pH 7.0, rinsed in 4.5 L sterile water for injection (SWFI) and equilibrated with 4.3 L of 10 mM Hepes, 25 mM NaCl, pH 7.0. The buffer exchanged protein (3 L at 94,960 hyaluronidase units/mL) was loaded onto the column. The flowthrough and first wash was 5830 mL at 75 hyaluronidase units/mL, indicating that nearly all of the product (99.8%) bound the resin. The column was washed with 4.2 L of 10 mM Hepes, 25 mM NaCl, pH 7.0, and 4.6 L of 10 mM Hepes, 50 mM NaCl, pH 7.0. The product was then eluted in 2.9 L of 10 mM Hepes, 400 mM NaCl, pH 7.0, yielding 2880 mL at 96,080 units/mL, and filtered through a 0.2 μ m filter.

[0252] A Phenyl Sepharose column with a 2.2 L column bed (height 28 cm, diameter 10 cm) was sanitized with 5.0 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then rinsed with 4.5 L SWFI and cleaned with 4.6 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate and rinsed again with 6.8 L SWFI. The column was then equilibrated in 4.6 L of 5 mM potassium phosphate, 0.5 mM ammonium sulfate. To the eluate from the Q Sepharose column, 9.6 mL of 1 M potassium phosphate monobasic, 9.6 mL 1 M potassium phosphate dibasic and 0.4 mL 1 mL CaCl₂ was added. This was then loaded onto the column and the flow through and chase (5 mM potassium phosphate, 0.5 mM ammonium sulfate) were collected, yielding 6905 mL at 36,280 units/mL. The product was filtered through a 0.2 μ m filter.

[0253] An Amino Phenyl Boronate column with a 2.2 L column bed (height 29 cm, diameter 10 cm) was sanitized with 3.8 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then rinsed with 5.0 L SWFI, cleaned with 5.0 L of 5

mM potassium phosphate, 0.5 M ammonium sulfate and rinsed again with 5.0 L SWFI. The column was then equilibrated with 5.0 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The flow through material from the Phenyl Sepharose column was loaded onto the Amino Phenyl Boronate column. The column was washed with 9.9 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0, then with 9.7 L of 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0 then with 9.9 L of 20 mM bicine, 100 mM NaCl, pH 9.0. The product was eluted with 5.0 L 50 mM Hepes, 100 mM NaCl, pH 7.0, resulting in 4460 mL at 48,400 units/mL, and filtered through a 0.2 µm filter.

[0254] A Hydroxyapatite column with a 1.1 L column bed (diameter 7 cm, height 28 cm) was sanitized with 2.7 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. The column was neutralized with 2.1 L of 200 mM potassium phosphate, pH 7.0, then equilibrated in 2.2 L of 5 mM potassium phosphate, 100 mM NaCl. To the eluate from the Amino Phenyl Boronate column, 11.2 mL of 1 M potassium phosphate monobasic, 11.2 mL 1 M potassium phosphate dibasic and 0.45 mL 1 mL CaCl₂ was added. This was then loaded onto the column and subsequently washed with 3.5 L of 5 mM potassium phosphate, 100 mM NaCl, 0.5 M ammonium sulfate, pH 7.0, then with 3.5 L of 10 mM potassium phosphate, 100 mM NaCl, 0.5 M ammonium sulfate, pH 7.0. The product was eluted with 1.4 L of 70 mM potassium phosphate, pH 7.0, resulting in 1260 mL at 152,560 units/mL, and filtered through a 0.2 µm filter.

[0255] The purified product was concentrated using a 0.05 ft² Millipore 30 kDa MWCO cartridge that had been equilibrated in 130 mM NaCl, 10 mM Hepes, pH 7.0. The product was concentrated from 1250 mL at 1.04/mg/mL to 120 mL and buffer exchanged 10× with the 130 mM NaCl, 10 mM Hepes, pH 7.0 buffer then filtered through a 0.2 µm filter. A₂₈₀ measurements were performed and indicated that the soluble rHuPH20 concentration of the remaining 118 ml was 11.45 mg/mL. An additional 17 mL of 130 mM NaCl, 10 mM Hepes, pH 7.0 buffer was added to bring the protein concentration to 10 mg/mL (Lot 056-122). Ten mL of the 10 mg/mL protein solution was diluted in the buffer to yield a 1 mg/mL solution (Lot 056-123). Both solutions were filtered through a 0.2 µm filter.

[0256] The formulated product was filled into 10 mL and 1 mL glass vials, the combined total of which yielded 1308 mg soluble rHuPH20. The vials were frozen at -80°C then transferred to -20°C for storage. Lot 056-122 and 056-123 were then characterized with respect to activity and purity. Lots 056-122 and 056-123 exhibited 1,376,992 units/mL and 129,412 units/mL enzyme activity, and 136,900 units/mg and 124,400 units/mg specific activity (calculated from enzyme activity and protein concentration). The purity of the soluble rHuPH20 samples was determined by SDS-PAGE, IsoElectric Focusing (IEF), reverse phase high pressure liquid chromatography (RP-HPLC), size-exclusion chromatography (SEC) and anion-exchange chromatography. As determined by RP-HPLC, purity of the two Lots was observed to be approximately 96.2%. As determined by SEC, purity of the two Lots was observed to be greater than 99%. Endotoxin levels were shown to be ≤0.8 EU/mL and 0.09 EU/mL for lots 056-122 and 056-123, respectively. Osmolarity was measured to be 265 mOsm/kg and 256 mOsm/kg for lots 056-122 and 056-123, respectively. The pH of each was 7.2.

D. Reproducibility of the production process of Gen 2 soluble rHuPH20 in 30 L Bioreactor Cell Culture

[0257] The process described above for Batch 2B2-20K.6 was used for a subsequent batch to demonstrate the reproducibility of the process. The process was modified slightly by the incorporation of a viral inactivation step immediately prior to the column chromatography steps.

[0258] A vial of 2B2 cells (1×10^7 cells) was thawed and cultured at 37°C, 7% CO₂ for 7. passages in CD CHO (Invitrogen, Carlsbad, CA) supplemented with 20 µM methotrexate and 40 mL/L GlutaMAX™-I (Invitrogen), after which it was expanded to 400 mL then 4.4 L in CD CHO medium supplemented with 40 mL/L GlutaMAX™-I and no methotrexate. A 36 L bioreactor (Bellco 1964 series) containing 20 L CD CHO medium supplemented with 800 mL GlutaMAX™-I, 100 mg recombinant human insulin and 300 mg gentamicin sulfate was inoculated with 3 L culture at an initial seeding density of 4.7×10^5 cell/mL. The agitation set point of the bioreactor was set to 80 RPM; temperature: 37°C; pH: 7.15; dissolved oxygen: 25%. The bioreactor received filtered air overlay and an air/oxygen/CO₂ sparge, as controlled by an Applikon ADI 1030 controller.

[0259] The culture was fed 4 times throughout the 13 day bioreactor run, at 127, 163, 208 and hours post inoculation. The feed media were filtered into the bioreactor via peristaltic pump. The temperature setpoint of the bioreactor was reduced from 37°C to 36.5 °C on day 7, to 36.0 °C on day 9 and finally to 35.5 °C on day 11. The content of each feed media and the bioreactor feed parameters throughout the run are provided in Tables 23 and 24, respectively.

Table 23. Feed Media formulations

Component	Initial bioreactor medium	Feed #1	Feed #2	Feed #3	Feed #4
CD CHO liquid medium (Invitrogen)	20 L	0	0	0	0
CD CHO AGT powder	0g	97.2 g	48.6 g	24.3 g	24.3 g
SWFI	0	700 mL	700 mL	600 mL	600 mL

(continued)

Component	Initial bioreactor medium	Feed #1	Feed #2	Feed #3	Feed #4
GlutaMAX™-I (Invitrogen)	800 mL	160 mL	80 mL	60 mL	40 mL
Yeastolate Ultrafiltrate (Invitrogen; 200g/L)	0	100 mL	200 mL	300 mL	300 mL
Dextrose (D-glucose)	0	40 g	40 g	60 g	50 g
Gentamicin	300 mg	0	0	0	0
rHulnsulin	100 mg	40 mg	0	0	0
Sodium butyrate	0	0	1.1 g	2.2 g	1.1 g

Table 24. Bioreactor Parameters

Hours	Viable cell density ($\times 10^5$ cells/mL)	% viability	pH	Hyaluronidase Units	Vol (L)	Glucose	Feed
0	4.7	98	7.28	113	24	8200	-
24	8.9	98	7.23	202	24	6160	-
50	19.3	97	7.15	332	24	5480	-
76	36.7	99	6.85	680	24	3620	-
120	73.6	99	6.76	1619	24	2100	Feed #1
145	84.3	99	6.75	2842	25	2660	-
165	98.8	99	6.87	3756	25	840	Feed #2
190	95.3	99	6.85	4773	26	1330	-
201	105	97	6.90	5484	26	270	Feed #3
214	95.9	93	6.82	6344	27	2590	-
242	81.2	81	6.75	7890	27	1350	Feed #4
268	51.9	48	6.65	10398	28	2500	-
287	38.4	41	6.70	11864	28	2170	-
308	31.6	31	6.66	12864	28	1850	Harvest

[0260] The bioreactor was harvested and filtered through a system that contained a series of Millipore Pod filters D0HC (0.5 m²) and A1HC stacks (0.1 m²), which - contain layers of graded-pore-size diatomaceous earth, followed by final filtration through capsule filter (Sartorius Sartobran 300) into 20 L storage bags. The harvest was performed using a peristaltic pump and completed in approximately 75 minutes, yielding approximately 30 L of harvested cell culture fluid (HCCF). This includes the 28 L bioreactor volume plus approximately 2 L PBS chase. The HCCF was supplemented with EDTA and Tris to final concentrations of 10 mM each, and a final pH of 7.5. The HCCF was then stored at 2-8°C before being concentrated and subjected to a buffer exchange.

[0261] To concentrate the protein, a Sartorius Slice system with 3 $\times$ 1.0 ft² Sartocon Slice crossflow cassettes (30 kDa MWCO) was first equilibrated in 20 mM Na₂SO₄, 10 mM Tris, pH 7.5. Thirty liters of HCCF was concentrated 10 $\times$ to 3 L and buffer exchanged 10 $\times$ with the 20 mM Na₂SO₄, 10 mM Tris, pH 7.5 buffer. The average flux rate during the concentration was 115 mL/minute and the average transmembrane pressure was 16 psig. The average flux rate during the diafiltration was 150 mL/minute and the average transmembrane pressure was 15 psig. The retentate was filtered through a 0.2 μ m vacuum filter systems into a 5 L storage bags for a final volume of 3.0 L. The retentate was then stored at 2-8°C.

[0262] Viral inactivation was performed by mixing 235 mL of a filtered solution of 10% w/w Triton X-100, 35 w/w Tributyl phosphate in SWFI with 2.15 L of roomtemperature concentrated and buffer exchanged protein in a glass spinner

flask stirring at 30–40 rpm. After 45 minutes, the protein solution was loaded onto the Q sepharose column (as described below). The loading took 24 minutes, which resulted in a total exposure time to the detergent solution of 69 minutes.

[0263] The Q sepharose column with a 1.1 L column bed, diameter 7 cm, height 28 cm was sanitized with 2.1 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then cleaned with 2.5 L of 10 mM Hepes, 400 mM NaCl, pH 7.0, rinsed in 4.5 L sterile water for injection (SWFI) and equilibrated with 4.5 L of 10 mM Hepes, 25 mM NaCl, pH 7.0. The buffer exchanged, viral inactivated protein (2385 mL at 133,040 hyaluronidase units/mL) was loaded onto the column. The column was washed with 4.5 L of 10 mM Hepes, 25 mM NaCl, pH 7.0, and 4.5 L of 10 mM Hepes, 50 mM NaCl, pH 7.0. The product was then eluted in 2.5 L of 10 mM Hepes, 400 mM NaCl, pH 7.0, yielding 2500 mL at 133,680 units/mL, and filtered through a 0.2 μ m filter.

[0264] A Phenyl Sepharose column with a 2.2 L column bed (height 28 cm, diameter 10 cm) was sanitized with 5.0 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then rinsed with 6.0 L SWFI and equilibrated in 4.6 L of 5 mM potassium phosphate, 0.5 mM ammonium sulfate. To elute the column, 9.6 mL of 1 M potassium phosphate monobasic, 9.6 mL 1 M potassium phosphate dibasic and 0.4 mL 1 M CaCl_2 was added. This was then loaded onto the column and the flow through and chase (5 mM potassium phosphate, 0.5 mM ammonium sulfate) were collected, yielding 6450 mL at 43,840 units/mL. The product was filtered through a 0.2 μ m filter.

[0265] An Amino Phenyl Boronate column with a 2.2 L column bed (height 29 cm, diameter 10 cm) was sanitized with 3.5 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. It was then rinsed with 5.0 L SWFI and equilibrated with 9.0 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The flow through material from the Phenyl Sepharose column was loaded onto the Amino Phenyl Boronate column. The column was washed with 9.9 L of 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0, then with 9.9 L of 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0. The product was eluted with 4.4 L 50 mM Hepes, 100 mM NaCl, pH 7.0, yielding 4389 mL at 33,840 units/mL, and filtered through a 0.2 μ m filter.

[0266] A Hydroxyapatite column with a 1.1 L column bed (diameter 7 cm, height 28 cm) was sanitized with 2.1 L 1.0 N NaOH and stored in 0.1 N NaOH prior to use. The column was neutralized with 3.6 L of 200 mM potassium phosphate, pH 7.0, then equilibrated in 3.2 L of 5 mM potassium phosphate, 100 mM NaCl. To the eluate from the Amino Phenyl Boronate column, 11 mL of 1 M potassium phosphate monobasic, 11 mL 1 M potassium phosphate dibasic and 0.44 mL 1 M CaCl_2 was added. This was then loaded onto the column and subsequently washed with 4.8 L of 5 mM potassium phosphate, 100 mM NaCl, 0.5 M ammonium sulfate, pH 7.0, then with 3.8 L of 10 mM potassium phosphate, 100 mM NaCl, 0.5 M ammonium sulfate, pH 7.0. The product was eluted with 1.5 L of 70 mM potassium phosphate, pH 7.0, resulting in 1500 mL at 114,320 units/mL, and filtered through a 0.2 μ m filter.

[0267] The purified product was concentrated using a 0.05 ft² Millipore 30 kDa MWCO cartridge that had been equilibrated in 130 mM NaCl, 10 mM Hepes, pH 7.0. The product was concentrated from 1500 mL at 0.961 mg/mL to 125 mL and buffer exchanged 10 $\times$ with the 130 mM NaCl, 10 mM Hepes, pH 7.0 buffer then filtered through a 0.2 μ m filter. A_{280} measurements were performed and indicated that the protein concentration of the remaining 122 mL was 11.431 mg/mL. An additional 17.5 mL of 130 mM NaCl, 10 mM Hepes, pH 7.0 buffer was added to bring the protein concentration to 10 mg/mL (Lot 056-135). Ten mL of the 10 mg/mL protein solution was diluted in the buffer to yield a 1 mg/mL solution (Lot 056-136). Both solutions were filtered through a 0.2 μ m filter.

[0268] The formulated product was filled into 5 mL and 1 mL glass vials, the combined total of which yielded 1324 mg soluble rHuPH20. The vials were frozen at -80°C then transferred to -20°C for storage. Lot 056-135 and 056-136 were then characterized with respect to activity and purity. Lots 056-135 and 056-136 exhibited 1,301,010 units/mL and 127,661 units/mL enzyme activity, and 121,600 units/mg and 127,700 units/mg specific activity (calculated from enzyme activity and protein concentration). The purity of the soluble rHuPH20 samples was determined by SDS-PAGE, IsoElectric Focusing (IEF), reverse phase high pressure liquid chromatography (RP-HPLC), size-exclusion chromatography (SEC) and anion-exchange chromatography. As determined by RP-HPLC, purity of the two Lots was observed to be between 93.5% and 93.7%. As determined by SEC, purity of the two Lots was observed to be greater than 99%. Endotoxin levels were shown to be $\leq$ 0.84 EU/mL and 0.09 EU/mL for lots 056-135 and 056-136, respectively. Osmolarity was measured to be 255 mOsm/kg and 260 mOsm/kg for lots 056-135 and 056-136, respectively. The pH of each was 7.2.

EXAMPLE 8

A. Production of Gen2 soluble rHuPH20 in 300 L Bioreactor Cell Culture

[0269] The production and purification methods detailed in Example 7, above, were scaled-up for production using a 400 L bioreactor. A vial of 2B2 cells (1×10^7 cells) was thawed and expanded from shaker flasks through 36L spinner flasks in CD CHO (Invitrogen, Carlsbad, CA) supplemented with 20 μ M methotrexate and 8 mM GlutaMAX™-I (Invitrogen). Briefly, the a vial of cells was thawed in a 37°C water bath, media was added and the cells were centrifuged. The cells were re-suspended in a 125 mL shake flask with 20 mL of fresh media and placed in a 37°C, 7% CO₂ incubator. The cells were expanded up to 40 mL in the 125 mL shake flask. When the cell density reached greater than 1.5×10^6

cells/mL, the culture was expanded into a 125 mL spinner flask in a 100 mL culture volume. The flask was incubated at 37°C, 7% CO₂. When the cell density reached greater than 1.5×10^6 cells/mL, the culture was expanded into a 250 mL spinner flask in 200 mL culture volume, and the flask was incubated at 37°C, 7% CO₂. When the cell density reached greater than 1.5×10^6 cells/mL, the culture was expanded into a 1 L spinner flask in 800 mL culture volume and incubated at 37°C, 7% CO₂. When the cell density reached greater than 1.5×10^6 cells/mL the culture was expanded into a 6 L spinner flask in 5000 mL culture volume and incubated at 37°C, 7% CO₂. When the cell density reached greater than 1.5×10^6 cells/mL the culture was expanded into a 36 L spinner flask in 32 L culture volume and incubated at 37°C, 7% CO₂.

[0270] A 400 L reactor was sterilized by steam at 121°C for 30 minutes and 230 mL of CD CHO media supplemented with 8 mM GlutaMAX™-I and 5mg/L rHuInsulin was added. Before use, the reactor was checked for contamination. Approximately 30 L cells were transferred from the 36L spinner flasks to the 400 L bioreactor (Braun) at an inoculation density of 4.0×10^5 viable cells per ml and a total volume of 260L. Parameters were temperature setpoint, 37°C; Impeller Speed 40-55 RPM; Vessel Pressure: 3 psi; Air Sparge 0.5- 1.5 L/Min.; Air Overlay: 3 L/ min. The reactor was sampled daily for cell counts, pH verification, media analysis, protein production and retention. Also, during the run nutrient feeds were added. At 120 hrs (day 5), 10.4L of Feed #1 Medium (4× CD CHO + 33 g/L Glucose + 160 mL/L GlutaMAX™-I + 16.6 g/L Yeastolate + 33 mg/L rHuInsulin) was added. At 168 hours (day 7), 10.8 L of Feed #2 (2× CD CHO + 33 g/L Glucose + 80 mL/L GlutaMAX™-I + 33.4 g/L Yeastolate + 0.92 g/L Sodium Butyrate) was added, and culture temperature was changed to 36.5°C. At 216 hours (day 9), 10.8 L of Feed #3 (1 × CD CHO + 50 g/L Glucose + 50 mL/L GlutaMAX™-I + 50 g/L Yeastolate + 1.80 g/L Sodium Butyrate) was added, and culture temperature was changed to 36° C. At 264 hours (day 11), 10.8 L of Feed #4 (1 × CD CHO + 33 g/L Glucose + 33 mL/L GlutaMAX™-I + 50 g/L Yeastolate + 0.92 g/L Sodium Butyrate) was added, and culture temperature was changed to 35.5° C. The addition of the feed media was observed to dramatically enhance the production of soluble rHuPH20 in the final stages of production. The reactor was harvested at 14 days or when the viability of the cells dropped below 40%. The process resulted in a final productivity of 17,000 Units per ml with a maximal cell density of 12 million cells/mL. At harvest, the culture was sampled for mycoplasma, bioburden, endotoxin and viral *in vitro* and *in vivo*, TEM for viral particles and enzyme activity.

[0271] The culture was pumped by a peristaltic pump through four Millistak filtration system modules (Millipore) in parallel, each containing a layer of diatomaceous earth graded to 4-8 μm and a layer of diatomaceous earth graded to 1.4-1.1 μm, followed by a cellulose membrane, then through a second single Millistak filtration system (Millipore) containing a layer of diatomaceous earth graded to 0.4-0.11 μm and a layer of diatomaceous earth graded to <0.1 μm, followed by a cellulose membrane, and then through a 0.22 μm final filter into a sterile single use flexible bag with a 350 L capacity. The harvested cell culture fluid was supplemented with 10 mM EDTA and 10 mM Tris to a pH of 7.5. The culture was concentrated 10× with a model tangential flow filtration (TFF) apparatus using four Sartoslice TFF 30 kDa molecular weight cut-off (MWCO) polyether sulfone (PES) filters (Sartorius), followed by a 10× buffer exchange with 10 mM Tris, 20 mM Na₂SO₄, pH 7.5 into a 0.22 μm final filter into a 50 L sterile storage bag.

[0272] The concentrated, diafiltered harvest was inactivated for virus. Prior to viral inactivation, a solution of 10% Triton X-100, 3% tri (n-butyl) phosphate (TNBP) was prepared. The concentrated, diafiltered harvest was exposed to 1% Triton X-100, 0.3% TNBP for 1 hour in a 36 L glass reaction vessel immediately prior to purification on the Q column.

B. Purification of Gen2 sHuPH20

[0273] A Q Sepharose (Pharmacia) ion exchange column (9 L resin, H= 29 cm, D= 20 cm) was prepared. Wash samples were collected for a determination of pH, conductivity and endotoxin (LAL) assay. The column was equilibrated with 5 column volumes of 10 mM Tris, 20 mM Na₂SO₄, pH 7.5. Following viral inactivation, the concentrated, diafiltered harvest was loaded onto the Q column at a flow rate of 100 cm/hr. The column was washed with 5 column volumes of 10 mM Tris, 20 mM Na₂SO₄, pH 7.5 and 10 mM Hepes, 50 mM NaCl, pH 7.0. The protein was eluted with 10 mM Hepes, 400 mM NaCl, pH 7.0 into a 0.22 μm final filter into sterile bag. The eluate sample was tested for bioburden, protein concentration and enzyme activity. A₂₈₀ absorbance reading were taken at the beginning and end of the exchange.

[0274] Phenyl-Sepharose (Pharmacia) hydrophobic interaction chromatography was next performed. A Phenyl-Sepharose (PS) column (19-21 L resin, H=29 cm, D= 30 cm) was prepared. The wash was collected and sampled for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate, 0.1 mM CaCl₂, pH 7.0. The protein eluate from above was supplemented with 2M ammonium sulfate, 1 M potassium phosphate and 1 M CaCl₂ stock solutions to yield final concentrations of 5 mM, 0.5 M and 0.1 mM, respectively. The protein was loaded onto the PS column at a flow rate of 100 cm/hr. 5 mM potassium phosphate, 0.5 M ammonium sulfate and 0.1 mM CaCl₂ pH 7.0 was added at 100 cm/hr. The flow through was passed through a 0.22 μm final filter into a sterile bag. The flow through was sampled for bioburden, protein concentration and enzyme activity.

[0275] An aminophenyl boronate column (ProMedics; 21 L resin, H=29 cm, D= 30 cm) was prepared. The wash was collected and sampled for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 column

volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The PS purified protein was loaded onto the aminophenyl boronate column at a flow rate of 100 cm/hr. The column was washed with 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0. The column was washed with 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0. The column was washed with 20 mM bicine, 100 mM sodium chloride, pH 9.0. The protein was eluted with 50 mM Hepes, 100 mM NaCl, pH 6.9 and passed through a sterile filter into a sterile bag. The eluted sample was tested for bioburden, protein concentration and enzyme activity.

[0276] The hydroxyapatite (HAP) column (BioRad; 13 L resin, H=20 cm, D= 30 cm) was prepared. The wash was collected and test for pH, conductivity and endotoxin (LAL assay). The column was equilibrated with 5 mM potassium phosphate, 100 mM NaCl, 0.1 mM CaCl₂, pH 7.0. The aminophenyl boronate purified protein was supplemented to final concentrations of 5 mM potassium phosphate and 0.1 mM CaCl₂ and loaded onto the HAP column at a flow rate of 100 cm/hr. The column was washed with 5 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM CaCl₂. The column was next washed with 10 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM CaCl₂. The protein was eluted with 70 mM potassium phosphate, pH 7.0 and passed through a 0.22 μm sterile filter into a sterile bag. The eluted sample was tested for bioburden, protein concentration and enzyme activity.

[0277] The HAP purified protein was then passed through a viral removal filter. The sterilized Virosart filter (Sartorius) was first prepared by washing with 2 L of 70 mM potassium phosphate, pH 7.0. Before use, the filtered buffer was sampled for pH and conductivity. The HAP purified protein was pumped via a peristaltic pump through the 20 nM viral removal filter. The filtered protein in 70 mM potassium phosphate, pH 7.0 was passed through a 0.22 μm final filter into a sterile bag. The viral filtered sample was tested for protein concentration, enzyme activity, oligosaccharide, monosaccharide and sialic acid profiling (as described in Examples 9 to 10, below).

[0278] The protein in the filtrate was then concentrated to 10 mg/mL using a 10 kD molecular weight cut off (MWCO) Sartocoon Slice tangential flow filtration (TFF) system (Sartorius). The filter was first prepared by washing with 10 mM histidine, 130 mM NaCl, pH 6.0 and the permeate was sampled for pH and conductivity. Following concentration, the concentrated protein was sampled and tested for protein concentration and enzyme activity. A 6x buffer exchange was performed on the concentrated protein into the final buffer: 10 mM histidine, 130 mM NaCl, pH 6.0. Following buffer exchange, the concentrated protein was passed through a 0.22 μm filter into a 20 L sterile storage bag. The protein was sampled and tested for protein concentration, enzyme activity, free sulfhydryl groups, oligosaccharide profiling and osmolality (as described in Examples 9 to 10, below).

[0279] The sterile filtered bulk protein was then aseptically dispensed at 20 mL into 30 mL sterile Teflon vials (Nalgene). The vials were then flash frozen and stored at - 20 ± 5°C. Production and purification of soluble rHuPH20 using this method yielded approximately 11 and 15 grams, with specific activity of 95,000 units/mg to 120,000 units/mg.

C. Comparison of production and purification of Gen1 and Gen2 sHuPH20

[0280] The production and purification of Gen2 soluble rHuPH20 in a 300L bioreactor cell culture contained some changes in the protocols compared to the production and purification Gen1 soluble rHuPH20 in a 100L bioreactor cell culture (described in Example 4). Table 25 sets forth exemplary differences, in addition to simple scale up changes, between the methods.

Table 25. Exemplary differences between Gen1 and Gen2 soluble rHuPH20 production and purification using the 100L and 300L bioreactor cell culture methods

Process Difference	Gen1 soluble rHuPH20	Gen2 soluble rHuPH20
Cell line	3D35M	2B2
Media used to expand cell	Contains 0.10 μM	Contains 20 μM
inoculum	methotrexate (0.045 mg/L)	methotrexate (9 mg/L)
Media in 6L cultures onwards	Contains 0.10 μM methotrexate	Contains no methotrexate
36 L spinner flask	No instrumentation 20 L operating volume.	Equipped with instrumentation that monitors and controls pH, dissolved oxygen, sparge and overlay gas flow rate. 32 L operating volume
Final operating volume in bioreactor	Approx. 100 L in a 125 L bioreactor (initial culture volume + 65 L)	Approx. 300L in a 400L bioreactor (initial culture volume + 260L)

(continued)

Process Difference	Gen1 soluble rHuPH20	Gen2 soluble rHuPH20
Culture media in final bioreactor	No rHuInsulin	5.0 mg/L rHuInsulin
Media feed volume	Scaled at 4% of the bioreactor cell culture volume i.e. 3.4, 3.5 and 3.7 L, resulting in a target bioreactor volume of ~92 L.	Scaled at 4% of the bioreactor cell culture volume i.e. 10.4, 10.8, 11.2 and 11.7 L, resulting in a target bioreactor volume of ~303L.
Media feed	Feed #1 Medium: CD CHO + 50 g/L Glucose + 8 mM GlutaMAX™-I Feed #2 (CD CHO + 50 g/L Glucose + 8 mM GlutaMAX™-I + 1.1 g/L Sodium Butyrate Feed #3: CD CHO + 50 g/L Glucose + 8 mM GlutaMAX™-I + 1.1 g/L Sodium Butyrate	Feed #1 Medium: 4× CD CHO + 33 g/L Glucose + 32 mM GlutaMAX™-I + 16.6 g/L Yeastolate + 33 mg/L rHuInsulin Feed #2: 2× CD CHO + 33 g/L Glucose + 16 mM GlutaMAX™-I + 33.4 g/L Yeastolate + 0.92 g/L Sodium Butyrate Feed #3: 1× CD CHO + 50 g/L Glucose + 10 mM GlutaMAX™-I + 50 g/L Yeastolate + 1.80 g/L Sodium Butyrate Feed #4: 1× CD CHO + 33 g/L Glucose + 6.6 mM GlutaMAX™-I + 50 g/L Yeastolate + 0.92 g/L Sodium Butyrate
Filtration of bioreactor cell culture	Four polyethersulfone filters (8.0 μm, 0.65 μm,	1st stage - Four modules in parallel, each with a layer
	0.22 μm and 0.22 μm) in series	of diatomaceous earth graded to 4-8 μm and a layer of diatomaceous earth graded to 1.4-1.1 μm, followed by a cellulose membrane.
		2nd stage -single module containing a layer of diatomaceous earth graded to 0.4-0.11 μm and a layer of diatomaceous earth graded to <0.1 μm, followed by a cellulose membrane.
	100 L storage bag	3rd stage - 0.22 μm polyethersulfone filter 300L storage bag Harvested cell culture is supplemented with 10 mM EDTA, 10 mM Tris to a pH of 7.5.
Concentration and buffer exchange prior to chromatography	Concentrate with 2 TFF with Millipore Spiral Polyethersulfone 30K MWCO Filter Buffer Exchange the Concentrate 6× with 10 mM Hepes, 25 mM NaCl, pH 7.0 20L sterile storage bag	Concentrate using four Sartorius Sartoslice TFF 30K MWCO Filter Buffer Exchange the Concentrate 10× with 10 mM Tris, 20 mM Na2SO4, pH 7.5 50L sterile storage bag
Viral inactivation prior to chromatography	None	Viral inactivation performed with the addition of a 1% Triton X-100, 0.3% Tributyl Phosphate, pH 7.5,
1st purification step (Q sepharose)	No absorbance reading	A280 measurements at the beginning and end
Viral filtration after chromatography	Pall DV-20 filter (20 nm)	Sartorius Virosart filter (20 nm)
Concentration and buffer exchange after chromatography	Hepes/saline pH 7.0 buffer Protein concentrated to 1 mg/ml	Histidine/saline, pH 6.0 buffer Protein concentrated to 10

(continued)

Process Difference	Gen1 soluble rHuPH20	Gen2 soluble rHuPH20
		mg/ml
Vial filling	5 mL and 1 mL fill volumes Stored at $\leq 30^{\circ}\text{C}$ Glass/rubber stopper	20 mL fill volumes Stored at $\leq 20^{\circ}\text{C}$ Teflon/screw cap
Soluble rHuPH20 yield	Approx. 400-700 mg	Approx. 11-25 g

EXAMPLE 9**Determination of enzymatic activity of soluble rHuPH20**

[0281] Enzymatic activity of soluble rHuPH20 in samples such as cell cultures, purification fractions and purified solutions was determined using a turbidometric assay, which based on the formation of an insoluble precipitate when hyaluronic acid binds with serum albumin. The activity is measured by incubating soluble rHuPH20 with sodium hyaluronate (hyaluronic acid) for a set period of time (10 minutes) and then precipitating the undigested sodium hyaluronate with the addition of acidified serum albumin. The turbidity of the resulting sample is measured at 640 nm after a 30 minute development period. The decrease in turbidity resulting from enzyme activity on the sodium hyaluronate substrate is a measure of the soluble rHuPH20 enzymatic activity. The method is run using a calibration curve generated with dilutions of a soluble rHuPH20 assay working reference standard, and sample activity measurements are made relative to this calibration curve.

[0282] Dilutions of the sample were prepared in Enzyme Diluent Solutions. The Enzyme Diluent Solution was prepared by dissolving 33.0 ± 0.05 mg of hydrolyzed gelatin in 25.0 mL of the 50 mM PIPES Reaction Buffer (140 mM NaCl, 50 mM PIPES, pH 5.5) and 25.0 mL of SWFI, and diluting 0.2 mL of 25% Buminat solution into the mixture and vortexing for 30 seconds. This was performed within 2 hours of use and stored on ice until needed. The samples were diluted to an estimated 1-2 U/mL. Generally, the maximum dilution per step did not exceed 1:100 and the initial sample size for the first dilution was not be less than 20 μL . The minimum sample volumes needed to perform the assay were: In-process Samples, FPLC Fractions: 80 μL ; Tissue Culture Supernatants: 1 mL; Concentrated Material 80 μL ; Purified or Final Step Material: 80 μL . The dilutions were made in triplicate in a Low Protein Binding 96-well plate, and 30 μL of each dilution was transferred to Optilux black/clear bottom plates (BD BioSciences).

[0283] Dilutions of known soluble rHuPH20 with a concentration of 2.5 U/mL were prepared in Enzyme Diluent Solution to generate a standard curve and added to the Optilux plate in triplicate. The dilutions included 0 U/mL, 0.25 U/mL, 0.5 U/mL, 1.0 U/mL, 1.5 U/mL, 2.0 U/mL, and 2.5 U/mL. "Reagent blank" wells that contained 60 μL of Enzyme Diluent Solution were included in the plate as a negative control. The plate was then covered and warmed on a heat block for 5 minutes at 37°C . The cover was removed and the plate was shaken for 10 seconds. After shaking, the plate was returned to the plate to the heat block and the MULTIDROP 384 Liquid Handling Device was primed with the warm 0.25mg/mL sodium hyaluronate solution (prepared by dissolving 100 mg of sodium hyaluronate (LifeCore Biomedical) in 20.0 mL of SWFI. This was mixed by gently rotating and/or rocking at $2-8^{\circ}\text{C}$ for 2-4 hours, or until completely dissolved). The reaction plate was transferred to the MULTIDROP 384 and the reaction was initiated by pressing the start key to dispense 30 μL sodium hyaluronate into each well. The plate was then removed from the MULTIDROP 384 and shaken for 10 seconds before being transferred to a heat block with the plate cover replaced. The plate was incubated at 37°C for 10 minutes

[0284] The MULTIDROP 384 was prepared to stop the reaction by priming the machine with Serum Working Solution and changing the volume setting to 240 μL . (25 mL of Serum Stock Solution [1 volume of Horse Serum (Sigma) was diluted with 9 volumes of 500 mM Acetate Buffer Solution and the pH was adjusted to 3.1 with hydrochloric acid] in 75 mL of 500 mM Acetate Buffer Solution). The plate was removed from the heat block and placed onto the MULTIDROP 384 and 240 μL of serum Working Solutions was dispensed into the wells. The plate was removed and shaken on a plate reader for 10 seconds. After a further 15 minutes, the turbidity of the samples was measured at 640 nm and the enzyme activity (in U/mL) of each sample was determined by fitting to the standard curve.

[0285] Specific activity (Units/mg) was calculated by dividing the enzyme activity (U/ml) by the protein concentration (mg/mL).

EXAMPLE 10**Determination of sialic acid and monosaccharide content**

[0286] The sialic acid and monosaccharide content of soluble rHuPH20 can be assessed by reverse phase liquid chromatography (RPLC) following hydrolysis with trifluoroacetic acid. In one example, the sialic acid and monosaccharide content of purified hyaluronidase lot # HUB0701E (1.2 mg/mL; produced and purified essentially as described in Example 8) was determined. Briefly, 100 µg sample was hydrolyzed with 40 % (v/v) trifluoroacetic acid at 100°C for 4 hours in duplicate. Following hydrolysis, the samples were dried down and resuspended in 300 µL water. A 45 µL aliquot from each re-suspended sample was transferred to a new tube and dried down, and 10 µL of a 10 mg/mL sodium acetate solution was added to each. The released monosaccharides were fluorescently labeled by the addition of 50 µL of a solution containing 30 mg/mL 2-aminobenzoic acid, 20 mg/mL sodium cyanoborohydride, approximately 40 mg/mL sodium acetate and 20 mg/mL boric acid in methanol. The mixture was incubated for 30 minutes at 80 °C in the dark. The derivitization reaction was quenched by the addition of 440 µL of mobile phase A (0.2% (v/v) n-butylamine, 0.5% (v/v) phosphoric acid, 1% (v/v) tetrahydrofuran). A matrix blank of water also was hydrolyzed and derivitized as described for the hyaluronidase sample as a negative control. The released monosaccharides were separated by RPLC using an Octadecyl (C₁₈) reverse phase column (4.6 × 250 mm, 5 µm particle size; J.T. Baker) and monitored by fluorescence detection (360 nm excitation, 425 nm emission). Quantitation of the monosaccharide content was made by comparison of the chromatograms from the hyaluronidase sample with chromatograms of monosaccharide standards including *N*-D-glucosamine (GlcN), *N*-D-galactosamine (GalN), galactose, fucose and mannose. Table 26 presents the molar ratio of each monosaccharide per hyaluronidase molecule.

Table 26. Monosaccharide content of soluble rHuPH20

Lot	Replicate	GlcN	GalN	Galactose	Mannose	Fucose
HUB0701E	1	14.28	0.07*	6.19	25.28	2.69
	2	13.66	0.08*	6.00	24.34	2.61
	Average	13.97	0.08*	6.10	24.81	2.65
* GalN results were below the limit of detection						

EXAMPLE 11**C-terminal heterogeneity of soluble rHuPH20 from 3D35M and 2B2 cells**

[0287] C-terminal sequencing was performed on two lots of sHuPH20 produced and purified from 3D35M cells in a 100 L bioreactor volume (Lot HUA0505MA) and 2B2 cells in a 300L bioreactor volume (Lot HUB0701EB). The lots were separately digested with endoproteinase Asp-N, which specifically cleaves peptide bonds N-terminally at aspartic and cysteic acid. This releases the C-terminal portion of the soluble rHuPH20 at the aspartic acid at position 431 of SEQ ID NO:4. The C-terminal fragments were separated and characterized to determine the sequence and abundance of each population in Lot HUA0505MA and Lot HUB0701EB.

[0288] It was observed that the soluble rHuPH20 preparations from 3D35M cells and 2B2 cells displayed heterogeneity, and contained polypeptides that differed from one another in their C-terminal sequence (Tables 27 and 28). This heterogeneity is likely the result of C-terminal cleavage of the expressed 447 amino acid polypeptide (SEQ ID NO:4) by peptidases present in the cell culture medium or other solutions during the production and purification process. The polypeptides in the soluble rHuPH20 preparations have amino acid sequences corresponding to amino acids 1-447, 1-446, 1-445, 1-444 and 1-443 of the soluble rHuPH20 sequence set forth SEQ ID NO:4. The full amino acid sequence of each of these polypeptides is forth in SEQ ID NOS: 4 to 8, respectively. As noted in tables 27 and 28, the abundance of each polypeptide in the soluble rHuPH20 preparations from 3D35M cells and 2B2 cells differs.

Table 27. Analysis of C-terminal fragments from Lot HUA0505MA

Fragment	Amino acid position (relative to SEQ ID NO: 4)	Sequence	Theor. Mass	Exp. Mass	Error	Elution time	Abundance
D28a	431-447	DAFKLPPMETEEPQIFY (SEQ ID NO:66)	2053.97	2054.42	0.45	99.87	0.2%
D28b	431-446	DAFKLPPMETEEPQIF (SEQ ID NO:67)	1890.91	1891.28	0.37	97.02	18.4%
D28c	431-445	DAFKLPPMETEEPQI (SEQ ID NO:68)	1743.84	1744.17	0.33	86.4	11.8%
D28d	431-444	DAFKLPPMETEEPQ (SEQ ID NO:69)	1630.70	1631.07	0.32	74.15	56.1%
D28e	431-443	DAFKLPPMETEEP (SEQ ID NO:70)	1502.70	1502.98	0.28	77.36	13.6%
D28f	431-442	DAFKLPPMETEE (SEQ ID NO:71)	1405.64	ND	N/A	N/A	0.0%

Table 28. Analysis of C-terminal fragments from Lot HUB0701EB

Fragment	Amino acid position (relative to SEQ ID NO: 4)	Sequence	Theor. Mass	Exp. Mass	Error	Elution time	Abundance
D28a	431-477	DAFKLPPMETEEPQIFY (SEQ ID NO:66)	2053.97	2054.42	0.45	99.89	1.9%
D28b	431-446	DAFKLPPMETEEPQIF (SEQ ID NO:67)	1890.91	1891.36	0.45	96.92	46.7%
D28c	431-445	DAFKLPPMETEEPQI (SEQ ID NO:68)	1743.84	1744.24	0.40	85.98	16.7%
D28d	431-444	DAFKLPPMETEEPQ (SEQ ID NO:69)	1630.70	1631.14	0.39	73.9	27.8%
D28e	431-443	DAFKLPPMETEEP (SEQ ID NO:70)	1502.70	1503.03	0.33	77.02	6.9%
D28f	431-442	DAFKLPPMETEE (SEQ ID NO:71)	1405.64	ND	N/A	N/A	0.0%

EXAMPLE 12**Production and purification of soluble rHuPH20 in 2500 L Bioreactor Cell Culture**

[0289] The production and purification of soluble rHuPH20 can be scaled up from a 300 L batch-fed bioreactor process (described in Example 8) to a 2500 L batch-fed bioreactor process. Like production of rHuPH20 in a 300 L bioreactor cell culture, the production of rHuPH20 in a 2500 L bioreactor cell culture is performed by first thawing and expanding a vial of 2B2 cells, culturing in a bioreactor, harvesting and clarifying the culture, concentrating and buffer-exchanging the harvest, followed by viral inactivation. The rHuPH20 is then purified from the concentrate using a series of purification steps that utilize Q sepharose, Phenyl sepharose, aminophenyl boronate and hydroxyapatite boronate, followed by viral filtration.

1. Cell culture expansion

[0290] To generate higher cell numbers required for seeding the 2500 L bioreactor cell culture compared to the 300 L culture, the cell culture is serially expanded through a 125 mL shaker flask, a 250 mL shaker, a 1 L shaker flask, two 2 L shaker flasks, six 2 L shaker flasks, a 25 L WAVE Bioreactor™ (GE Healthcare Life Sciences), a 100 L WAVE Bioreactor™, and a 600 L stirred tank seed bioreactor (ABEC, Inc. Bethlehem, PA; Stainless Technology division). At each expansion, the target seeding density is 4×10^5 cells/mL. The temperature throughout the expansion is 37°C (or between 36°C and 38°C) with 7% CO₂ (or between 6-8 % CO₂). The flasks are agitated at approximately 110 RPM (or 90-130 RPM), the 25 L and 100 L WAVE Bioreactor™ are rocked at 20 RPM (or 15-25 or 18-22 RPM, respectively) and the 600 L seed bioreactor is agitated at 90 RPM (or 85-95 RPM).

[0291] First, a vial of 2B2 cells (1×10^7 cells) from the working cell bank is thawed in a 37°C water bath for approximately 2 minutes (preferably no more than 5 minutes) before media is added and the cells are centrifuged. The cells are resuspended to approximately 25 mL (or between 20-30 mL) with fresh media (CD CHO AGT™ with 40 mL/L (8mM) GlutaMAX™-I and 20 µM methotrexate in a 125 mL shaker flask and placed in a 37°C, 7% CO₂ incubator. When the cell density reaches approximately 8×10^5 cells/mL, the culture is transferred into a 250 mL shake flask in a 50 mL culture volume (or 45-55 mL). Following incubation, when the cell density reaches approximately 1.6×10^6 cells/mL, the culture is expanded into a 1 L flask in 200 mL culture volume (or 190-210 mL) and incubated. When the cell density in the 1 L flask reaches approximately 1.6×10^6 cells/mL, the culture is expanded into 2 × 2 L flasks, each with a total culture volume of approximately 400 mL (or between 350-450 mL per flask), and incubated. When the cell density in the 2 L flasks reaches approximately 1.2×10^6 cells/mL, the culture is expanded into 6 × 2 L flasks, each with a total culture volume of approximately 400 mL (or between 350-450 mL per flask), and incubated. When the cell density in the 2 L flasks reaches approximately 2.5×10^6 cells/mL, the culture is expanded into a 25L WAVE Bioreactor™, with a total culture volume of approximately 15 L (or between 14-16 L) and incubated with an air flow of 1.5L/minute.

[0292] When the cell density in the 25 L WAVE Bioreactor™ reaches approximately 2.2×10^6 cells/mL, the culture is expanded into a 100 L WAVE Bioreactor™, with a total culture volume of approximately 80 L (or between 75-85 L), using CD-CHO AGT™ media that is supplemented with 3.6 g/L methotrexate, 40 mL/L GlutaMAX™-I and 1 mL/L 1N NaOH, and incubated with an air flow of 1.5L/minute. When the cell density in the 100 L WAVE Bioreactor™ reaches approximately 2.6×10^6 cells/mL, the culture is expanded into a 600 L seed bioreactor ABEC, Inc. Bethlehem, PA; Stainless Technology division) with a total culture volume of approximately 480 L (or between 440-520 L) using CD-CHO AGT™ media that is supplemented with 40 mL/L GlutaMAX™-I and incubated until the cell density in the 600 L bioreactor reaches approximately 1.6×10^6 cells/mL.

2. rHuPH20 production

[0293] A 3500 L bioreactor with a total volume of 3523 L and a working volume of 500-2500 L (ABEC, Inc, Bethlehem, PA) is used for high yield production of rHuPH20. Following sterilization, approximately 1800-2000 L CD-CHO AGT™ media containing 24.3 g/L powdered CD-CHO AGT™, supplemented with 40 mL/L GlutaMAX™-I and 5 mg/L rHuInsulin is added to the bioreactor. Parameters are set to: temperature setpoint, 37°C; Impeller Speed 75 RPM; Vessel Pressure: 5 psi; Air Sparge 18 L/ min; dissolved oxygen: 25%; pH ≤ 7.2 . Before use, the reactor is checked for contamination. Approximately between 300-500 L of cells (depending on cell count) from the 600 L seed bioreactor culture are inoculated into the cell culture medium in the 3500 L bioreactor at an inoculation density of 4.0×10^5 viable cells per mL, to reach a total volume of 2100 L. During the 14 day cell incubation, the bioreactor is sampled daily for cell viability, cell density, pH verification, and enzymatic activity. Temperature and dissolved oxygen also are monitored closely.

[0294] Nutrient feeds are added during the 14 day bioreactor run, each at approximately 4% v/v. At day 5, approximately 84 L (or 4 % v/v) of Feed #1 Medium (81 g/L powdered CD-CHO AGT™ + 33 g/L Glucose + 13.3 mL/L GlutaMAX™-I + 83.3 g/L Yeastolate + 33 mg/L rHuInsulin) is added. At day 7, approximately 87 L (or 4 % v/v) of Feed #2 (40.5 g/L powdered CD-CHO AGT™ + 33 g/L Glucose + 66.7 mL/L GlutaMAX™-I + 166.7 g/L Yeastolate + 0.92 g/L Sodium butyrate) is added, and culture temperature is changed to 36.5°C. At day 9, approximately 91 L (or 4 % v/v) of Feed #3 (20.3 g/L powdered CD-CHO AGT™ + 50 g/L Glucose + 50 mL/L GlutaMAX™-I + 250 g/L Yeastolate + 1.8 g/L Sodium butyrate) is added, and culture temperature is changed to 36° C. At day 11, approximately 94 L (or 4 % v/v) of Feed #4 (20.3 g/L powdered CD-CHO AGT™ + 33.3 g/L Glucose + 33.3 mL/L GlutaMAX™-I + 250 g/L Yeastolate + 0.92 g/L Sodium butyrate) is added, and culture temperature is changed to 35.5° C. The reactor is harvested at 14 days, yielding 2400-2600 L harvest (typically approximately 2500 L).

[0295] The culture is pressure transferred through 20 Millistak filtration system modules (Millipore) in parallel, each containing a layer of diatomaceous earth graded to 4-8 µm and a layer of diatomaceous earth graded to 1.4-1.1 µm, followed by a cellulose membrane, then through a second Millistak filtration system (Millipore) containing 10 modules, each with a layer of diatomaceous earth graded to 0.4-0.11 µm and a layer of diatomaceous earth graded to <0.1 µm, followed by a cellulose membrane, and then through a 0.22 µm final filter into a sterile single use flexible bag with a 350

L capacity. The harvested cell culture fluid is supplemented with 10 mM EDTA and 10 mM Tris, pH 8.4, to a target pH of 7.5. The culture is concentrated 10× with a tangential flow filtration (TFF) apparatus (Pall) using 18-21 m² of Sartoslice TFF 30 kDa molecular weight cut-off (MWCO) polyether sulfone (PES) filters (Sartorius), followed by a 10× buffer exchange with 10 mM Tris, 20 mM Na₂SO₄, pH 7.5 into a 0.22 μm final filter into a 350 L sterile storage bag.

[0296] The concentrated, diafiltered harvest is inactivated for virus. Prior to viral inactivation, a solution of 10% Triton X-100, 3% tri (n-butyl) phosphate (TNBP) was prepared. The concentrated, diafiltered harvest was exposed to 1% Triton X-100, 0.3% TNBP up to 2 hours in 500 L stainless steel reaction vessels immediately prior to purification on the Q column.

B. Purification of Gen2 rHuPH20

[0297] A Q Sepharose (Pharmacia) ion exchange column (81 L resin, H= 26 cm, D= 63 cm) is prepared. The column is equilibrated with 5 column volumes of 10 mM Tris, 20 mM Na₂SO₄, pH 7.5. Following viral inactivation, the concentrated, diafiltered, viral-inactivated harvest of approximately 250 L is loaded onto the Q column at a flow rate of 150 cm/hr. The column is washed with 5 column volumes of 10 mM Tris, 20 mM Na₂SO₄, pH 7.5 and 10 mM Hepes, 50 mM NaCl, pH 7.0. The protein is eluted with 10 mM Hepes, 400 mM NaCl, pH 7.0 into a 0.22 μm final filter into sterile bag. The eluate sample is tested for bioburden, protein concentration and enzyme activity. A₂₈₀ absorbance reading were taken at the beginning and end of the exchange.

[0298] Phenyl-Sepharose (Pharmacia) hydrophobic interaction chromatography is next performed. A Phenyl-Sepharose (PS) column (176 L resin, H=35 cm, D= 80 cm) is prepared. The column is equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate, 0.1 mM CaCl₂, pH 7.0. The protein eluate from above is supplemented with 2M ammonium sulfate, 1 M potassium phosphate and 1 M CaCl₂ stock solutions to yield final concentrations of 5 mM, 0.5 M and 0.1 mM, respectively. The protein is loaded onto the PS column at a flow rate of 100 cm/hr. 5 mM potassium phosphate, 0.5 M ammonium sulfate and 0.1 mM CaCl₂ pH 7.0 was added at 100 cm/hr. The flow through is passed through a 0.22 μm final filter into a sterile bag. The flow through is sampled for bioburden, protein concentration and enzyme activity.

[0299] An aminophenyl boronate column (ProMedics; 176 L resin, H=35 cm, D= 80 cm) is then prepared. The column is equilibrated with 5 column volumes of 5 mM potassium phosphate, 0.5 M ammonium sulfate. The PS-purified protein is loaded onto the aminophenyl boronate column at a flow rate of 50 cm/hr. The flow rate was increased to 100 cm/hr for the remainder of the process. The column was first washed with 5 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0, then 20 mM bicine, 0.5 M ammonium sulfate, pH 9.0, and then with 20 mM bicine, 100 mM sodium chloride, pH 9.0. The protein is eluted with 50 mM Hepes, 100 mM NaCl, pH 6.9 and passed through a sterile filter into a sterile bag. The eluted sample is tested for bioburden, protein concentration and enzyme activity.

[0300] The hydroxyapatite (HAP) column (BioRad; 116 L resin, H=23 cm, D= 80 cm) is prepared. The column is equilibrated with 5 mM potassium phosphate, 100 mM NaCl, 0.1 mM CaCl₂, pH 7.0. The aminophenyl boronate purified protein is supplemented to final concentrations of 5 mM potassium phosphate and 0.1 mM CaCl₂ and loaded onto the HAP column at a flow rate of 100 cm/hr. The column is first washed with 5 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM CaCl₂, then 10 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1 mM CaCl₂. The protein is eluted with 70 mM potassium phosphate, pH 7.0 and passed through a 0.22 μm sterile filter into a sterile bag. The eluted sample is tested for bioburden, protein concentration and enzyme activity.

[0301] The HAP purified protein is then passed through a viral removal filter. The sterilized Viosart filter (Sartorius) is first prepared by washing with 2 L of 70 mM potassium phosphate, pH 7.0. The HAP purified protein is pumped via a peristaltic pump through the 20 nM viral removal filter. The filtered protein in 70 mM potassium phosphate, pH 7.0 is then passed through a 0.22 μm final filter into a sterile bag. The viral filtered sample is tested for protein concentration, enzyme activity, oligosaccharide, monosaccharide and sialic acid profiling (as described in Examples 9 to 10, below).

[0302] The protein in the filtrate was then concentrated 8-12× using three 10 kD molecular weight cut off (MWCO) Sartoon PES cassettes, each with a filter surface area of 0.7 m², for a total surface area of 2.1 m². Following concentration, the concentrated protein was sampled and tested for protein concentration and enzyme activity. A 10× diafiltration is then performed on the concentrated protein. This can be performed one of two ways: 1) using a 20 mM histidine, 130 mM NaCl, pH 6.5 buffer and 1% polysorbate 80; or 2) using a 10 mM histidine, 130 mM NaCl, pH 6.5 buffer. The concentrated, diafiltered bulk protein is at a concentration of approximately 10 mg/mL. Following buffer exchange, the concentrated protein is passed through a 0.22 μm filter into a 20 L sterile storage bag.

[0303] The sterile filtered bulk protein is then aseptically dispensed at 400 mL into 1 L sterile PFA Nalgene bottles. The bottles are then flash frozen in a liquid nitrogen bath and stored at less than -20 °C for the bulk protein that does not contain the polysorbate 80, and less than -70 °C for the bulk protein that does contain the polysorbate 80.

[0304] Table 29 sets forth some exemplary differences between production of rHuPH20 in a 300 L and 2500 L bioreactor culture

Table 29. Exemplary differences between production of rHuPH20 in a 300 L and 2500 L bioreactor culture

Process Difference	300 L cell culture	2500 L cell culture
Cell line	2B2	2B2
Media used to expand cell inoculum	Contains 20 μ M methotrexate (9 mg/L)	Contains 20 μ M methotrexate (9 mg/L)
Cell expansion	Expanded through 125 mL flask, 250 mL flask, 1 L flask, 6 L flask and 36 L flask.	Expanded through 125 mL flask, 250 mL flask, 1 L flask, 2 $\times$ 2 L shaker flasks, 6 $\times$ 2 L shaker flasks, 25 L WAVE Bioreactor™, 100 L WAVE Bioreactor™, and a 600 L stirred tank seed bioreactor
Final operating volume in bioreactor	Approx. 300L in a 400L bioreactor	Approx. 2500 L in a 3500 L bioreactor
Culture media in at bioreactor inoculation	CD CHO with 5.0 mg/L rHuInsulin 40 mL/L (8 mM) GlutaMAX™-I	CD CHO AGT™ with 5.0 mg/L rHuInsulin 40 mL/L (8 mM) GlutaMAX™-I
Media feed volume	Scaled at 4% of the bioreactor cell culture volume i.e. approximately 10.4, 10.8, 11.2 and 11.7 L, resulting in a target bioreactor volume of ~300 L.	Scaled at 4% of the bioreactor cell culture volume i.e. approximately 84, 87, 91, and 94 L, resulting in a target bioreactor volume of ~2500 L.
Media feed	Feed #1 Medium: 4 $\times$ CD CHO + 33 g/L Glucose + 32 mM GlutaMAX™-I + 16.6 g/L Yeastolate + 33 mg/L rHuInsulin Feed #2: 2 $\times$ CD CHO + 33 g/L Glucose + 16 mM GlutaMAX™-I + 33.4 g/L Yeastolate + 0.92 g/L Sodium Butyrate Feed #3: 1 $\times$ CD CHO + 50 g/L Glucose + 10 mM GlutaMAX™-I + 50 g/L Yeastolate + 1.80 g/L Sodium Butyrate Feed #4: 1 $\times$ CD CHO + 33 g/L Glucose + 6.7 mM GlutaMAX™-I + 50 g/L Yeastolate + 0.92 g/L Sodium Butyrate	Feed #1 Medium: 81 g/L powdered CD-CHO AGT™ + 33 g/L Glucose + 26.6 mM GlutaMAX™-I + 83.3 g/L Yeastolate + 33 mg/L rHuInsulin Feed #2: 40.5 g/L powdered CD-CHO AGT™ + 33 g/L Glucose + 13.4 mM GlutaMAX™-I + 166.7 g/L Yeastolate + 0.92 g/L Sodium butyrate Feed #3: 20.3 g/L powdered CD-CHO AGT™ + 50 g/L Glucose + 10 mM GlutaMAX™-I + 250 g/L Yeastolate + 1.8g/L Sodium butyrate Feed #4: 20.3 g/L powdered CD-CHO AGT™ + 33.3 g/L Glucose + 6.7 mM GlutaMAX™-I + 250 g/L Yeastolate + 0.92 g/L Sodium butyrate
Filtration of bioreactor cell culture	1 st stage - 4 modules in parallel, each with a layer of diatomaceous earth graded to 4-8 μ m and a layer of diatomaceous earth graded to 1.4-1.1 μ m, followed by a cellulose membrane. 2 nd stage -single module containing a layer of diatomaceous earth graded to 0.4-0.11 μ m and a layer of diatomaceous earth graded to <0.1 μ m, followed by a cellulose membrane. 3 rd stage - 0.22 μ m polyethersulfone filter 300L storage bag	1 st stage - 20 modules in parallel, each with a layer of diatomaceous earth graded to 4-8 μ m and a layer of diatomaceous earth graded to 1.4-1.1 μ m, followed by a cellulose membrane. 2 nd stage -10 modules containing a layer of diatomaceous earth graded to 0.4-0.11 μ m and a layer of diatomaceous earth graded to <0.1 μ m, followed by a cellulose membrane. 3 rd stage - 0.22 μ m polyethersulfone filter 350L storage bag

(continued)

	Process Difference	300 L cell culture	2500 L cell culture
5	Concentration and buffer exchange prior to chromatography	Concentrate using four Sartorius Sartoslice TFF 30K MWCO Filter	Concentrate with 2 TFF with Millipore Spiral Polyethersulfone 30K MWCO Filter
10		Buffer Exchange the Concentrate 10× with 10 mM Tris, 20 mM Na ₂ SO ₄ , pH 7.5 50L sterile storage bag	Buffer Exchange the Concentrate 6× with 10 mM Hepes, 25 mM NaCl, pH 7.0 20L sterile storage bag
15	Q sepharose column	9 L resin, H= 29 cm, D= 20 cm	81 L resin, H= 26 cm, D= 63 cm
	A Phenyl-Sephadex (PS) column	19-21 L resin, H=29 cm, D= 30 cm	176 L resin, H=35 cm, D= 80 cm
20	aminophenyl boronate column	21 L resin, H=29 cm, D= 30 cm	176 L resin, H=35 cm, D= 80 cm
	hydroxyapatite (HAP) column	13 L resin, H=20 cm, D= 30 cm	116 L resin, H=23 cm, D= 80 cm
25	Protein Concentration	Single 10 kD MWCO Sartoclon Slice tangential flow filtration (TFF)	Three 10 kD molecular weight cut off (MWCO) Sartoclon PES cassettes
30		6× buffer exchange with 10 mM histidine, 130 mM NaCl, pH 6.0	10× diafiltration with: 1) 20 mM histidine, 130 mM NaCl, pH 6.5 buffer and 1% polysorbate 80; or 2) 10 mM histidine, 130 mM NaCl, pH 6.5 buffer.
35	Vial filling	20 mL fill volumes Stored at ≤20°C	400 mL fill volumes Stored at ≤20°C if protein does not contain polysorbate 80, or ≤70°C if protein does contain polysorbate 80

SEQUENCE LISTING

[0305]

<110> Halozyme, Inc.

<120> LARGE-SCALE PRODUCTION OF SOLUBLE
HYALURONIDASE

<130> 225788

<150> US 61/068,622

<151> 2008-03-06

<160> 71

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 509

<212> PRT

<213> Homo sapiens

<220>

<223> precursor human PH20

5

<400> 1

Met Gly Val Leu Lys Phe Lys His Ile Phe Phe Arg Ser Phe Val Lys
 1 5 10 15
 Ser Ser Gly Val Ser Gln Ile Val Phe Thr Phe Leu Leu Ile Pro Cys
 20 25 30
 Cys Leu Thr Leu Asn Phe Arg Ala Pro Pro Val Ile Pro Asn Val Pro
 35 40 45
 Phe Leu Trp Ala Trp Asn Ala Pro Ser Glu Phe Cys Leu Gly Lys Phe
 50 55 60
 Asp Glu Pro Leu Asp Met Ser Leu Phe Ser Phe Ile Gly Ser Pro Arg
 65 70 75 80
 Ile Asn Ala Thr Gly Gln Gly Val Thr Ile Phe Tyr Val Asp Arg Leu
 85 90 95
 Gly Tyr Tyr Pro Tyr Ile Asp Ser Ile Thr Gly Val Thr Val Asn Gly
 100 105 110
 Gly Ile Pro Gln Lys Ile Ser Leu Gln Asp His Leu Asp Lys Ala Lys
 115 120 125
 Lys Asp Ile Thr Phe Tyr Met Pro Val Asp Asn Leu Gly Met Ala Val
 130 135 140
 Ile Asp Trp Glu Glu Trp Arg Pro Thr Trp Ala Arg Asn Trp Lys Pro
 145 150 155 160
 Lys Asp Val Tyr Lys Asn Arg Ser Ile Glu Leu Val Gln Gln Gln Asn
 165 170 175
 Val Gln Leu Ser Leu Thr Glu Ala Thr Glu Lys Ala Lys Gln Glu Phe
 180 185 190
 Glu Lys Ala Gly Lys Asp Phe Leu Val Glu Thr Ile Lys Leu Gly Lys
 195 200 205
 Leu Leu Arg Pro Asn His Leu Trp Gly Tyr Tyr Leu Phe Pro Asp Cys
 210 215 220
 Tyr Asn His His Tyr Lys Lys Pro Gly Tyr Asn Gly Ser Cys Phe Asn
 225 230 235 240

40

45

50

55

	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
					245					250					255	
5	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
				260					265					270		
	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
			275					280					285			
	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
		290					295					300				
10	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
	305					310					315				320	
	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
				325						330					335	
15	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
				340					345					350		
	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
			355				360						365			
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
20		370					375					380				
	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
	385					390				395					400	
	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
				405					410						415	
25	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
			420						425					430		
	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
			435				440						445			
30	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
		450					455					460				
	Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile
	465					470				475					480	
	Phe	Tyr	Asn	Ala	Ser	Pro	Ser	Thr	Leu	Ser	Ala	Thr	Met	Phe	Ile	Val
35				485						490					495	
	Ser	Ile	Leu	Phe	Leu	Ile	Ile	Ser	Ser	Val	Ala	Ser	Leu			
				500					505							

<210> 2

40 <211> 474

<212> PRT

<213> Homo sapiens

<220>

45 <223> Mature PH20

<400> 2

50

55

EP 4 269 578 B1

	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp
	1				5					10					15	
	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
				20					25					30		
5	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
			35					40					45			
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
10	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75				80	
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile

15

20

25

30

35

40

45

50

55

				85				90				95				
	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp
				100					105					110		
5	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
			115					120					125			
	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu
		130					135					140				
10	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
	145					150					155					160
	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
				165						170					175	
	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
			180						185					190		
15	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
		195						200					205			
	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
		210					215					220				
20	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
	225					230					235					240
	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
				245						250					255	
	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
			260						265					270		
25	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
		275						280					285			
	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
		290				295						300				
30	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
	305					310					315					320
	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
				325						330					335	
	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
35				340					345					350		
	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
			355					360					365			
	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly
		370					375					380				
40	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys
	385					390					395					400
	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp
				405						410					415	
45	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala
			420						425					430		
	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile	Phe	Tyr	Asn
			435					440					445			
	Ala	Ser	Pro	Ser	Thr	Leu	Ser	Ala	Thr	Met	Phe	Ile	Val	Ser	Ile	Leu
50		450					455					460				
	Phe	Leu	Ile	Ile	Ser	Ser	Val	Ala	Ser	Leu						
	465					470										

<210> 3

55 <211> 482

<212> PRT

<213> Homo sapiens

<220>

<223> precursor soluble rHuPH20

<400> 3

5

10

15

20

25

30

35

40

45

50

55

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
5	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
			20						25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
		35						40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
	50					55						60				
10	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65				70						75				80	
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
				85					90					95		
15	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
			100					105						110		
	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
		115						120					125			
20	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
	130						135					140				
	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155				160	
	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
				165						170					175	
25	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
			180					185						190		
	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
		195					200					205				
30	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
	210					215						220				
	Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
	225				230						235				240	
	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
				245						250					255	
35	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
			260						265					270		
	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
		275					280						285			
40	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
	290					295						300				
	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
	305					310					315				320	
45	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
				325						330					335	
	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
			340						345					350		
	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
		355					360						365			
50	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
	370					375						380				
	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
	385					390					395				400	
55	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
				405						410					415	

EP 4 269 578 B1

	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
				420					425					430		
	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
5			435					440					445			
	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
		450					455					460				
	Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile
	465					470					475					480
10	Phe	Tyr														

	<210> 4
	<211> 447
	<212> PRT
15	<213> Homo sapiens
	<220>
	<223> soluble rHuPH20 1-447
20	<400> 4

25

30

35

40

45

50

55

EP 4 269 578 B1

	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp
	1				5					10					15	
5	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
			20						25					30		
	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
		35						40					45			
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
10	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75					80
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile
					85					90					95	
15	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp
				100					105					110		
	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
			115					120					125			
	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu
20		130					135					140				
	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
	145					150					155					160
	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
					165					170					175	
25	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
				180					185					190		
	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
			195					200					205			
30	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
		210					215					220				
	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
	225					230					235					240
	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
					245					250					255	
35	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
				260					265					270		
	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr

40

45

50

55

EP 4 269 578 B1

[illegible]

25

<210>	5
<211>	446
<212>	PRT
<213>	Homo sapiens

30 <220>
 <223> soluble rHuPH20 1-446

 <400> 5

35

40

45

50

55

EP 4 269 578 B1

	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp
	1				5					10					15	
5	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
			20						25					30		
	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
		35						40					45			
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
10	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75					80
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile
					85					90					95	
15	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp
				100					105					110		
	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
			115					120					125			
	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu
20		130					135					140				
	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
	145					150					155					160
	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
				165						170					175	
25	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
				180					185					190		

30

35

40

45

50

55

	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
			195					200					205			
	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
		210					215				220					
5	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
		225				230					235					240
	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
				245						250					255	
10	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
				260					265					270		
	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
			275					280					285			
	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
15		290					295					300				
	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
		305				310					315					320
	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
					325					330					335	
20	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
				340					345					350		
	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
			355					360					365			
25	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly
		370					375					380				
	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys
		385				390					395					400
	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp
30					405					410					415	
	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala
				420					425					430		
	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile	Phe		
			435					440					445			

35

<210> 6

<211> 445

<212> PRT

<213> Homo sapiens

40

<220>

<223> soluble rHuPH20 1-445

45

<400> 6

50

55

EP 4 269 578 B1

	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp
	1				5					10					15	
	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
				20					25					30		
5	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
			35					40					45			
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
10	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75					80
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile
					85					90					95	
15	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp

15

20

25

30

35

40

45

50

55

				100				105				110				
	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
				115				120				125				
5	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu
		130					135					140				
	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
	145					150					155					160
	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
10				165						170						175
	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
				180					185					190		
	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
		195						200					205			
15	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
		210					215					220				
	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
	225					230					235					240
20	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
				245						250						255
	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
				260					265					270		
	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
25			275					280				285				
	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
		290				295						300				
	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
	305				310						315					320
30	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
				325						330					335	
	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
			340						345					350		
35	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
			355					360					365			
	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly
		370					375					380				
40	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys
	385				390						395					400
	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp
				405						410					415	
	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala
			420						425					430		
45	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile			
			435					440					445			

<210> 7

<211> 444

50 <212> PRT

<213> Homo sapiens

<220>

<223> soluble rHuPH20 1-444

55

<400> 7

EP 4 269 578 B1

Leu Asn Phe Arg Ala Pro Pro Val Ile Pro Asn Val Pro Phe Leu Trp
1 5 10 15

5

10

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
			20						25					30		
	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
			35					40					45			
5	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75					80
10	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile
					85					90					95	
	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp
				100					105					110		
	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
				115				120					125			
15	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu
		130					135					140				
	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
	145					150					155					160
20	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
					165					170					175	
	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
				180					185					190		
	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
			195					200					205			
25	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
		210					215					220				
	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
	225					230					235					240
30	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
					245					250					255	
	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
				260					265					270		
35	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
			275					280					285			
	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
		290				295						300				
	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
	305					310					315					320
40	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
					325					330					335	
	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
				340					345					350		
45	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
			355					360					365			
	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly
		370					375					380				
	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys
	385					390					395					400
50	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp
					405					410					415	
	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala
				420					425					430		
55	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln				
			435					440								

<210> 8
<211> 443
<212> PRT
<213> Homo sapiens
5

<220>
<223> soluble rHuPH20 1-443

<400> 8
10

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp
	1				5					10					15	
	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
				20					25					30		
5	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
			35					40					45			
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
10	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75					80
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile
					85					90					95	
	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp
15				100					105					110		
	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
			115					120					125			
	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu
		130					135					140				
20	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
	145					150					155					160
	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
					165					170					175	
25	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
				180					185					190		
	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
		195						200					205			
	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
30		210					215					220				
	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
	225					230					235					240
	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
					245					250					255	
35	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
				260					265					270		
	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
			275					280					285			
40	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
		290					295					300				
	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
	305					310					315					320
	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
					325					330					335	
45	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
				340					345					350		
	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
			355					360					365			
50	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly

55

EP 4 269 578 B1

		370		375		380													
	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys			
	385					390					395					400			
5	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp			
					405					410					415				
	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala			
				420					425					430					
10	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro								
			435				440												

<210> 9
 <211> 442
 <212> PRT
 15 <213> Homo sapiens

 <220>
 <223> soluble rHuPH20 1-442

 20 <400> 9

25

30

35

40

45

50

55

	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp
	1				5					10					15	
5	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
				20					25					30		
	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
		35						40					45			
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
10	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75					80
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile
				85						90					95	
15	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp
				100					105					110		
	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
			115					120					125			
	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu
20		130					135					140				
	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
	145				150						155					160
	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
				165						170					175	
25	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
			180						185					190		
	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
			195					200					205			
30	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
		210					215					220				
	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
	225					230					235					240
	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
				245						250					255	
35	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
			260						265					270		
	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
			275					280					285			

40

45

50

55

	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
		290					295					300				
5	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
	305					310					315					320
	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
					325					330					335	
	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
				340					345					350		
10	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
			355					360					365			
	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly
		370					375					380				
15	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys
	385					390					395					400
	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp
					405					410					415	
20	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala
				420					425					430		
	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu						
			435					440								

<210> 10
 <211> 450
 <212> PRT
 <213> Bos taurus

 <220>
 <223> hyaluronidase

 <400> 10

35

40

45

50

55

EP 4 269 578 B1

	Met	Arg	Pro	Phe	Ser	Leu	Glu	Val	Ser	Leu	His	Leu	Pro	Trp	Ala	Met
	1				5					10					15	
	Ala	Ala	His	Leu	Leu	Pro	Val	Cys	Thr	Leu	Phe	Leu	Asn	Leu	Leu	Ser
5				20					25					30		
	Met	Thr	Gln	Gly	Ser	Arg	Asp	Pro	Val	Val	Pro	Asn	Gln	Pro	Phe	Thr
		35					40					45				
	Thr	Ile	Trp	Asn	Ala	Asn	Thr	Glu	Trp	Cys	Met	Lys	Lys	His	Gly	Val
	50					55					60					
10	Asp	Val	Asp	Ile	Ser	Ile	Phe	Asp	Val	Val	Thr	Asn	Pro	Gly	Gln	Thr
	65				70					75					80	
	Phe	Arg	Gly	Pro	Asn	Met	Thr	Ile	Phe	Tyr	Ser	Ser	Gln	Leu	Gly	Thr
				85					90					95		
15	Tyr	Pro	Tyr	Tyr	Thr	Ser	Ala	Gly	Glu	Pro	Val	Phe	Gly	Gly	Leu	Pro
			100					105					110			
	Gln	Asn	Ala	Ser	Leu	Asn	Ala	His	Leu	Ala	Arg	Thr	Phe	Gln	Asp	Ile
		115				120						125				
	Leu	Ala	Ala	Met	Pro	Glu	Pro	Arg	Phe	Ser	Gly	Leu	Ala	Val	Ile	Asp
20		130				135					140					
	Trp	Glu	Ala	Trp	Arg	Pro	Arg	Trp	Ala	Phe	Asn	Trp	Asp	Thr	Lys	Asp
	145				150					155					160	
	Ile	Tyr	Arg	Gln	Arg	Ser	Arg	Ala	Leu	Val	Gln	Lys	Gln	His	Pro	Asp
				165				170						175		
25	Trp	Leu	Ala	Pro	Arg	Val	Glu	Ala	Ala	Ala	Gln	Asp	Gln	Phe	Glu	Gly
			180					185					190			
	Ala	Ala	Glu	Glu	Trp	Met	Ala	Gly	Thr	Leu	Lys	Leu	Gly	Gln	Ala	Leu

30

35

40

45

50

55

EP 4 269 578 B1

				195				200				205				
	Arg	Pro	Gln	Gly	Leu	Trp	Gly	Phe	Tyr	Asn	Phe	Pro	Glu	Cys	Tyr	Asn
		210					215					220				
5	Tyr	Asp	Phe	Lys	Ser	Pro	Asn	Tyr	Thr	Gly	Arg	Cys	Pro	Leu	Asn	Ile
	225					230					235				240	
	Cys	Ala	Gln	Asn	Asp	Gln	Leu	Gly	Trp	Leu	Trp	Gly	Gln	Ser	Arg	Ala
				245						250					255	
	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Pro	Ala	Ala	Leu	Glu	Gly	Thr	Lys	Lys
10				260					265					270		
	Thr	Gln	Met	Phe	Val	Gln	His	Arg	Val	Ala	Glu	Ala	Phe	Arg	Val	Ala
			275					280					285			
	Ala	Gly	Ala	Gly	Asp	Pro	Lys	Leu	Pro	Val	Leu	Pro	Tyr	Met	Gln	Leu
		290					295					300				
15	Phe	Tyr	Asp	Met	Thr	Asn	His	Phe	Leu	Pro	Ala	Glu	Glu	Leu	Glu	His
	305					310					315				320	
	Ser	Leu	Gly	Glu	Ser	Ala	Ala	Gln	Gly	Ala	Ala	Gly	Val	Val	Leu	Trp
					325						330				335	
	Val	Ser	Trp	Leu	Ser	Thr	Ser	Thr	Lys	Glu	Ser	Cys	Gln	Ala	Ile	Lys
20				340					345					350		
	Glu	Tyr	Val	Asp	Thr	Thr	Leu	Gly	Pro	Ser	Ile	Leu	Asn	Val	Thr	Ser
			355					360					365			
	Gly	Ala	Arg	Leu	Cys	Ser	Gln	Val	Leu	Cys	Ser	Gly	His	Gly	Arg	Cys
25							375					380				
	Ala	Arg	Arg	Pro	Ser	Tyr	Pro	Lys	Ala	Arg	Leu	Ile	Leu	Asn	Ser	Thr
	385					390					395				400	
	Ser	Phe	Ser	Ile	Lys	Pro	Thr	Pro	Gly	Gly	Gly	Pro	Leu	Thr	Leu	Gln
					405					410					415	
30	Gly	Ala	Leu	Ser	Leu	Glu	Asp	Arg	Leu	Arg	Met	Ala	Val	Glu	Phe	Glu
				420					425					430		
	Cys	Arg	Cys	Tyr	Arg	Gly	Trp	Arg	Gly	Thr	Arg	Cys	Glu	Gln	Trp	Gly
			435					440					445			
35	Met	Trp														
		450														

<210> 11

<211> 331

<212> PRT

40 <213> *Vespula vulgaris*

<220>

<223> hyaluronidase A

45 <400> 11

50

55

EP 4 269 578 B1

	Ser	Glu	Arg	Pro	Lys	Arg	Val	Phe	Asn	Ile	Tyr	Trp	Asn	Val	Pro	Thr
	1				5					10					15	
	Phe	Met	Cys	His	Gln	Tyr	Asp	Leu	Tyr	Phe	Asp	Glu	Val	Thr	Asn	Phe
5				20					25					30		
	Asn	Ile	Lys	Arg	Asn	Ser	Lys	Asp	Phe	Gln	Gly	Asp	Lys	Ile	Ala	
			35				40					45				
	Ile	Phe	Tyr	Asp	Pro	Gly	Glu	Phe	Pro	Ala	Leu	Leu	Ser	Leu	Lys	Asp
		50				55						60				
10	Gly	Lys	Tyr	Lys	Lys	Arg	Asn	Gly	Gly	Val	Pro	Gln	Glu	Gly	Asn	Ile
	65				70					75					80	
	Thr	Ile	His	Leu	Gln	Lys	Phe	Ile	Glu	Asn	Leu	Asp	Lys	Ile	Tyr	Pro
				85					90					95		
15																
	Asn	Arg	Asn	Phe	Ser	Gly	Ile	Gly	Val	Ile	Asp	Phe	Glu	Arg	Trp	Arg
				100					105					110		
	Pro	Ile	Phe	Arg	Gln	Asn	Trp	Gly	Asn	Met	Lys	Ile	His	Lys	Asn	Phe
			115					120					125			
20	Ser	Ile	Asp	Leu	Val	Arg	Asn	Glu	His	Pro	Thr	Trp	Asn	Lys	Lys	Met
		130					135					140				
	Ile	Glu	Leu	Glu	Ala	Ser	Lys	Arg	Phe	Glu	Lys	Tyr	Ala	Arg	Phe	Phe
	145				150						155				160	
	Met	Glu	Glu	Thr	Leu	Lys	Leu	Ala	Lys	Lys	Thr	Arg	Lys	Gln	Ala	Asp
25				165						170					175	
	Trp	Gly	Tyr	Tyr	Gly	Tyr	Pro	Tyr	Cys	Phe	Asn	Met	Ser	Pro	Asn	Asn
			180						185					190		
	Leu	Val	Pro	Glu	Cys	Asp	Val	Thr	Ala	Met	His	Glu	Asn	Asp	Lys	Met
			195				200						205			
30	Ser	Trp	Leu	Phe	Asn	Asn	Gln	Asn	Val	Leu	Leu	Pro	Ser	Val	Tyr	Val
		210				215						220				
	Arg	Gln	Glu	Leu	Thr	Pro	Asp	Gln	Arg	Ile	Gly	Leu	Val	Gln	Gly	Arg
	225				230						235				240	
35	Val	Lys	Glu	Ala	Val	Arg	Ile	Ser	Asn	Asn	Leu	Lys	His	Ser	Pro	Lys
				245						250				255		
	Val	Leu	Ser	Tyr	Trp	Trp	Tyr	Val	Tyr	Gln	Asp	Glu	Thr	Asn	Thr	Phe
			260						265					270		
	Leu	Thr	Glu	Thr	Asp	Val	Lys	Lys	Thr	Phe	Gln	Glu	Ile	Val	Ile	Asn
40			275				280						285			
	Gly	Gly	Asp	Gly	Ile	Ile	Ile	Trp	Gly	Ser	Ser	Ser	Asp	Val	Asn	Ser
		290				295						300				
	Leu	Ser	Lys	Cys	Lys	Arg	Leu	Gln	Asp	Tyr	Leu	Leu	Thr	Val	Leu	Gly
	305				310						315				320	
45	Pro	Ile	Ala	Ile	Asn	Val	Thr	Glu	Ala	Val	Asn					
				325						330						

<210> 12

<211> 340

50 <212> PRT

<213> *Vespula vulgaris*

<220>

<223> hyaluronidase B

55

<400> 12

	Asp	Arg	Thr	Ile	Trp	Pro	Lys	Lys	Gly	Phe	Ser	Ile	Tyr	Trp	Asn	Ile
	1				5					10					15	
5	Pro	Thr	His	Phe	Cys	His	Asn	Phe	Gly	Val	Tyr	Phe	Lys	Glu	Leu	Lys
				20					25					30		
	Gln	Phe	Asn	Ile	Lys	Tyr	Asn	Ser	Met	Asn	Asn	Phe	Arg	Gly	Glu	Thr
			35				40						45			
	Ile	Ser	Leu	Phe	Tyr	Asp	Pro	Gly	Asn	Phe	Pro	Ser	Met	Val	Leu	Leu
		50				55					60					
10	Lys	Asn	Gly	Thr	Tyr	Glu	Ile	Arg	Asn	Glu	Gly	Val	Pro	Gln	Lys	Gly
	65					70					75				80	
	Asn	Leu	Thr	Ile	His	Leu	Glu	Gln	Phe	Thr	Lys	Glu	Leu	Asp	Glu	Ile
				85						90				95		
15	Tyr	Pro	Lys	Lys	Ile	Ala	Gly	Gly	Ile	Gly	Val	Ile	His	Phe	His	Asn
				100					105					110		
	Trp	Arg	Pro	Ile	Phe	Arg	Arg	Asn	Val	Asp	Asn	Leu	Lys	Ile	Asn	Lys
20				115				120				125				
	Asp	Ile	Ser	Ile	Asp	Leu	Val	Arg	Lys	Glu	His	Pro	Lys	Trp	Asp	Lys
		130					135					140				
	Ser	Met	Ile	Glu	Lys	Glu	Ala	Ser	Asn	Arg	Phe	Glu	Thr	Ser	Ala	Lys
	145					150					155				160	
25	Ile	Phe	Met	Glu	Lys	Thr	Leu	Lys	Leu	Ala	Lys	Glu	Ile	Arg	Lys	Lys
				165						170					175	
	Thr	Glu	Trp	Gly	Tyr	His	Gly	Tyr	Pro	His	Cys	Leu	Ser	Gly	Ser	Thr
				180					185					190		
30	Asp	Lys	Pro	Ser	Phe	Asp	Cys	Asp	Ala	Leu	Ser	Met	Ser	Glu	Asn	Asp
			195					200					205			
	Lys	Met	Ser	Trp	Leu	Phe	Asn	Asn	Gln	Asn	Val	Leu	Leu	Pro	Ser	Ile
		210					215					220				
	Tyr	Leu	Lys	Asn	Val	Leu	Lys	Pro	Asp	Glu	Lys	Ile	His	Leu	Val	Gln
	225					230					235				240	
35	Glu	Arg	Leu	Lys	Glu	Ala	Ile	Arg	Ile	Ser	Lys	Asn	Phe	Lys	His	Leu
				245						250					255	
	Pro	Lys	Val	Leu	Pro	Tyr	Trp	Trp	Tyr	Thr	Tyr	Gln	Asp	Lys	Glu	Ser
				260					265					270		
40	Ile	Phe	Leu	Thr	Glu	Ala	Asp	Val	Lys	Asn	Thr	Phe	Lys	Glu	Ile	Leu
			275					280					285			
	Thr	Asn	Gly	Ala	Asp	Gly	Ile	Ile	Ile	Trp	Gly	Val	Ser	Tyr	Glu	Leu
		290					295					300				
45	Thr	Asp	Arg	Lys	Arg	Cys	Glu	Lys	Leu	Lys	Glu	Tyr	Leu	Met	Lys	Ile
	305					310					315				320	
	Leu	Gly	Pro	Ile	Ala	Phe	Lys	Val	Thr	Lys	Ala	Val	Lys	Glu	Asn	Thr
					325					330					335	
	Pro	Leu	Asn	Phe												
				340												

<210> 13

<211> 382

<212> PRT

<213> Apis mellifera

55

<220>

<223> hyaluronidase

<400> 13

5	Met	Ser	Arg	Pro	Leu	Val	Ile	Thr	Glu	Gly	Met	Met	Ile	Gly	Val	Leu
	1				5					10				15		
	Leu	Met	Leu	Ala	Pro	Ile	Asn	Ala	Leu	Leu	Leu	Gly	Phe	Val	Gln	Ser
				20					25					30		
	Thr	Pro	Asp	Asn	Asn	Lys	Thr	Val	Arg	Glu	Phe	Asn	Val	Tyr	Trp	Asn
			35					40					45			
10	Val	Pro	Thr	Phe	Met	Cys	His	Lys	Tyr	Gly	Leu	Arg	Phe	Glu	Glu	Val
		50					55					60				
	Ser	Glu	Lys	Tyr	Gly	Ile	Leu	Gln	Asn	Trp	Met	Asp	Lys	Phe	Arg	Gly
	65				70						75					80
15	Glu	Glu	Ile	Ala	Ile	Leu	Tyr	Asp	Pro	Gly	Met	Phe	Pro	Ala	Leu	Leu
				85						90					95	
	Lys	Asp	Pro	Asn	Gly	Asn	Val	Val	Ala	Arg	Asn	Gly	Gly	Val	Pro	Gln
				100					105					110		
	Leu	Gly	Asn	Leu	Thr	Lys	His	Leu	Gln	Val	Phe	Arg	Asp	His	Leu	Ile
20			115					120					125			
	Asn	Gln	Ile	Pro	Asp	Lys	Ser	Phe	Pro	Gly	Val	Gly	Val	Ile	Asp	Phe
		130					135					140				
25	Glu	Ser	Trp	Arg	Pro	Ile	Phe	Arg	Gln	Asn	Trp	Ala	Ser	Leu	Gln	Pro
	145					150					155					160
	Tyr	Lys	Lys	Leu	Ser	Val	Glu	Val	Val	Arg	Arg	Glu	His	Pro	Phe	Trp
				165						170					175	
	Asp	Asp	Gln	Arg	Val	Glu	Gln	Glu	Ala	Lys	Arg	Arg	Phe	Glu	Lys	Tyr
30				180					185					190		
	Gly	Gln	Leu	Phe	Met	Glu	Glu	Thr	Leu	Lys	Ala	Ala	Lys	Arg	Met	Arg
			195					200					205			
	Pro	Ala	Ala	Asn	Trp	Gly	Tyr	Tyr	Ala	Tyr	Pro	Tyr	Cys	Tyr	Asn	Leu
		210				215						220				
35	Thr	Pro	Asn	Gln	Pro	Ser	Ala	Gln	Cys	Glu	Ala	Thr	Thr	Met	Gln	Glu
	225					230					235					240
	Asn	Asp	Lys	Met	Ser	Trp	Leu	Phe	Glu	Ser	Glu	Asp	Val	Leu	Leu	Pro
				245						250				255		
40	Ser	Val	Tyr	Leu	Arg	Trp	Asn	Leu	Thr	Ser	Gly	Glu	Arg	Val	Gly	Leu
				260					265					270		
	Val	Gly	Gly	Arg	Val	Lys	Glu	Ala	Leu	Arg	Ile	Ala	Arg	Gln	Met	Thr
			275					280					285			
	Thr	Ser	Arg	Lys	Lys	Val	Leu	Pro	Tyr	Tyr	Trp	Tyr	Lys	Tyr	Gln	Asp
45			290				295					300				
	Arg	Arg	Asp	Thr	Asp	Leu	Ser	Arg	Ala	Asp	Leu	Glu	Ala	Thr	Leu	Arg
	305					310					315					320
	Lys	Ile	Thr	Asp	Leu	Gly	Ala	Asp	Gly	Phe	Ile	Ile	Trp	Gly	Ser	Ser
				325						330					335	
50	Asp	Asp	Ile	Asn	Thr	Lys	Ala	Lys	Cys	Leu	Gln	Phe	Arg	Glu	Tyr	Leu
				340					345					350		
	Asn	Asn	Glu	Leu	Gly	Pro	Ala	Val	Lys	Arg	Ile	Ala	Leu	Asn	Asn	Asn
			355					360					365			
55	Ala	Asn	Asp	Arg	Leu	Thr	Val	Asp	Val	Ser	Val	Asp	Gln	Val		
			370				375					380				

<210> 14

<211> 331

<212> PRT

<213> Dolichovespula maculata

<220>

5 <223> hyaluronidase

<400> 14

10 Ser Glu Arg Pro Lys Arg Val Phe Asn Ile Tyr Trp Asn Val Pro Thr
 1 5 10 15
 Phe Met Cys His Gln Tyr Gly Leu Tyr Phe Asp Glu Val Thr Asn Phe
 20 25 30
 Asn Ile Lys His Asn Ser Lys Asp Asp Phe Gln Gly Asp Lys Ile Ser
 35 40 45
 15 Ile Phe Tyr Asp Pro Gly Glu Phe Pro Ala Leu Leu Pro Leu Lys Glu
 50 55 60
 Gly Asn Tyr Lys Ile Arg Asn Gly Gly Val Pro Gln Glu Gly Asn Ile
 65 70 75 80
 20 Thr Ile His Leu Gln Arg Phe Ile Glu Asn Leu Asp Lys Thr Tyr Pro
 85 90 95
 Asn Arg Asn Phe Asn Gly Ile Gly Val Ile Asp Phe Glu Arg Trp Arg

 25 Pro Ile Phe Arg Gln Asn Trp Gly Asn Met Met Ile His Lys Lys Phe
 100 105 110
 115 120 125
 Ser Ile Asp Leu Val Arg Asn Glu His Pro Phe Trp Asp Lys Lys Met
 130 135 140
 30 Ile Glu Leu Glu Ala Ser Lys Arg Phe Glu Lys Tyr Ala Arg Leu Phe
 145 150 155 160
 Met Glu Glu Thr Leu Lys Leu Ala Lys Lys Thr Arg Lys Gln Ala Asp
 165 170 175
 Trp Gly Tyr Tyr Gly Tyr Pro Tyr Cys Phe Asn Met Ser Pro Asn Asn
 180 185 190
 35 Leu Val Pro Asp Cys Asp Ala Thr Ala Met Leu Glu Asn Asp Lys Met
 195 200 205
 Ser Trp Leu Phe Asn Asn Gln Asn Val Leu Leu Pro Ser Val Tyr Ile
 210 215 220
 40 Arg His Glu Leu Thr Pro Asp Gln Arg Val Gly Leu Val Gln Gly Arg
 225 230 235 240
 Val Lys Glu Ala Val Arg Ile Ser Asn Asn Leu Lys His Ser Pro Lys
 245 250 255
 45 Val Leu Ser Tyr Trp Trp Tyr Val Tyr Gln Asp Asp Thr Asn Thr Phe
 260 265 270
 Leu Thr Glu Thr Asp Val Lys Lys Thr Phe Gln Glu Ile Ala Ile Asn
 275 280 285
 Gly Gly Asp Gly Ile Ile Ile Trp Gly Ser Ser Ser Asp Val Asn Ser
 290 295 300
 50 Leu Ser Lys Cys Lys Arg Leu Arg Glu Tyr Leu Leu Thr Val Leu Gly
 305 310 315 320
 Pro Ile Thr Val Asn Val Thr Glu Thr Val Asn
 325 330

55

<210> 15

<211> 367

<212> PRT

<213> Polistes annularis

<220>

<223> hyaluronidase

5

<400> 15

Tyr Val Ser Leu Ser Pro Asp Ser Val Phe Asn Ile Ile Thr Asp Asp
 1 5 10 15
 Ile Ser His Gln Ile Leu Ser Arg Ser Asn Cys Glu Arg Ser Lys Arg
 20 25 30
 Pro Lys Arg Val Phe Ser Ile Tyr Trp Asn Val Pro Thr Phe Met Cys
 35 40 45
 His Gln Tyr Gly Met Asn Phe Asp Glu Val Thr Asp Phe Asn Ile Lys
 50 55 60
 His Asn Ser Lys Asp Asn Phe Arg Gly Glu Thr Ile Ser Ile Tyr Tyr
 65 70 75 80
 Asp Pro Gly Lys Phe Pro Ala Leu Met Pro Leu Lys Asn Gly Asn Tyr
 85 90 95
 Glu Glu Arg Asn Gly Gly Val Pro Gln Arg Gly Asn Ile Thr Ile His
 100 105 110
 Leu Gln Gln Phe Asn Glu Asp Leu Asp Lys Met Thr Pro Asp Lys Asn
 115 120 125
 Phe Gly Gly Ile Gly Val Ile Asp Phe Glu Arg Trp Lys Pro Ile Phe
 130 135 140
 Arg Gln Asn Trp Gly Asn Thr Glu Ile His Lys Lys Tyr Ser Ile Glu
 145 150 155 160
 Leu Val Arg Lys Glu His Pro Lys Trp Ser Glu Ser Met Ile Glu Ala
 165 170 175
 Glu Ala Thr Lys Lys Phe Glu Lys Tyr Ala Arg Tyr Phe Met Glu Glu
 180 185 190
 Thr Leu Lys Leu Ala Lys Lys Thr Arg Lys Arg Ala Lys Trp Gly Tyr
 195 200 205
 Tyr Gly Phe Pro Tyr Cys Tyr Asn Val Thr Pro Asn Asn Pro Gly Pro
 210 215 220
 Asp Cys Asp Ala Lys Ala Thr Ile Glu Asn Asp Arg Leu Ser Trp Met
 225 230 235 240
 Tyr Asn Asn Gln Glu Ile Leu Phe Pro Ser Val Tyr Val Arg His Glu
 245 250 255
 Gln Lys Pro Glu Glu Arg Val Tyr Leu Val Gln Gly Arg Ile Lys Glu
 260 265 270
 Ala Val Arg Ile Ser Asn Asn Leu Glu His Ser Pro Ser Val Leu Ala
 275 280 285
 Tyr Trp Trp Tyr Val Tyr Gln Asp Lys Met Asp Ile Tyr Leu Ser Glu
 290 295 300
 Thr Asp Val Glu Lys Thr Phe Gln Glu Ile Val Thr Asn Gly Gly Asp
 305 310 315 320
 Gly Ile Ile Ile Trp Gly Ser Ser Ser Asp Val Asn Ser Leu Ser Lys
 325 330 335
 Cys Lys Arg Leu Arg Glu Tyr Leu Leu Asn Thr Leu Gly Pro Phe Ala
 340 345 350
 Val Asn Val Thr Glu Thr Val Asn Gly Arg Ser Ser Leu Asn Phe
 355 360 365

<210> 16
 <211> 462
 <212> PRT
 <213> Mus musculus

5

<220>
 <223> hyaluronidase

10

<400> 16

Met Leu Gly Leu Thr Gln His Ala Gln Lys Val Trp Arg Met Lys Pro
 1 5 10 15
 Phe Ser Pro Glu Val Ser Pro Gly Ser Ser Pro Ala Thr Ala Gly His
 20 25 30
 Leu Leu Arg Ile Ser Thr Leu Phe Leu Thr Leu Leu Glu Leu Ala Gln
 35 40 45
 Val Cys Arg Gly Ser Val Val Ser Asn Arg Pro Phe Ile Thr Val Trp
 50 55 60
 Asn Gly Asp Thr His Trp Cys Leu Thr Glu Tyr Gly Val Asp Val Asp
 65 70 75 80
 Val Ser Val Phe Asp Val Val Ala Asn Lys Glu Gln Ser Phe Gln Gly
 85 90 95
 Ser Asn Met Thr Ile Phe Tyr Arg Glu Glu Leu Gly Thr Tyr Pro Tyr
 100 105 110
 Tyr Thr Pro Thr Gly Glu Pro Val Phe Gly Gly Leu Pro Gln Asn Ala

30

35

40

45

50

55

		115				120				125				
	Ser	Leu	Val	Thr	His	Leu	Ala	His	Thr	Phe	Gln	Asp	Ile	Lys
		130					135				140			
5	Met	Pro	Glu	Pro	Asp	Phe	Ser	Gly	Leu	Ala	Val	Ile	Asp	Trp
	145					150					155			160
	Trp	Arg	Pro	Arg	Trp	Ala	Phe	Asn	Trp	Asp	Ser	Lys	Asp	Ile
					165					170				175
	Gln	Arg	Ser	Met	Glu	Leu	Val	Gln	Ala	Glu	His	Pro	Asp	Trp
10				180					185				190	
	Thr	Leu	Val	Glu	Ala	Ala	Ala	Lys	Asn	Gln	Phe	Gln	Glu	Ala
		195						200				205		
	Ala	Trp	Met	Ala	Gly	Thr	Leu	Gln	Leu	Gly	Gln	Val	Leu	Arg
	210						215				220			
15	Gly	Leu	Trp	Gly	Tyr	Tyr	Gly	Phe	Pro	Asp	Cys	Tyr	Asn	Asn
	225				230						235			240
	Leu	Ser	Leu	Asn	Tyr	Thr	Gly	Gln	Cys	Pro	Val	Phe	Val	Arg
				245					250					255
20	Asn	Asp	Gln	Leu	Gly	Trp	Leu	Trp	Asn	Gln	Ser	Tyr	Ala	Leu
			260						265				270	
	Ser	Ile	Tyr	Leu	Pro	Ala	Ala	Leu	Met	Gly	Thr	Gly	Lys	Ser
		275					280					285		
	Tyr	Val	Arg	His	Arg	Val	Gln	Glu	Ala	Leu	Arg	Val	Ala	Ile
25		290				295					300			
	Arg	Asp	Pro	His	Val	Pro	Val	Met	Pro	Tyr	Val	Gln	Ile	Phe
	305				310					315				320
	Met	Thr	Asp	Tyr	Leu	Pro	Leu	Glu	Glu	Leu	Glu	His	Ser	Leu
				325					330					335
30	Glu	Ser	Ala	Ala	Gln	Gly	Val	Ala	Gly	Ala	Val	Leu	Trp	Leu
			340					345					350	
	Asp	Lys	Thr	Ser	Thr	Lys	Glu	Ser	Cys	Gln	Ala	Ile	Lys	Ala
		355					360					365		
35	Asp	Ser	Thr	Leu	Gly	Pro	Phe	Ile	Val	Asn	Val	Thr	Ser	Ala
		370				375					380			
	Leu	Cys	Ser	Glu	Ala	Leu	Cys	Ser	Gly	His	Gly	Arg	Cys	Val
	385				390					395				400
	Pro	Ser	Tyr	Pro	Glu	Ala	Leu	Leu	Thr	Leu	Asn	Pro	Ala	Ser
				405					410					415
40	Ile	Glu	Leu	Thr	His	Asp	Gly	Arg	Pro	Pro	Ser	Leu	Lys	Gly
			420					425					430	
	Ser	Leu	Lys	Asp	Arg	Ala	Gln	Met	Ala	Met	Lys	Phe	Arg	Cys
		435					440				445			
45	Tyr	Arg	Gly	Trp	Arg	Gly	Lys	Trp	Cys	Asp	Lys	Arg	Gly	Met
		450				455					460			

<210> 17

<211> 473

50 <212> PRT

<213> Mus musculus

<220>

<223> Hyaluronidase 2

55

<400> 17

EP 4 269 578 B1

Met	Arg	Ala	Gly	Leu	Gly	Pro	Ile	Ile	Thr	Leu	Ala	Leu	Val	Leu	Glu
1				5					10					15	

5

10

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Val	Ala	Trp	Ala	Gly	Glu	Leu	Lys	Pro	Thr	Ala	Pro	Pro	Ile	Phe	Thr
				20					25					30		
	Gly	Arg	Pro	Phe	Val	Val	Ala	Trp	Asn	Val	Pro	Thr	Gln	Glu	Cys	Ala
			35					40					45			
5	Pro	Arg	His	Lys	Val	Pro	Leu	Asp	Leu	Arg	Ala	Phe	Asp	Val	Lys	Ala
		50					55					60				
	Thr	Pro	Asn	Glu	Gly	Phe	Phe	Asn	Gln	Asn	Ile	Thr	Thr	Phe	Tyr	Tyr
	65				70						75				80	
	Asp	Arg	Leu	Gly	Leu	Tyr	Pro	Arg	Phe	Asp	Ala	Ala	Gly	Thr	Ser	Val
10					85					90					95	
	His	Gly	Gly	Val	Pro	Gln	Asn	Gly	Ser	Leu	Cys	Ala	His	Leu	Pro	Met
			100					105						110		
	Leu	Lys	Glu	Ser	Val	Glu	Arg	Tyr	Ile	Gln	Thr	Gln	Glu	Pro	Gly	Gly
		115						120					125			
15	Leu	Ala	Val	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Val	Trp	Val	Arg	Asn
		130					135					140				
	Trp	Gln	Glu	Lys	Asp	Val	Tyr	Arg	Gln	Ser	Ser	Arg	Gln	Leu	Val	Ala
	145				150						155				160	
	Ser	Arg	His	Pro	Asp	Trp	Pro	Ser	Asp	Arg	Val	Met	Lys	Gln	Ala	Gln
20					165					170					175	
	Tyr	Glu	Phe	Glu	Phe	Ala	Ala	Arg	Gln	Phe	Met	Leu	Asn	Thr	Leu	Arg
			180						185					190		
	Tyr	Val	Lys	Ala	Val	Arg	Pro	Gln	His	Leu	Trp	Gly	Phe	Tyr	Leu	Phe
		195					200					205				
25	Pro	Asp	Cys	Tyr	Asn	His	Asp	Tyr	Val	Gln	Asn	Trp	Glu	Ser	Tyr	Thr
		210					215					220				
	Gly	Arg	Cys	Pro	Asp	Val	Glu	Val	Ala	Arg	Asn	Asp	Gln	Leu	Ala	Trp
	225				230					235					240	
30	Leu	Trp	Ala	Glu	Ser	Thr	Ala	Leu	Phe	Pro	Ser	Val	Tyr	Leu	Asp	Glu
				245						250					255	
	Thr	Leu	Ala	Ser	Ser	Val	His	Ser	Arg	Asn	Phe	Val	Ser	Phe	Arg	Val
			260						265					270		
	Arg	Glu	Ala	Leu	Arg	Val	Ala	His	Thr	His	His	Ala	Asn	His	Ala	Leu
		275					280						285			
35	Pro	Val	Tyr	Val	Phe	Thr	Arg	Pro	Thr	Tyr	Thr	Arg	Gly	Leu	Thr	Gly
		290					295					300				
	Leu	Ser	Gln	Val	Asp	Leu	Ile	Ser	Thr	Ile	Gly	Glu	Ser	Ala	Ala	Leu
	305				310					315					320	
40	Gly	Ser	Ala	Gly	Val	Ile	Phe	Trp	Gly	Asp	Ser	Glu	Asp	Ala	Ser	Ser
				325						330					335	
	Met	Glu	Thr	Cys	Gln	Tyr	Leu	Lys	Asn	Tyr	Leu	Thr	Gln	Leu	Leu	Val
			340						345					350		
	Pro	Tyr	Ile	Val	Asn	Val	Ser	Trp	Ala	Thr	Gln	Tyr	Cys	Ser	Trp	Thr
		355					360						365			
45	Gln	Cys	His	Gly	His	Gly	Arg	Cys	Val	Arg	Arg	Asn	Pro	Ser	Ala	Asn
		370					375					380				
	Thr	Phe	Leu	His	Leu	Asn	Ala	Ser	Ser	Phe	Arg	Leu	Val	Pro	Gly	His
	385				390					395					400	
	Thr	Pro	Ser	Glu	Pro	Gln	Leu	Arg	Pro	Glu	Gly	Gln	Leu	Ser	Glu	Ala
50				405						410					415	
	Asp	Leu	Asn	Tyr	Leu	Gln	Lys	His	Phe	Arg	Cys	Gln	Cys	Tyr	Leu	Gly
			420						425					430		
	Trp	Gly	Gly	Glu	Gln	Cys	Gln	Arg	Asn	Tyr	Lys	Gly	Ala	Ala	Gly	Asn
		435					440						445			
55	Ala	Ser	Arg	Ala	Trp	Ala	Gly	Ser	His	Leu	Thr	Ser	Leu	Leu	Gly	Leu
		450					455					460				

Val Ala Val Ala Leu Thr Trp Thr Leu
465 470

5 <210> 18
<211> 412
<212> PRT
<213> Mus musculus

10 <220>
<223> hyaluronidase 3

<400> 18

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Met	Ile	Met	His	Leu	Gly	Leu	Met	Met	Val	Val	Gly	Leu	Thr	Leu	Cys
	1				5					10					15	
	Leu	Met	His	Gly	Gln	Ala	Leu	Leu	Gln	Val	Pro	Glu	His	Pro	Phe	Ser
5				20					25					30		
	Val	Val	Trp	Asn	Val	Pro	Ser	Ala	Arg	Cys	Lys	Ala	His	Phe	Gly	Val
		35						40					45			
	His	Leu	Pro	Leu	Asp	Ala	Leu	Gly	Ile	Val	Ala	Asn	His	Gly	Gln	His
		50					55					60				
10	Phe	His	Gly	Gln	Asn	Ile	Ser	Ile	Phe	Tyr	Lys	Asn	Gln	Phe	Gly	Leu
	65				70					75						80
	Tyr	Pro	Tyr	Phe	Gly	Pro	Arg	Gly	Thr	Ala	His	Asn	Gly	Gly	Ile	Pro
				85						90					95	
	Gln	Ala	Val	Ser	Leu	Asp	His	His	Leu	Ala	Arg	Ala	Ala	His	Gln	Ile
15				100					105					110		
	Leu	His	Ser	Leu	Gly	Ser	Ser	Phe	Ala	Gly	Leu	Ala	Val	Leu	Asp	Trp
			115					120					125			
	Glu	Glu	Trp	Tyr	Pro	Leu	Trp	Ala	Gly	Asn	Trp	Gly	Pro	His	Arg	Gln
20		130					135					140				
	Val	Tyr	Leu	Ala	Ala	Ser	Trp	Val	Trp	Thr	Gln	Gln	Met	Phe	Pro	Gly
	145					150					155					160
	Leu	Asp	Pro	Gln	Glu	Gln	Leu	His	Lys	Ala	His	Thr	Ser	Phe	Glu	Gln
				165						170					175	
25	Ala	Ala	Arg	Ala	Leu	Met	Glu	Tyr	Thr	Leu	Gln	Leu	Gly	Arg	Thr	Leu
				180					185					190		
	Arg	Pro	Ser	Gly	Leu	Trp	Gly	Phe	Tyr	Arg	Tyr	Pro	Ala	Cys	Gly	Asn
			195					200					205			
	Gly	Trp	His	Lys	Met	Ala	Ser	Asn	Tyr	Thr	Gly	His	Cys	His	Ala	Ala
30		210					215					220				
	Ile	Thr	Thr	Gln	Asn	Thr	Gln	Leu	Arg	Trp	Leu	Trp	Ala	Ala	Ser	Ser
	225				230						235					240
	Ala	Leu	Phe	Pro	Ser	Ile	Tyr	Leu	Pro	Pro	Arg	Leu	Pro	Leu	Ala	Tyr
				245						250					255	
35	Arg	Gln	Ala	Phe	Val	Arg	His	Arg	Leu	Glu	Glu	Ala	Phe	Arg	Val	Ala
				260					265					270		
	Leu	Leu	Glu	His	Ser	His	Pro	Leu	Pro	Val	Leu	Ala	Tyr	Ser	Arg	Leu
			275					280					285			
40	Thr	His	Arg	Ser	Ser	Gly	Arg	Phe	Leu	Ser	Leu	Asp	Asp	Leu	Met	Gln
		290					295					300				
	Thr	Ile	Gly	Val	Ser	Ala	Ala	Leu	Gly	Thr	Ala	Gly	Val	Val	Leu	Trp
	305					310					315					320
	Gly	Asp	Leu	Ser	Phe	Ser	Ser	Ser	Glu	Glu	Lys	Cys	Trp	Arg	Leu	His
45				325						330					335	
	Asp	Tyr	Leu	Val	Gly	Thr	Leu	Gly	Pro	Tyr	Val	Ile	Asn	Val	Thr	Lys
				340					345					350		
50	Ala	Asp	Met	Ala	Cys	Ser	His	Gln	Arg	Cys	His	Gly	His	Gly	Arg	Cys
			355					360					365			
	Ala	Arg	Lys	Asp	Pro	Gly	Gln	Met	Glu	Ala	Phe	Leu	His	Leu	Gln	Pro
		370					375					380				
	Asp	Asp	Ser	Leu	Gly	Ala	Trp	Asn	Ser	Phe	Arg	Cys	His	Cys	Tyr	Ser
55		385				390					395					400
	Gly	Trp	Ala	Gly	Pro	Thr	Cys	Leu	Glu	Pro	Lys	Pro				
				405						410						

<210> 19
 <211> 435
 <212> PRT
 <213> Sus scrofa

5

<220>
 <223> hyaluronidase

10

<400> 19

	Met	Ala	Ala	His	Leu	Leu	Pro	Ile	Cys	Thr	Leu	Phe	Leu	Asn	Leu	Leu	
	1				5					10					15		
	Ser	Val	Ala	Gln	Gly	Ser	Arg	Asp	Pro	Val	Val	Leu	Asn	Arg	Pro	Phe	
15				20					25					30			
	Thr	Thr	Ile	Trp	Asn	Ala	Asn	Thr	Gln	Trp	Cys	Leu	Lys	Arg	His	Gly	
			35					40					45				
	Val	Asp	Val	Asp	Val	Ser	Val	Phe	Glu	Val	Val	Val	Asn	Pro	Gly	Gln	
		50					55					60					
20	Thr	Phe	Arg	Gly	Pro	Asn	Met	Thr	Ile	Phe	Tyr	Ser	Ser	Gln	Leu	Gly	
	65					70					75					80	
	Thr	Tyr	Pro	Tyr	Tyr	Thr	Ser	Ala	Gly	Glu	Pro	Val	Phe	Gly	Gly	Leu	
					85					90					95		
	Pro	Gln	Asn	Ala	Ser	Leu	Asp	Val	His	Leu	Asn	Arg	Thr	Phe	Lys	Asp	
25				100					105					110			
	Ile	Leu	Ala	Ala	Met	Pro	Glu	Ser	Asn	Phe	Ser	Gly	Leu	Ala	Val	Ile	
			115				120						125				
	Asp	Trp	Glu	Ala	Trp	Arg	Pro	Arg	Trp	Ala	Phe	Asn	Trp	Asp	Ala	Lys	
30			130				135					140					
	Asp	Ile	Tyr	Arg	Gln	Arg	Ser	Arg	Ala	Leu	Val	Gln	Lys	Gln	His	Pro	
	145					150					155					160	
	Asp	Trp	Pro	Ala	Pro	Trp	Val	Glu	Ala	Ala	Ala	Gln	Asp	Gln	Phe	Gln	
					165					170					175		
35	Glu	Ala	Ala	Gln	Thr	Trp	Met	Ala	Gly	Thr	Leu	Lys	Leu	Gly	Gln	Thr	
				180					185					190			
	Leu	Arg	Pro	His	Gly	Leu	Trp	Gly	Phe	Tyr	Gly	Phe	Pro	Asp	Cys	Tyr	
			195					200					205				
	Asn	Tyr	Asp	Phe	Gln	Ser	Ser	Asn	Tyr	Thr	Gly	Gln	Cys	Pro	Pro	Gly	
40		210				215						220					
	Val	Ser	Ala	Gln	Asn	Asp	Gln	Leu	Gly	Trp	Leu	Trp	Gly	Gln	Ser	Arg	
	225					230					235					240	
	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Pro	Ser	Ala	Leu	Glu	Gly	Thr	Asn	
					245					250					255		
45	Lys	Thr	Gln	Leu	Tyr	Val	Gln	His	Arg	Val	Asn	Glu	Ala	Phe	Arg	Val	
				260					265					270			
	Ala	Ala	Ala	Ala	Gly	Asp	Pro	Asn	Leu	Pro	Val	Leu	Pro	Tyr	Ala	Gln	
				275				280					285				

50

55

	Ile	Phe	His	Asp	Met	Thr	Asn	Arg	Leu	Leu	Ser	Arg	Glu	Glu	Leu	Glu
		290					295				300					
5	His	Ser	Leu	Gly	Glu	Ser	Ala	Ala	Gln	Gly	Ala	Ala	Gly	Val	Val	Leu
	305					310					315					320
	Trp	Val	Ser	Trp	Glu	Asn	Thr	Arg	Thr	Lys	Glu	Ser	Cys	Gln	Ser	Ile
					325					330					335	
	Lys	Glu	Tyr	Val	Asp	Thr	Thr	Leu	Gly	Pro	Phe	Ile	Leu	Asn	Val	Thr
				340					345					350		
10	Ser	Gly	Ala	Leu	Leu	Cys	Ser	Gln	Ala	Val	Cys	Ser	Gly	His	Gly	Arg
			355					360					365			
	Cys	Val	Arg	Arg	Pro	Ser	His	Thr	Glu	Ala	Leu	Pro	Ile	Leu	Asn	Pro
			370				375					380				
15	Ser	Ser	Phe	Ser	Ile	Lys	Pro	Thr	Pro	Gly	Gly	Gly	Pro	Leu	Thr	Leu
	385					390					395					400
	Gln	Gly	Ala	Leu	Ser	Leu	Lys	Asp	Arg	Val	Gln	Met	Ala	Glu	Glu	Phe
				405						410					415	
	Gln	Cys	Arg	Cys	Tyr	Pro	Gly	Trp	Arg	Gly	Thr	Trp	Cys	Glu	Gln	Gln
20				420					425					430		
	Gly	Thr	Arg													
			435													

<210> 20
 <211> 419
 <212> PRT
 <213> Sus scrofa

 <220>
 <223> hyaluronidase 3

 <400> 20

35

40

45

50

55

EP 4 269 578 B1

	Met	Thr	Met	Gln	Leu	Gly	Leu	Ala	Leu	Val	Leu	Gly	Val	Ala	Met	Cys
	1				5					10					15	
	Leu	Gly	Cys	Gly	Gln	Pro	Leu	Leu	Arg	Ala	Pro	Glu	Arg	Pro	Phe	Cys
5				20					25					30		
	Val	Leu	Trp	Asn	Val	Pro	Ser	Ala	Arg	Cys	Lys	Ala	Arg	Phe	Gly	Val
		35						40					45			
	His	Leu	Pro	Leu	Glu	Ala	Leu	Gly	Ile	Thr	Ala	Asn	His	Gly	Gln	Arg
		50					55					60				
10	Phe	His	Gly	Gln	Asn	Ile	Thr	Ile	Phe	Tyr	Lys	Ser	Gln	Leu	Gly	Leu
	65				70					75						80
	Tyr	Pro	Tyr	Phe	Gly	Pro	Arg	Gly	Thr	Ala	His	Asn	Gly	Gly	Ile	Pro
				85						90					95	
15	Gln	Ala	Val	Ser	Leu	Asp	His	His	Leu	Ala	Arg	Ala	Ala	Tyr	Gln	Ile
				100					105					110		
	His	Arg	Ser	Leu	Arg	Pro	Gly	Phe	Thr	Gly	Leu	Ala	Val	Leu	Asp	Trp
			115				120						125			
	Glu	Glu	Trp	Cys	Pro	Leu	Trp	Ala	Gly	Asn	Trp	Gly	Arg	Arg	Gln	Ala
20		130					135					140				
	Tyr	Gln	Ala	Ala	Ser	Cys	Ala	Trp	Ala	Gln	Arg	Val	Tyr	Pro	Asn	Leu
	145				150					155						160
	Asp	Pro	Gln	Glu	Gln	Leu	Cys	Lys	Ala	Arg	Ala	Gly	Phe	Glu	Glu	Ala
				165						170					175	
25	Ala	Arg	Ala	Leu	Met	Glu	Asp	Thr	Leu	Arg	Leu	Gly	Arg	Met	Leu	Arg
				180					185					190		
	Pro	His	Gly	Leu	Trp	Gly	Phe	Tyr	His	Tyr	Pro	Ala	Cys	Gly	Asn	Gly
30				195				200				205				
	Trp	His	Gly	Thr	Ala	Ser	Asn	Tyr	Thr	Gly	His	Cys	His	Ala	Ala	Ala
		210					215					220				
	Leu	Ala	Arg	Asn	Thr	Gln	Leu	Tyr	Trp	Leu	Trp	Ala	Ala	Ser	Ser	Ala
	225				230						235					240
35	Leu	Phe	Pro	Ser	Ile	Tyr	Leu	Pro	Pro	Gly	Leu	Pro	Pro	Ala	Tyr	His
				245						250					255	
	Gln	Ala	Phe	Val	Arg	Tyr	Arg	Leu	Glu	Glu	Ala	Phe	Arg	Val	Ala	Leu
				260					265					270		
	Val	Gly	His	Pro	His	Pro	Leu	Pro	Val	Leu	Ala	Tyr	Ala	Arg	Leu	Thr
40			275				280						285			
	His	Arg	Asn	Ser	Gly	Arg	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Gln	Thr
		290				295					300					
	Ile	Gly	Val	Ser	Ala	Ala	Leu	Gly	Ala	Ser	Gly	Val	Val	Leu	Trp	Gly
	305				310					315						320
45	Asp	Leu	Ser	Phe	Ser	Ser	Ser	Glu	Glu	Glu	Cys	Trp	His	Leu	Arg	Gly
				325						330					335	
	Tyr	Leu	Val	Gly	Thr	Leu	Gly	Pro	Tyr	Val	Ile	Asn	Val	Thr	Arg	Ala
				340					345					350		
50	Ala	Met	Ala	Cys	Ser	His	Gln	Arg	Cys	His	Gly	His	Gly	Arg	Cys	Ala
			355					360					365			
	Trp	Gln	Asp	Pro	Gly	Gln	Leu	Lys	Val	Phe	Leu	His	Leu	His	Pro	Gly
		370				375						380				
	Gly	Ser	Pro	Gly	Ala	Trp	Glu	Ser	Phe	Ser	Cys	Arg	Cys	Tyr	Trp	Gly
55		385			390					395						400
	Trp	Ala	Gly	Pro	Thr	Cys	Gln	Glu	Pro	Arg	Pro	Glu	Leu	Gly	Pro	Glu
				405						410					415	
	Glu	Ala	Thr													

<210> 21
 <211> 449
 <212> PRT
 <213> Rattus norvegicus

5

<220>
 <223> hyaluronidase 1

10

<400> 21

	Met	Lys	Pro	Phe	Ser	Pro	Glu	Val	Ser	Pro	Asp	Pro	Cys	Pro	Ala	Thr
	1				5					10					15	
	Ala	Ala	His	Leu	Leu	Arg	Thr	Tyr	Thr	Leu	Phe	Leu	Thr	Leu	Leu	Glu
15				20					25					30		
	Leu	Ala	Gln	Gly	Cys	Arg	Gly	Ser	Met	Val	Ser	Asn	Arg	Pro	Phe	Ile
		35						40					45			
	Thr	Val	Trp	Asn	Ala	Asp	Thr	His	Trp	Cys	Leu	Lys	Asp	His	Gly	Val
		50					55					60				
20	Asp	Val	Asp	Val	Ser	Val	Phe	Asp	Val	Val	Ala	Asn	Lys	Glu	Gln	Asn
	65					70					75					80
	Phe	Gln	Gly	Pro	Asn	Met	Thr	Ile	Phe	Tyr	Arg	Glu	Glu	Leu	Gly	Thr
				85						90					95	
25	Tyr	Pro	Tyr	Tyr	Thr	Pro	Thr	Gly	Glu	Pro	Val	Phe	Gly	Gly	Leu	Pro
				100					105					110		
	Gln	Asn	Ala	Ser	Leu	Val	Thr	His	Leu	Ala	His	Ala	Phe	Gln	Asp	Ile
			115					120					125			

30

35

40

45

50

55

	Lys	Ala	Ala	Met	Pro	Glu	Pro	Asp	Phe	Ser	Gly	Leu	Ala	Val	Ile	Asp
	130						135					140				
	Trp	Glu	Ala	Trp	Arg	Pro	Arg	Trp	Ala	Phe	Asn	Trp	Asp	Ser	Lys	Asp
5	145					150					155					160
	Ile	Tyr	Gln	Gln	Arg	Ser	Met	Glu	Leu	Val	Arg	Ala	Glu	His	Pro	Asp
					165						170					175
	Trp	Pro	Glu	Thr	Leu	Val	Glu	Ala	Glu	Ala	Gln	Gly	Gln	Phe	Gln	Glu
				180						185					190	
10	Ala	Ala	Glu	Ala	Trp	Met	Ala	Gly	Thr	Leu	Gln	Leu	Gly	Gln	Val	Leu
			195					200					205			
	Arg	Pro	Arg	Gly	Leu	Trp	Gly	Tyr	Tyr	Gly	Phe	Pro	Asp	Cys	Tyr	Asn
	210						215					220				
	Tyr	Asp	Phe	Leu	Ser	Pro	Asn	Tyr	Thr	Gly	Gln	Cys	Ser	Leu	Ser	Ile
15	225					230					235					240
	His	Asp	Gln	Asn	Asp	Gln	Leu	Gly	Trp	Leu	Trp	Asn	Gln	Ser	Tyr	Ala
				245						250					255	
	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Pro	Ala	Ala	Leu	Met	Gly	Thr	Gly	Lys
			260						265					270		
20	Ser	Gln	Met	Tyr	Val	Arg	Tyr	Arg	Val	Gln	Glu	Ala	Phe	Arg	Leu	Ala
		275					280						285			
	Leu	Val	Ser	Arg	Asp	Pro	His	Val	Pro	Ile	Met	Pro	Tyr	Val	Gln	Ile
	290					295						300				
25	Phe	Tyr	Glu	Lys	Thr	Asp	Tyr	Leu	Leu	Pro	Leu	Glu	Glu	Leu	Glu	His
	305					310					315					320
	Ser	Leu	Gly	Glu	Ser	Ala	Ala	Gln	Gly	Ala	Ala	Gly	Ala	Val	Leu	Trp
				325					330						335	
	Ile	Ser	Ser	Glu	Lys	Thr	Ser	Thr	Lys	Glu	Ser	Cys	Gln	Ala	Ile	Lys
30				340					345					350		
	Ala	Tyr	Met	Asp	Ser	Thr	Leu	Gly	Pro	Phe	Ile	Leu	Asn	Val	Thr	Ser
		355						360					365			
	Ala	Ala	Leu	Leu	Cys	Ser	Glu	Ala	Leu	Cys	Ser	Gly	Arg	Gly	Arg	Cys
	370						375					380				
35	Val	Arg	His	Pro	Ser	Tyr	Pro	Glu	Ala	Leu	Leu	Thr	Leu	Ser	Pro	Ala
	385					390					395					400
	Ser	Phe	Ser	Ile	Glu	Pro	Thr	His	Asp	Gly	Arg	Pro	Leu	Ser	Leu	Lys
				405						410					415	
40	Gly	Thr	Leu	Ser	Leu	Lys	Asp	Arg	Ala	Gln	Met	Ala	Met	Lys	Phe	Lys
			420						425				430			
	Cys	Arg	Cys	Tyr	Arg	Gly	Trp	Ser	Gly	Glu	Trp	Cys	Lys	Lys	Gln	Asp
			435					440					445			
45	Met															

<210> 22

<211> 473

<212> PRT

<213> Rattus norvegicus

<220>

<223> hyaluronidase 2

<400> 22

EP 4 269 578 B1

Met	Arg	Ala	Gly	Leu	Gly	Pro	Ile	Ile	Thr	Leu	Ala	Leu	Val	Leu	Glu
1				5					10					15	
Val	Ala	Trp	Ala	Ser	Glu	Leu	Lys	Pro	Thr	Ala	Pro	Pro	Ile	Phe	Thr

5

10

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

			20				25				30						
	Gly	Arg	Pro	Phe	Val	Val	Ala	Trp	Asn	Val	Pro	Thr	Gln	Glu	Cys	Ala	
			35					40					45				
5	Pro	Arg	His	Lys	Val	Pro	Leu	Asp	Leu	Arg	Ala	Phe	Asp	Val	Glu	Ala	
		50					55					60					
	Thr	Pro	Asn	Glu	Gly	Phe	Phe	Asn	Gln	Asn	Ile	Thr	Thr	Phe	Tyr	Tyr	
	65				70					75					80		
	Asp	Arg	Leu	Gly	Leu	Tyr	Pro	Arg	Phe	Asp	Ala	Ala	Gly	Met	Ser	Val	
				85						90					95		
10	His	Gly	Gly	Val	Pro	Gln	Asn	Gly	Ser	Leu	Cys	Ala	His	Leu	Pro	Met	
			100					105						110			
	Leu	Lys	Glu	Ala	Val	Glu	Arg	Tyr	Ile	Gln	Thr	Gln	Glu	Pro	Ala	Gly	
		115						120					125				
15	Leu	Ala	Val	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Val	Trp	Val	Arg	Asn	
		130					135					140					
	Trp	Gln	Glu	Lys	Asp	Val	Tyr	Arg	Gln	Ser	Ser	Arg	Gln	Leu	Val	Ala	
	145				150						155					160	
	Ser	Arg	His	Pro	Asp	Trp	Pro	Ser	Asp	Arg	Ile	Val	Lys	Gln	Ala	Gln	
				165					170						175		
20	Tyr	Glu	Phe	Glu	Phe	Ala	Ala	Arg	Gln	Phe	Met	Leu	Asn	Thr	Leu	Arg	
			180						185					190			
	Tyr	Val	Lys	Ala	Val	Arg	Pro	Gln	His	Leu	Trp	Gly	Phe	Tyr	Leu	Phe	
		195					200					205					
25	Pro	Asp	Cys	Tyr	Asn	His	Asp	Tyr	Val	Gln	Asn	Trp	Asp	Ser	Tyr	Thr	
		210					215					220					
	Gly	Arg	Cys	Pro	Asp	Val	Glu	Val	Ala	Gln	Asn	Asp	Gln	Leu	Ala	Trp	
	225				230					235					240		
	Leu	Trp	Ala	Glu	Asn	Thr	Ala	Leu	Phe	Pro	Ser	Val	Tyr	Leu	Asp	Lys	
				245					250					255			
30	Thr	Leu	Ala	Ser	Ser	Lys	His	Ser	Arg	Asn	Phe	Val	Ser	Phe	Arg	Val	
			260					265						270			
	Gln	Glu	Ala	Leu	Arg	Val	Ala	His	Thr	His	His	Ala	Asn	His	Ala	Leu	
		275					280						285				
35	Pro	Val	Tyr	Val	Phe	Thr	Arg	Pro	Thr	Tyr	Thr	Arg	Arg	Leu	Thr	Glu	
		290					295					300					
	Leu	Asn	Gln	Met	Asp	Leu	Ile	Ser	Thr	Ile	Gly	Glu	Ser	Ala	Ala	Leu	
	305				310						315					320	
	Gly	Ser	Ala	Gly	Val	Ile	Phe	Trp	Gly	Asp	Ser	Val	Tyr	Ala	Ser	Ser	
				325					330					335			
40	Met	Glu	Asn	Cys	Gln	Asn	Leu	Lys	Lys	Tyr	Leu	Thr	Gln	Thr	Leu	Val	
			340					345					350				
	Pro	Tyr	Ile	Val	Asn	Val	Ser	Trp	Ala	Thr	Gln	Tyr	Cys	Ser	Trp	Thr	
		355					360						365				
45	Gln	Cys	His	Gly	His	Gly	Arg	Cys	Val	Arg	Arg	Asn	Pro	Ser	Ala	Ser	
		370				375						380					
	Thr	Phe	Leu	His	Leu	Ser	Pro	Ser	Ser	Phe	Arg	Leu	Val	Pro	Gly	Arg	
	385				390					395					400		
	Thr	Pro	Ser	Glu	Pro	Gln	Leu	Arg	Pro	Glu	Gly	Glu	Leu	Ser	Glu	Asp	
				405						410				415			
50	Asp	Leu	Ser	Tyr	Leu	Gln	Met	His	Phe	Arg	Cys	His	Cys	Tyr	Leu	Gly	
			420					425					430				
	Trp	Gly	Gly	Glu	Gln	Cys	Gln	Trp	Asn	His	Lys	Arg	Ala	Ala	Gly	Asp	
		435					440					445					
55	Ala	Ser	Arg	Ala	Trp	Ala	Gly	Ala	His	Leu	Ala	Ser	Leu	Leu	Gly	Leu	
		450				455					460						
	Val	Ala	Met	Thr	Leu	Thr	Trp	Thr	Leu								

465

470

5 <210> 23
 <211> 412
 <212> PRT
 <213> Rattus norvegicus

10 <220>
 <223> hyaluronidase 3

 <400> 23

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Met	Ile	Thr	Gln	Leu	Gly	Leu	Thr	Leu	Val	Val	Gly	Leu	Thr	Leu	Cys
	1				5					10					15	
	Leu	Val	His	Val	Gln	Ala	Leu	Leu	Gln	Val	Pro	Glu	Phe	Pro	Phe	Ser
5				20					25					30		
	Val	Leu	Trp	Asn	Val	Pro	Ser	Ala	Arg	Cys	Lys	Thr	Arg	Phe	Gly	Val
		35						40					45			
	His	Leu	Pro	Leu	Asp	Ala	Leu	Gly	Ile	Ile	Ala	Asn	His	Gly	Gln	Arg
		50					55					60				
10	Phe	His	Gly	Gln	Asn	Ile	Thr	Ile	Phe	Tyr	Lys	Asn	Gln	Phe	Gly	Leu
	65				70					75						80
	Tyr	Pro	Tyr	Phe	Gly	Pro	Arg	Gly	Thr	Ala	His	Asn	Gly	Gly	Ile	Pro
				85						90					95	
	Gln	Ala	Val	Ser	Leu	Asp	His	His	Leu	Ala	Gln	Ala	Ala	His	Gln	Ile
15				100					105					110		
	Leu	His	Asn	Leu	Gly	Ser	Ser	Phe	Ala	Gly	Leu	Ala	Val	Leu	Asp	Trp
			115					120					125			
	Glu	Glu	Trp	Tyr	Pro	Leu	Trp	Ala	Gly	Asn	Trp	Gly	Thr	His	Arg	Gln
20		130					135					140				
	Val	Tyr	Gln	Ala	Ala	Ser	Trp	Ala	Trp	Ala	Gln	Met	Phe	Pro	Asp	
	145				150					155					160	
	Leu	Asn	Pro	Gln	Glu	Gln	Leu	His	Lys	Ala	Gln	Thr	Gly	Phe	Glu	Gln
				165					170						175	
25	Ala	Ala	Arg	Ala	Leu	Met	Glu	His	Thr	Leu	Arg	Leu	Gly	Gln	Met	Leu
				180					185					190		
	Arg	Pro	His	Gly	Leu	Trp	Gly	Phe	Tyr	Arg	Tyr	Pro	Val	Cys	Gly	Asn
			195					200					205			
	Gly	Trp	His	Asn	Met	Ala	Ser	Asn	Tyr	Thr	Gly	His	Cys	His	Pro	Ala
30		210					215					220				
	Ile	Ile	Thr	Arg	Asn	Thr	Gln	Leu	Arg	Trp	Leu	Trp	Ala	Ala	Ser	Ser
	225				230						235					240
	Ala	Leu	Phe	Pro	Ser	Ile	Tyr	Leu	Pro	Pro	Arg	Leu	Pro	Pro	Ala	Tyr
				245					250						255	
35	His	Gln	Thr	Phe	Val	Arg	His	Arg	Leu	Glu	Glu	Ala	Phe	Arg	Val	Ala
				260				265						270		
	Leu	Thr	Gly	His	Ala	His	Pro	Leu	Pro	Val	Leu	Ala	Tyr	Val	Arg	Leu
			275				280						285			
40	Thr	His	Arg	Ser	Ser	Gly	Arg	Phe	Leu	Ser	Leu	Asp	Asp	Leu	Met	Gln
		290					295					300				
	Thr	Ile	Gly	Val	Ser	Ala	Ala	Leu	Gly	Ala	Ala	Gly	Val	Val	Leu	Trp
	305					310					315					320
	Gly	Asp	Leu	Ser	Val	Ser	Ser	Ser	Glu	Glu	Glu	Cys	Trp	Arg	Leu	His
45				325						330					335	
	Asp	Tyr	Leu	Val	Gly	Thr	Leu	Gly	Pro	Tyr	Val	Ile	Asn	Val	Thr	Lys
				340					345					350		
50	Ala	Ala	Thr	Ala	Cys	Ser	His	Gln	Arg	Cys	His	Gly	His	Gly	Arg	Cys
			355					360					365			
	Ser	Trp	Lys	Asp	Pro	Gly	Gln	Met	Glu	Ala	Phe	Leu	His	Leu	Gln	Pro
		370					375					380				
	Asp	Asp	Asn	Leu	Gly	Ala	Trp	Lys	Ser	Phe	Arg	Cys	Arg	Cys	Tyr	Leu
55					390						395					400
	Gly	Trp	Ser	Gly	Pro	Thr	Cys	Leu	Glu	Pro	Lys	Pro				
					405					410						

<210> 24
 <211> 545
 <212> PRT
 <213> Oryctolagus cuniculus

5

<220>
 <223> PH20

10

<400> 24

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Gly	Ser	Ala	Val	Glu
	1				5					10					15	
	Leu	Ser	Gly	Val	Phe	Gln	Ile	Val	Phe	Ile	Phe	Leu	Leu	Ile	Pro	Cys
15				20					25					30		
	Cys	Leu	Thr	Ala	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
		35					40					45				
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Thr	Glu	Phe	Cys	Leu	Gly	Lys	Ser
		50				55					60					
20	Gly	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Leu	Phe	Gly	Ser	Pro	Arg
	65				70					75					80	
	Lys	Asn	Lys	Thr	Gly	Gln	Gly	Ile	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
				85					90					95		
	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Pro	His	Thr	Gly	Ala	Ile	Val	His	Gly
25				100				105						110		
	Arg	Ile	Pro	Gln	Leu	Gly	Pro	Leu	Gln	Gln	His	Leu	Thr	Lys	Leu	Arg
		115					120					125				
	Gln	Glu	Ile	Leu	Tyr	Tyr	Met	Pro	Lys	Asp	Asn	Val	Gly	Leu	Ala	Val
30		130					135				140					
	Ile	Asp	Trp	Glu	Glu	Trp	Leu	Pro	Thr	Trp	Leu	Arg	Asn	Trp	Lys	Pro
	145				150					155					160	
	Lys	Asp	Ile	Tyr	Arg	Ile	Lys	Ser	Ile	Glu	Leu	Val	Lys	Ser	Gln	His
				165					170						175	
35	Pro	Gln	Tyr	Asn	His	Ser	Tyr	Ala	Thr	Glu	Lys	Ala	Lys	Arg	Asp	Phe
				180				185						190		
	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Met	Glu	Glu	Thr	Leu	Lys	Leu	Gly	Arg
		195					200					205				
	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
40		210					215					220				
	Tyr	Asn	His	His	Tyr	Asp	Lys	Pro	Asn	Leu	Tyr	Lys	Gly	Ser	Cys	Phe
	225				230					235					240	
	Asp	Ile	Glu	Lys	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Lys	Glu
				245					250					255		
45	Ser	Thr	Ala	Leu	Phe	Pro	Ser	Val	Tyr	Leu	Thr	Ser	Arg	Ala	Arg	Ser
				260				265						270		
	Ala	Thr	Ala	Leu	Ser	Lys	Leu	Tyr	Val	Val	Arg	Asn	Arg	Val	His	Glu
		275					280					285				
50	Ala	Ile	Arg	Val	Ser	Lys	Ile	Pro	Asp	Asp	Lys	Ser	Pro	Leu	Pro	Asn

55

EP 4 269 578 B1

		290				295				300							
		Phe	Val	Tyr	Thr	Arg	Leu	Val	Phe	Thr	Asp	Gln	Ile	Phe	Gln	Phe	Leu
		305					310					315					320
5		Ser	His	His	Asp	Leu	Val	Tyr	Thr	Ile	Gly	Glu	Ile	Val	Ala	Leu	Gly
						325					330						335
		Ala	Ser	Gly	Ile	Val	Val	Trp	Gly	Ser	Gln	Ser	Leu	Ala	Arg	Ser	Met
					340					345					350		
		Lys	Ser	Cys	Leu	His	Leu	Asp	Asn	Tyr	Met	Lys	Thr	Ile	Leu	Asn	Pro
10				355					360					365			
		Tyr	Leu	Ile	Asn	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Asn	Gln	Val	Leu
			370					375					380				
		Cys	Gln	Glu	Gln	Gly	Val	Cys	Thr	Arg	Lys	Asn	Trp	Asn	Pro	Asn	Asp
		385				390						395					400
15		Tyr	Leu	His	Leu	Asn	Pro	Gly	Asn	Phe	Ala	Ile	Gln	Leu	Gly	Ser	Asn
					405					410							415
		Gly	Thr	Tyr	Lys	Val	Asp	Gly	Lys	Pro	Thr	Leu	Thr	Asp	Leu	Glu	Gln
					420					425					430		
		Phe	Ser	Lys	Asn	Phe	Gln	Cys	Ser	Cys	Tyr	Thr	Asn	Leu	Asn	Cys	Lys
20				435					440					445			
		Glu	Arg	Thr	Asp	Met	Asn	Asn	Val	Arg	Thr	Val	Asn	Val	Cys	Ala	Val
			450					455					460				
		Glu	Asn	Val	Cys	Ile	Asp	Thr	Asn	Val	Gly	Pro	Gln	Ala	Val	Thr	Tyr
25						470					475						480
		Ala	Pro	Lys	Glu	Lys	Lys	Asp	Val	Ala	His	Ile	Leu	Ser	Asn	Thr	Thr
					485					490							495
		Ser	Ile	Asn	Ser	Ser	Thr	Thr	Met	Ser	Leu	Pro	Phe	Pro	Arg	Lys	His
				500					505					510			
30		Val	Ser	Gly	Cys	Leu	Leu	Val	Leu	Cys	Met	Tyr	Ser	Gln	Tyr	Leu	Asn
			515					520					525				
		Ile	Cys	Tyr	Arg	Leu	Val	Ala	Ile	Gly	Ile	Gln	His	Gly	Tyr	Tyr	Leu
			530					535					540				
		Lys															
35		545															

<210> 25
 <211> 476
 <212> PRT
 40 <213> Ovis aries

 <220>
 <223> hyaluronidase 2

 45 <400> 25

 50

 55

EP 4 269 578 B1

	Met	Trp	Thr	Gly	Leu	Gly	Pro	Ala	Val	Thr	Leu	Ala	Leu	Val	Leu	Val
	1				5					10					15	
	Val	Ala	Trp	Ala	Thr	Glu	Leu	Lys	Pro	Thr	Ala	Pro	Pro	Ile	Phe	Thr
				20					25					30		
5	Gly	Arg	Pro	Phe	Val	Val	Ala	Trp	Asp	Val	Pro	Thr	Gln	Asp	Cys	Gly
			35					40					45			
	Pro	Arg	His	Lys	Met	Pro	Leu	Asp	Pro	Lys	Asp	Met	Lys	Ala	Phe	Asp
		50					55					60				
10	Val	Gln	Ala	Ser	Pro	Asn	Glu	Gly	Phe	Val	Asn	Gln	Asn	Ile	Thr	Ile
	65					70					75					80
	Phe	Tyr	Arg	Asp	Arg	Leu	Gly	Met	Tyr	Pro	His	Phe	Asn	Ser	Val	Gly
					85					90					95	

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Arg	Ser	Val	His	Gly	Gly	Val	Pro	Gln	Asn	Gly	Ser	Leu	Trp	Val	His
				100					105					110		
	Leu	Glu	Met	Leu	Lys	Gly	His	Val	Glu	His	Tyr	Ile	Arg	Thr	Gln	Glu
5			115					120					125			
	Pro	Ala	Gly	Leu	Ala	Val	Ile	Asp	Trp	Glu	Asp	Trp	Arg	Pro	Val	Trp
			130				135					140				
	Val	Arg	Asn	Trp	Gln	Asp	Lys	Asp	Val	Tyr	Arg	Arg	Leu	Ser	Arg	Gln
	145				150						155					160
10	Leu	Val	Ala	Ser	His	His	Pro	Asp	Trp	Pro	Pro	Glu	Arg	Ile	Val	Lys
					165					170					175	
	Glu	Ala	Gln	Tyr	Glu	Phe	Glu	Phe	Ala	Ala	Arg	Gln	Phe	Met	Leu	Glu
				180					185					190		
	Thr	Leu	Arg	Phe	Val	Lys	Ala	Phe	Arg	Pro	Arg	His	Leu	Trp	Gly	Phe
15			195					200					205			
	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His	Asp	Tyr	Val	Gln	Asn	Trp	Glu
			210				215					220				
	Thr	Tyr	Thr	Gly	Arg	Cys	Pro	Asp	Val	Glu	Val	Ser	Arg	Asn	Asp	Gln
	225				230						235					240
20	Leu	Ser	Trp	Leu	Trp	Ala	Glu	Ser	Thr	Ala	Leu	Phe	Pro	Ser	Val	Tyr
				245						250					255	
	Leu	Glu	Glu	Thr	Leu	Ala	Ser	Ser	Thr	His	Gly	Arg	Asn	Phe	Val	Ser
				260					265					270		
25	Phe	Arg	Val	Gln	Glu	Ala	Leu	Arg	Val	Ala	Asp	Val	His	His	Ala	Asn
			275					280					285			
	His	Ala	Leu	Pro	Val	Tyr	Val	Phe	Thr	Arg	Pro	Thr	Tyr	Ser	Arg	Gly
			290				295					300				
	Leu	Thr	Gly	Leu	Ser	Glu	Met	Asp	Leu	Ile	Ser	Thr	Ile	Gly	Glu	Ser
30	305					310					315					320
	Ala	Ala	Leu	Gly	Ala	Ala	Gly	Val	Ile	Leu	Trp	Gly	Asp	Ala	Gly	Phe
				325						330					335	
	Thr	Thr	Ser	Asn	Glu	Thr	Cys	Arg	Arg	Leu	Lys	Asp	Tyr	Leu	Thr	Arg
				340				345						350		
35	Ser	Leu	Val	Pro	Tyr	Val	Val	Asn	Val	Ser	Trp	Ala	Ala	Gln	Tyr	Cys
			355					360					365			
	Ser	Trp	Ala	Gln	Cys	His	Gly	His	Gly	Arg	Cys	Val	Arg	Arg	Asp	Pro
			370				375					380				
40	Asn	Ala	His	Thr	Phe	Leu	His	Leu	Ser	Ala	Ser	Ser	Phe	Arg	Leu	Val
	385					390					395					400
	Pro	Ser	His	Ala	Pro	Asp	Glu	Pro	Arg	Leu	Arg	Pro	Glu	Gly	Glu	Leu
				405						410					415	
	Ser	Trp	Ala	Asp	Arg	Asn	His	Leu	Gln	Thr	His	Phe	Arg	Cys	Gln	Cys
45				420					425				430			
	Tyr	Leu	Gly	Trp	Gly	Gly	Glu	Gln	Cys	Gln	Trp	Asp	Arg	Arg	Arg	Ala
			435					440				445				
	Ala	Gly	Gly	Ala	Ser	Gly	Ala	Trp	Ala	Gly	Ser	His	Leu	Thr	Gly	Leu
			450				455					460				
50	Leu	Ala	Val	Ala	Val	Leu	Ala	Phe	Thr	Trp	Thr	Ser				
	465					470					475					

<210> 26

<211> 414

55 <212> PRT

<213> Pongo pygmaeus

<220>

<223> hyaluronidase 3

<400> 26

5

10

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Met	Thr	Thr	Arg	Leu	Gly	Pro	Ala	Leu	Val	Leu	Gly	Val	Ala	Leu	Cys
	1				5					10					15	
5	Leu	Gly	Cys	Gly	Gln	Pro	Leu	Pro	Gln	Val	Pro	Glu	Arg	Pro	Phe	Ser
				20					25					30		
	Val	Leu	Trp	Asn	Val	Pro	Ser	Ala	His	Cys	Lys	Ser	Arg	Phe	Gly	Val
		35						40					45			
	His	Leu	Pro	Leu	Asn	Ala	Leu	Gly	Ile	Ile	Ala	Asn	Arg	Gly	Gln	His
		50					55					60				
10	Phe	His	Gly	Gln	Asn	Met	Thr	Ile	Phe	Tyr	Lys	Asn	Gln	Leu	Gly	Leu
	65					70					75					80
	Tyr	Pro	Tyr	Phe	Gly	Pro	Lys	Gly	Thr	Ala	His	Asn	Gly	Gly	Ile	Pro
				85						90					95	
15	Gln	Ala	Leu	Pro	Leu	Asp	Arg	His	Leu	Ala	Leu	Ala	Ala	Tyr	Gln	Ile
				100					105					110		
	His	His	Ser	Leu	Arg	Pro	Gly	Phe	Ala	Gly	Pro	Ala	Val	Leu	Asp	Trp
			115					120					125			
	Glu	Glu	Trp	Cys	Pro	Leu	Trp	Ala	Gly	Asn	Trp	Gly	Arg	Arg	Arg	Ala
		130					135					140				
20	Tyr	Gln	Ala	Ala	Ser	Trp	Ala	Trp	Ala	Gln	Gln	Val	Phe	Pro	Asp	Leu
	145					150					155					160
	Asp	Pro	Gln	Glu	Gln	Leu	Tyr	Lys	Ala	Tyr	Thr	Gly	Phe	Glu	Gln	Ala
				165						170					175	
25	Ala	Arg	Ala	Leu	Met	Glu	Asp	Thr	Leu	Arg	Val	Ala	Gln	Ala	Leu	Arg
				180					185					190		
	Pro	His	Gly	Leu	Trp	Gly	Phe	Tyr	His	Tyr	Pro	Ala	Cys	Gly	Asn	Gly
			195					200					205			
30	Trp	His	Ser	Met	Ala	Ser	Asn	Tyr	Thr	Gly	Arg	Cys	His	Ala	Ala	Thr
		210					215					220				
	Leu	Ala	Arg	Asn	Thr	Gln	Leu	His	Trp	Leu	Trp	Ala	Ala	Ser	Ser	Ala
	225				230						235					240
	Leu	Phe	Pro	Ser	Ile	Tyr	Leu	Pro	Pro	Arg	Leu	Pro	Pro	Ala	His	His
				245						250					255	
35	Gln	Ala	Phe	Val	Arg	His	Arg	Leu	Glu	Glu	Ala	Phe	Arg	Val	Ala	Leu
				260					265					270		
	Val	Gly	His	Leu	Pro	Val	Leu	Ala	Tyr	Val	Arg	Leu	Thr	His	Arg	Arg
			275					280					285			
40	Ser	Gly	Arg	Phe	Leu	Ser	Gln	Asp	Asp	Leu	Val	Gln	Thr	Ile	Gly	Val
		290					295					300				
	Ser	Ala	Ala	Leu	Gly	Ala	Gly	Val	Val	Leu	Trp	Gly	Asp	Leu	Ser	
	305				310					315					320	
	Leu	Ser	Ser	Ser	Glu	Glu	Glu	Cys	Trp	His	Leu	His	Asp	Tyr	Leu	Val
				325						330					335	
45	Asp	Thr	Leu	Gly	Pro	Tyr	Gly	Ile	Asn	Val	Thr	Arg	Ala	Ala	Met	Ala
				340					345					350		
	Cys	Ser	His	Gln	Arg	Cys	His	Gly	His	Gly	Arg	Cys	Ala	Arg	Arg	Asp
			355					360					365			
50	Pro	Gly	Gln	Met	Glu	Ala	Phe	Leu	His	Leu	Trp	Pro	Asp	Gly	Ser	Leu
		370					375					380				
	Gly	Asp	Trp	Lys	Ser	Phe	Ser	Cys	His	Cys	Tyr	Trp	Gly	Trp	Ala	Gly
	385				390						395					400
55	Pro	Thr	Cys	Gln	Glu	Pro	Arg	Leu	Gly	Pro	Lys	Glu	Ala	Val		
				405						410						

<210> 27

<211> 510
 <212> PRT
 <213> Macaca fascicularis

5 <220>
 <223> PH20

<400> 27

```

10      Met Gly Val Leu Lys Phe Lys His Ile Phe Phe Arg Ser Phe Val Lys
        1      5      10      15
        Ser Ser Gly Val Ser Gln Ile Val Phe Thr Phe Leu Leu Ile Pro Cys
        20      25      30
15      Cys Leu Thr Leu Asn Phe Arg Ala Pro Pro Ile Ile Pro Asn Val Pro
        35      40      45
        Phe Leu Trp Ala Trp Asn Ala Pro Ser Glu Phe Cys Leu Gly Lys Phe
        50      55      60
        Asn Glu Pro Leu Asp Met Ser Leu Phe Thr Leu Met Gly Ser Pro Arg
        65      70      75      80
20      Ile Asn Val Thr Gly Gln Gly Val Thr Ile Phe Tyr Val Asp Arg Leu
        85      90      95
        Gly Tyr Tyr Pro Tyr Ile Asp Leu Thr Thr Gly Val Thr Val His Gly
        100      105      110
25      Gly Ile Pro Gln Lys Val Ser Leu Gln Asp His Leu Asp Lys Ser Lys
        115      120      125
        Gln Asp Ile Leu Phe Tyr Met Pro Val Asp Asn Leu Gly Met Ala Val
        130      135      140
30      Ile Asp Trp Glu Glu Trp Arg Pro Thr Trp Ala Arg Asn Trp Lys Pro
        145      150      155      160
        Lys Asp Val Tyr Lys Asn Arg Ser Ile Glu Leu Val Gln Gln Gln Asn
        165      170      175
        Val Gln Leu Ser Leu Pro Gln Ala Thr Asp Lys Ala Lys Gln Glu Phe
        180      185      190
35      Glu Lys Ala Gly Lys Asp Phe Met Leu Glu Thr Ile Lys Leu Gly Arg
        195      200      205
        Ser Leu Arg Pro Asn His Leu Trp Gly Tyr Tyr Leu Phe Pro Asp Cys
        210      215      220
40      Tyr Asn His His Tyr Arg Lys Pro Gly Tyr Asn Gly Ser Cys Phe Asp
        225      230      235      240
        Val Glu Ile Lys Arg Asn Asp Asp Leu Ser Trp Leu Trp Asn Glu Ser
        245      250      255
45      Thr Ala Leu Tyr Pro Ser Ile Tyr Leu Asn Thr Gln Gln Ser Val Val
        260      265      270
        Val Ala Thr Leu Tyr Val Arg Asn Arg Val Arg Glu Ala Ile Arg Val
        275      280      285
        Ser Lys Ile Pro Asp Ala Lys Asn Pro Leu Pro Val Phe Val Tyr Ala
        290      295      300
50      Arg Leu Val Phe Thr Asp Gln Val Leu Lys Phe Leu Ser Arg Glu Glu
        305      310      315      320
        Leu Val Ser Thr Leu Gly Glu Thr Val Ala Leu Gly Ala Ser Gly Ile
        325      330      335
55      Val Ile Trp Gly Ser Leu Ser Ile Thr Arg Ser Met Lys Ser Cys Leu
        340      345      350
        Leu Leu Asp Thr Tyr Met Glu Thr Ile Leu Asn Pro Tyr Ile Ile Asn
        355      360      365

```

	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
		370					375					380				
5	Gly	Val	Cys	Ile	Arg	Lys	Asp	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
	385					390					395					400
	Asn	Pro	Asp	Asn	Phe	Asp	Ile	Arg	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
					405					410					415	
	Val	His	Gly	Lys	Pro	Thr	Val	Glu	Asp	Leu	Glu	Glu	Phe	Ser	Glu	Lys
				420					425					430		
10	Phe	Tyr	Cys	Ser	Cys	Tyr	Thr	Asn	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
			435					440					445			
	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
		450					455					460				
15	Ile	Asp	Ala	Ser	Leu	Lys	Pro	Pro	Val	Glu	Thr	Glu	Gly	Ser	Pro	Pro
	465					470					475					480
	Ile	Phe	Tyr	Asn	Thr	Ser	Ser	Ser	Thr	Val	Ser	Thr	Thr	Met	Phe	Ile
					485					490					495	
20	Val	Asn	Ile	Leu	Phe	Leu	Ile	Ile	Ser	Ser	Val	Ala	Ser	Leu		
				500					505					510		

<210> 28

<211> 529

<212> PRT

25 <213> Cavia porcellus

<220>

<223> PH20

30 <400> 28

35

40

45

50

55

EP 4 269 578 B1

	Met	Gly	Ala	Phe	Thr	Phe	Lys	His	Ser	Phe	Phe	Gly	Ser	Phe	Val	Glu
	1				5					10					15	
	Cys	Ser	Gly	Val	Leu	Gln	Thr	Val	Phe	Ile	Phe	Leu	Leu	Ile	Pro	Cys
5			20						25					30		
	Cys	Leu	Ala	Asp	Lys	Arg	Ala	Pro	Pro	Leu	Ile	Pro	Asn	Val	Pro	Leu
			35					40					45			
	Leu	Trp	Val	Trp	Asn	Ala	Pro	Thr	Glu	Phe	Cys	Ile	Gly	Gly	Thr	Asn
	50						55					60				
10	Gln	Pro	Leu	Asp	Met	Ser	Phe	Phe	Ser	Ile	Val	Gly	Thr	Pro	Arg	Lys
	65					70					75					80
	Asn	Ile	Thr	Gly	Gln	Ser	Ile	Thr	Leu	Tyr	Tyr	Val	Asp	Arg	Leu	Gly
				85						90					95	
	Tyr	Tyr	Pro	Tyr	Ile	Asp	Pro	His	Thr	Gly	Ala	Ile	Val	His	Gly	Gly
15				100					105					110		
	Leu	Pro	Gln	Leu	Met	Asn	Leu	Gln	Gln	His	Leu	Arg	Lys	Ser	Arg	Gln
			115					120					125			
	Asp	Ile	Leu	Phe	Tyr	Met	Pro	Thr	Asp	Ser	Val	Gly	Leu	Ala	Val	Ile
20			130				135					140				
	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Thr	Arg	Asn	Trp	Arg	Pro	Lys
	145					150					155					160
	Asp	Ile	Tyr	Arg	Asn	Lys	Ser	Ile	Glu	Leu	Val	Lys	Ser	Gln	His	Pro
					165					170					175	
25	Gln	Tyr	Asn	His	Ser	Tyr	Ala	Val	Ala	Val	Ala	Lys	Arg	Asp	Phe	Glu
				180					185					190		
	Arg	Thr	Gly	Lys	Ala	Phe	Met	Leu	Glu	Thr	Leu	Lys	Leu	Gly	Lys	Ser
			195					200					205			
30	Leu	Arg	Pro	Ser	Ser	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr

EP 4 269 578 B1

	210		215		220	
	Asn Thr His Phe Thr Lys Pro Asn Tyr Asp Gly His Cys Pro Pro Ile					
	225		230		235	240
5	Glu Leu Gln Arg Asn Asn Asp Leu Gln Trp Leu Trp Asn Asp Ser Thr					
		245		250		255
	Ala Leu Tyr Pro Ser Val Tyr Leu Thr Ser Arg Val Arg Ser Ser Gln					
		260		265		270
10	Asn Gly Ala Leu Tyr Val Arg Asn Arg Val His Glu Ser Ile Arg Val					
		275		280		285
	Ser Lys Leu Met Asp Asp Lys Asn Pro Leu Pro Ile Tyr Val Tyr Ile					
		290		295		300
	Arg Leu Val Phe Thr Asp Gln Thr Thr Thr Phe Leu Glu Leu Asp Asp					
15	305		310		315	320
	Leu Val His Ser Val Gly Glu Ile Val Pro Leu Gly Val Ser Gly Ile					
		325		330		335
	Ile Ile Trp Gly Ser Leu Ser Leu Thr Arg Ser Leu Val Ser Cys Ile					
		340		345		350
20	Gly Leu Glu Asn Tyr Met Lys Gly Thr Leu Leu Pro Tyr Leu Ile Asn					
		355		360		365
	Val Thr Leu Ala Ala Lys Met Cys Gly Gln Val Leu Cys Lys Asn Gln					
		370		375		380
	Gly Ile Cys Thr Arg Lys Asp Trp Asn Thr Asn Thr Tyr Leu His Leu					
25	385		390		395	400
	Asn Ala Thr Asn Phe Asp Ile Glu Leu Gln Gln Asn Gly Lys Phe Val					
		405		410		415
	Val His Gly Lys Pro Ser Leu Glu Asp Leu Gln Glu Phe Ser Lys Asn					
		420		425		430
30	Phe His Cys Ser Cys Tyr Thr Asn Val Ala Cys Lys Asp Arg Leu Asp					
		435		440		445
	Val His Asn Val Arg Ser Val Asn Val Cys Thr Ala Asn Asn Ile Cys					
		450		455		460
35	Ile Asp Ala Val Leu Asn Phe Pro Ser Leu Asp Asp Asp Glu Pro					
	465		470		475	480
	Pro Ile Thr Asp Asp Thr Ser Gln Asn Gln Asp Ser Ile Ser Asp Ile					
		485		490		495
	Thr Ser Ser Ala Pro Pro Ser Ser His Ile Leu Pro Lys Asp Leu Ser					
40		500		505		510
	Trp Cys Leu Phe Leu Leu Ser Ile Phe Ser Gln His Trp Lys Tyr Leu					
		515		520		525
	Leu					

45 <210> 29
 <211> 512
 <212> PRT
 <213> Rattus norvegicus

50 <220>
 <223> PH20

<400> 29

55

EP 4 269 578 B1

	Met	Gly	Glu	Leu	Gln	Phe	Lys	Trp	Leu	Phe	Trp	Arg	Ser	Phe	Ala	Glu
	1				5					10					15	
5	Ser	Gly	Gly	Thr	Phe	Gln	Thr	Val	Leu	Ile	Phe	Leu	Phe	Ile	Pro	Tyr
				20					25					30		
10																
15																
20																
25																
30																
35																
40																
45																
50																
55																

EP 4 269 578 B1

	Ser	Leu	Thr	Val	Asp	Tyr	Arg	Ala	Thr	Pro	Val	Leu	Ser	Asp	Thr	Thr
			35					40					45			
	Phe	Val	Trp	Val	Trp	Asn	Val	Pro	Thr	Glu	Ala	Cys	Val	Glu	Asn	Val
		50				55						60				
5	Thr	Glu	Pro	Ile	Asp	Leu	Ser	Phe	Phe	Ser	Leu	Ile	Gly	Ser	Pro	Arg
	65					70					75					80
	Lys	Thr	Ala	Ile	Gly	Gln	Pro	Val	Thr	Leu	Phe	Tyr	Val	Asp	Arg	Leu
					85					90					95	
	Gly	Asn	Tyr	Pro	His	Ile	Asp	Ala	Gln	Gln	Thr	Glu	His	His	Gly	Gly
10				100					105					110		
	Ile	Pro	Gln	Lys	Gly	Asp	Leu	Thr	Thr	His	Leu	Val	Lys	Ala	Lys	Glu
			115					120					125			
	Asp	Val	Glu	Arg	Tyr	Ile	Pro	Thr	Asp	Lys	Leu	Gly	Leu	Ala	Ile	Ile
		130						135				140				
15	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Met	Arg	Asn	Trp	Thr	Pro	Lys
	145					150					155					160
	Asp	Ile	Tyr	Arg	Asn	Lys	Ser	Ile	Glu	Leu	Val	Gln	Ala	Ala	Asp	Pro
					165				170						175	
	Ala	Ile	Asn	Ile	Thr	Glu	Ala	Thr	Val	Arg	Ala	Lys	Ala	Gln	Phe	Glu
20				180					185					190		
	Gly	Ala	Ala	Lys	Glu	Phe	Met	Glu	Gly	Thr	Leu	Lys	Leu	Gly	Lys	His
			195					200					205			
	Ile	Arg	Pro	Lys	His	Leu	Trp	Gly	Phe	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr
		210					215					220				
25	Asn	Asn	Lys	Phe	Gln	Val	Asp	Asn	Tyr	Asp	Gly	Gln	Cys	Pro	Asp	Val
	225					230					235					240
	Glu	Lys	Lys	Arg	Asn	Asp	Asp	Leu	Asp	Trp	Leu	Trp	Lys	Glu	Ser	Thr
				245						250					255	
	Gly	Leu	Tyr	Pro	Ser	Val	Tyr	Leu	Lys	Lys	Asp	Leu	Lys	Ser	Ser	Arg
30				260					265					270		
	Lys	Ala	Thr	Leu	Tyr	Val	Arg	Tyr	Arg	Val	Leu	Glu	Ser	Ile	Arg	Val
		275						280					285			
	Ser	Lys	Val	Ser	Asp	Glu	Ser	Asn	Pro	Val	Pro	Ile	Phe	Val	Tyr	Ile
		290						295				300				
35	Arg	Leu	Val	Phe	Thr	Asp	His	Val	Ser	Glu	Tyr	Leu	Leu	Glu	Asp	Asp
	305					310					315					320
	Leu	Val	Asn	Thr	Ile	Gly	Glu	Ile	Val	Ala	Gln	Gly	Thr	Ser	Gly	Ile
				325						330					335	
	Ile	Ile	Trp	Asp	Ala	Met	Ser	Leu	Ala	Gln	Arg	Ser	Ala	Gly	Cys	Pro
40				340					345					350		
	Ile	Leu	Arg	Gln	Tyr	Met	Lys	Thr	Thr	Leu	Asn	Pro	Tyr	Ile	Val	Asn
		355						360					365			
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Thr	Leu	Cys	Lys	Glu	Lys
		370					375					380				
45	Gly	Met	Cys	Ser	Arg	Lys	Thr	Glu	Ser	Ser	Asp	Ala	Tyr	Leu	His	Leu
	385					390					395					400
	Asp	Pro	Ser	Ser	Phe	Ser	Ile	Asn	Val	Thr	Glu	Ala	Gly	Lys	Tyr	Glu
					405					410					415	
	Val	Leu	Gly	Lys	Pro	Glu	Val	Lys	Asp	Leu	Glu	Tyr	Phe	Ser	Glu	His
50				420					425					430		
	Phe	Lys	Cys	Ser	Cys	Phe	Ser	Lys	Met	Thr	Cys	Glu	Glu	Thr	Ser	Asp
		435						440				445				
	Met	Arg	Ser	Ile	Gln	Asp	Val	Asn	Val	Cys	Met	Gly	Asp	Asn	Val	Cys
		450					455					460				
55	Ile	Lys	Ala	Thr	Leu	Gly	Pro	Asn	Ser	Ala	Phe	His	Leu	Leu	Pro	Gly
	465					470					475					480

EP 4 269 578 B1

	Lys	Gly	Leu	Leu	Leu	Met	Thr	Thr	Leu	Ala	His	Ile	Leu	His	His	Leu
					485					490					495	
5	Pro	His	Asp	Ile	Phe	Val	Phe	Pro	Trp	Lys	Met	Leu	Val	Ser	Thr	Pro
				500					505					510		

<210> 30
 <211> 512
 <212> PRT
 10 <213> Mus musculus

<220>
 <223> PH20
 15 <400> 30

20

25

30

35

40

45

50

55

	Met	Gly	Glu	Leu	Arg	Phe	Lys	His	Leu	Phe	Trp	Gly	Ser	Phe	Val	Glu
	1				5					10					15	
	Ser	Gly	Gly	Thr	Phe	Gln	Thr	Val	Leu	Ile	Phe	Leu	Leu	Ile	Pro	Cys
5				20					25					30		
	Ser	Leu	Thr	Val	Asp	Tyr	Arg	Ala	Pro	Ile	Leu	Ser	Asn	Thr	Thr	
			35					40				45				
	Phe	Leu	Trp	Ile	Trp	Asn	Val	Pro	Thr	Glu	Arg	Cys	Val	Gly	Asn	Val
		50				55						60				
10	Asn	Asp	Pro	Ile	Asp	Leu	Ser	Phe	Phe	Ser	Leu	Ile	Gly	Ser	Pro	Arg
	65					70					75					80
	Lys	Thr	Ala	Thr	Gly	Gln	Pro	Val	Thr	Leu	Phe	Tyr	Val	Asp	Arg	Leu
					85					90					95	
15	Gly	Leu	Tyr	Pro	His	Ile	Asp	Ala	Asn	Gln	Ala	Glu	His	Tyr	Gly	Gly
				100					105					110		
	Ile	Pro	Gln	Arg	Gly	Asp	Tyr	Gln	Ala	His	Leu	Arg	Lys	Ala	Lys	Thr
			115					120					125			
	Asp	Ile	Glu	His	Tyr	Ile	Pro	Asp	Asp	Lys	Leu	Gly	Leu	Ala	Ile	Ile
20		130					135					140				
	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Leu	Arg	Asn	Trp	Lys	Pro	Lys
	145					150					155					160
	Asp	Asn	Tyr	Arg	Asn	Lys	Ser	Ile	Glu	Leu	Val	Gln	Ser	Thr	Asn	Pro
					165					170					175	
25	Gly	Leu	Ser	Ile	Thr	Glu	Ala	Thr	Gln	Lys	Ala	Ile	Gln	Gln	Phe	Glu
				180					185					190		
	Glu	Ala	Gly	Arg	Lys	Phe	Met	Glu	Gly	Thr	Leu	His	Leu	Gly	Lys	Phe
			195					200					205			
30	Leu	Arg	Pro	Asn	Gln	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr
		210					215					220				
	Asn	Asn	Lys	Phe	Gln	Asp	Pro	Lys	Tyr	Asp	Gly	Gln	Cys	Pro	Ala	Val
	225					230					235					240
	Glu	Lys	Lys	Arg	Asn	Asp	Asn	Leu	Lys	Trp	Leu	Trp	Lys	Ala	Ser	Thr
35					245					250					255	
	Gly	Leu	Tyr	Pro	Ser	Val	Tyr	Leu	Lys	Lys	Asp	Leu	Lys	Ser	Asn	Arg
				260					265					270		
	Gln	Ala	Thr	Leu	Tyr	Val	Arg	Tyr	Arg	Val	Val	Glu	Ala	Ile	Arg	Val
			275					280					285			
40	Ser	Lys	Val	Gly	Asn	Ala	Ser	Asp	Pro	Val	Pro	Ile	Phe	Val	Tyr	Ile
		290					295					300				
	Arg	Leu	Val	Phe	Thr	Asp	Arg	Thr	Ser	Glu	Tyr	Leu	Leu	Glu	Asp	Asp
	305					310					315				320	
45	Leu	Val	Asn	Thr	Ile	Gly	Glu	Ile	Val	Ala	Leu	Gly	Thr	Ser	Gly	Ile

					325					330				335		
	Ile	Ile	Trp	Asp	Ala	Met	Ser	Leu	Ala	Gln	Arg	Ala	Ala	Gly	Cys	Pro
				340					345					350		
5	Ile	Leu	His	Lys	Tyr	Met	Gln	Thr	Thr	Leu	Asn	Pro	Tyr	Ile	Val	Asn
			355					360					365			
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Thr	Leu	Cys	Asn	Glu	Lys
			370					375					380			
10	Gly	Met	Cys	Ser	Arg	Arg	Lys	Glu	Ser	Ser	Asp	Val	Tyr	Leu	His	Leu
	385					390					395					400
	Asn	Pro	Ser	His	Phe	Asp	Ile	Met	Leu	Thr	Glu	Thr	Gly	Lys	Tyr	Glu
				405						410					415	
	Val	Leu	Gly	Asn	Pro	Arg	Val	Gly	Asp	Leu	Glu	Tyr	Phe	Ser	Glu	His
			420						425					430		
15	Phe	Lys	Cys	Ser	Cys	Phe	Ser	Arg	Met	Thr	Cys	Lys	Glu	Thr	Ser	Asp
			435					440					445			
	Val	Lys	Asn	Val	Gln	Asp	Val	Asn	Val	Cys	Val	Gly	Asp	Asn	Val	Cys
		450				455					460					
20	Ile	Lys	Ala	Lys	Val	Glu	Pro	Asn	Pro	Ala	Phe	Tyr	Leu	Leu	Pro	Gly
	465					470					475					480
	Lys	Ser	Leu	Leu	Phe	Met	Thr	Thr	Leu	Gly	His	Val	Leu	Tyr	His	Leu
				485						490					495	
25	Pro	Gln	Asp	Ile	Phe	Val	Phe	Pro	Arg	Lys	Thr	Leu	Val	Ser	Thr	Pro
			500						505					510		

<210> 31

<211> 807

<212> PRT

30 <213> Staphylococcus aureus

<220>

<223> hyaluronidase

35 <400> 31

40

45

50

55

	Met	Thr	Tyr	Arg	Ile	Lys	Lys	Trp	Gln	Lys	Leu	Ser	Thr	Ile	Thr	Leu
	1				5					10					15	
5	Leu	Met	Ala	Gly	Val	Ile	Thr	Leu	Asn	Gly	Gly	Glu	Phe	Arg	Ser	Val
				20					25					30		
	Asp	Lys	His	Gln	Ile	Ala	Val	Ala	Asp	Thr	Asn	Val	Gln	Thr	Pro	Asp
			35					40					45			
	Tyr	Glu	Lys	Leu	Arg	Asn	Thr	Trp	Leu	Asp	Val	Asn	Tyr	Gly	Tyr	Asp
		50					55					60				
10	Lys	Tyr	Asp	Glu	Asn	Asn	Pro	Asp	Met	Lys	Lys	Lys	Phe	Asp	Ala	Thr
	65					70					75					80
	Glu	Lys	Glu	Ala	Thr	Asn	Leu	Leu	Lys	Glu	Met	Lys	Thr	Glu	Ser	Gly
					85					90					95	
15	Arg	Lys	Tyr	Leu	Trp	Ser	Gly	Ala	Glu	Thr	Leu	Glu	Thr	Asn	Ser	Ser
				100					105					110		
	His	Met	Thr	Arg	Thr	Tyr	Arg	Asn	Ile	Glu	Lys	Ile	Ala	Glu	Ala	Met
			115					120					125			
	Arg	Asn	Pro	Lys	Thr	Thr	Leu	Asn	Thr	Asp	Glu	Asn	Lys	Lys	Lys	Val
20		130					135					140				
	Lys	Asp	Ala	Leu	Glu	Trp	Leu	His	Lys	Asn	Ala	Tyr	Gly	Lys	Glu	Pro
	145					150					155					160
	Asp	Lys	Lys	Val	Lys	Glu	Leu	Ser	Glu	Asn	Phe	Thr	Lys	Thr	Thr	Gly
					165					170					175	

25

30

35

40

45

50

55

EP 4 269 578 B1

	Lys	Asn	Thr	Asn	Leu	Asn	Trp	Trp	Asp	Tyr	Glu	Ile	Gly	Thr	Pro	Lys
				180					185					190		
	Ser	Leu	Thr	Asn	Thr	Leu	Ile	Leu	Leu	Asn	Asp	Gln	Phe	Ser	Asn	Glu
			195					200					205			
5	Glu	Lys	Lys	Lys	Phe	Thr	Ala	Pro	Ile	Lys	Thr	Phe	Ala	Pro	Asp	Ser
		210					215					220				
	Asp	Lys	Ile	Leu	Ser	Ser	Val	Gly	Lys	Ala	Glu	Leu	Ala	Lys	Gly	Gly
	225					230					235					240
10	Asn	Leu	Val	Asp	Ile	Ser	Lys	Val	Lys	Leu	Leu	Glu	Cys	Ile	Ile	Glu
				245						250					255	
	Glu	Asp	Lys	Asp	Met	Met	Lys	Lys	Ser	Ile	Asp	Ser	Phe	Asn	Lys	Val
			260						265					270		
	Phe	Thr	Tyr	Val	Gln	Asp	Ser	Ala	Thr	Gly	Lys	Glu	Arg	Asn	Gly	Phe
			275					280					285			
15	Tyr	Lys	Asp	Gly	Ser	Tyr	Ile	Asp	His	Gln	Asp	Val	Pro	Tyr	Thr	Gly
		290					295					300				
	Ala	Tyr	Gly	Val	Val	Leu	Leu	Glu	Gly	Ile	Ser	Gln	Met	Met	Pro	Met
	305					310					315					320
20	Ile	Lys	Glu	Thr	Pro	Phe	Asn	Asp	Lys	Thr	Gln	Asn	Asp	Thr	Thr	Leu
					325					330					335	
	Lys	Ser	Trp	Ile	Asp	Asp	Gly	Phe	Met	Pro	Leu	Ile	Tyr	Lys	Gly	Glu
			340					345						350		
	Met	Met	Asp	Leu	Ser	Arg	Gly	Arg	Ala	Ile	Ser	Arg	Glu	Asn	Glu	Thr
			355				360						365			
25	Ser	His	Ser	Ala	Ser	Ala	Thr	Val	Met	Lys	Ser	Leu	Leu	Arg	Leu	Ser
		370					375					380				
	Asp	Ala	Met	Asp	Asp	Ser	Thr	Lys	Ala	Lys	Tyr	Lys	Lys	Ile	Val	Lys
	385					390					395					400
30	Ser	Ser	Val	Glu	Ser	Asp	Ser	Ser	Tyr	Lys	Gln	Asn	Asp	Tyr	Leu	Asn
				405						410					415	
	Ser	Tyr	Ser	Asp	Ile	Asp	Lys	Met	Lys	Ser	Leu	Met	Thr	Asp	Asn	Ser
			420						425					430		
	Ile	Ser	Lys	Asn	Gly	Leu	Thr	Gln	Gln	Leu	Lys	Ile	Tyr	Asn	Asp	Met
		435						440					445			
35	Asp	Arg	Val	Thr	Tyr	His	Asn	Lys	Asp	Leu	Asp	Phe	Ala	Phe	Gly	Leu
		450					455					460				
	Ser	Met	Thr	Ser	Lys	Asn	Val	Ala	Arg	Tyr	Glu	Ser	Ile	Asn	Gly	Glu
	465					470					475					480
40	Asn	Leu	Lys	Gly	Trp	His	Thr	Gly	Ala	Gly	Met	Ser	Tyr	Leu	Tyr	Asn
				485						490					495	
	Ser	Asp	Val	Lys	His	Tyr	His	Asp	Asn	Phe	Trp	Val	Thr	Ala	Asp	Met
			500						505					510		
	Lys	Arg	Leu	Ser	Gly	Thr	Thr	Thr	Leu	Asp	Asn	Glu	Ile	Leu	Lys	Asp
		515						520					525			
45	Thr	Asp	Asp	Lys	Lys	Ser	Ser	Lys	Thr	Phe	Val	Gly	Gly	Thr	Lys	Val
		530					535					540				
	Asp	Asp	Gln	His	Ala	Ser	Ile	Gly	Met	Asp	Phe	Glu	Asn	Gln	Asp	Lys
	545					550					555					560
50	Thr	Leu	Thr	Ala	Lys	Ser	Tyr	Phe	Ile	Leu	Asn	Asp	Lys	Ile	Val	
				565					570						575	
	Phe	Leu	Gly	Thr	Gly	Ile	Lys	Ser	Thr	Asp	Ser	Ser	Lys	Asn	Pro	Val
			580						585					590		
	Thr	Thr	Ile	Glu	Asn	Arg	Lys	Ala	Asn	Gly	Tyr	Thr	Leu	Tyr	Thr	Asp
		595						600					605			
55	Asp	Lys	Gln	Thr	Thr	Asn	Ser	Asp	Asn	Gln	Glu	Asn	Asn	Ser	Val	Phe
		610					615						620			

	Leu	Glu	Ser	Thr	Asp	Thr	Lys	Lys	Asn	Ile	Gly	Tyr	His	Phe	Leu	Asn
	625					630					635					640
	Lys	Pro	Lys	Ile	Thr	Val	Lys	Lys	Glu	Ser	His	Thr	Gly	Lys	Trp	Lys
5					645					650						655
	Glu	Ile	Asn	Lys	Ser	Gln	Lys	Asp	Thr	Gln	Lys	Thr	Asp	Glu	Tyr	Tyr
				660					665					670		
	Glu	Val	Thr	Gln	Lys	His	Ser	Asn	Ser	Asp	Asn	Lys	Tyr	Gly	Tyr	Val
			675					680					685			
10	Leu	Tyr	Pro	Gly	Leu	Ser	Lys	Asp	Val	Phe	Lys	Thr	Lys	Lys	Asp	Glu
	690						695					700				
	Val	Thr	Val	Val	Lys	Gln	Glu	Asp	Asp	Phe	His	Val	Val	Lys	Asp	Asn
	705					710					715					720
	Glu	Ser	Val	Trp	Ala	Gly	Val	Asn	Tyr	Ser	Asn	Ser	Thr	Gln	Thr	Phe
15					725					730						735
	Asp	Ile	Asn	Asn	Thr	Lys	Val	Glu	Val	Lys	Ala	Lys	Gly	Met	Phe	Ile
				740					745					750		
	Leu	Lys	Lys	Lys	Asp	Asp	Asn	Thr	Tyr	Glu	Cys	Ser	Phe	Tyr	Asn	Pro
20			755				760						765			
	Glu	Ser	Thr	Asn	Ser	Ala	Ser	Asp	Ile	Glu	Ser	Lys	Ile	Ser	Met	Thr
		770					775					780				
	Gly	Tyr	Ser	Ile	Thr	Asn	Lys	Asn	Thr	Ser	Thr	Ser	Asn	Glu	Ser	Gly
	785					790					795					800
25	Val	His	Phe	Glu	Leu	Thr	Lys									
					805											

<210> 32

<211> 371

30 <212> PRT

<213> Streptococcus pyogenes bacteriophage H4489A

<220>

<223> hyaluronidase

35

<400> 32

40

45

50

55

	Met	Thr	Glu	Asn	Ile	Pro	Leu	Arg	Val	Gln	Phe	Lys	Arg	Met	Ser	Ala
	1				5					10					15	
	Asp	Glu	Trp	Ala	Arg	Ser	Asp	Val	Ile	Leu	Leu	Glu	Gly	Glu	Ile	Gly
5				20					25					30		
	Phe	Glu	Thr	Asp	Thr	Gly	Phe	Ala	Lys	Phe	Gly	Asp	Gly	Gln	Asn	Thr
			35				40						45			
	Phe	Ser	Lys	Leu	Lys	Tyr	Leu	Thr	Gly	Pro	Lys	Gly	Pro	Lys	Gly	Asp
		50				55						60				
10	Thr	Gly	Leu	Gln	Gly	Lys	Thr	Gly	Gly	Thr	Gly	Pro	Arg	Gly	Pro	Ala
	65				70						75					80
	Gly	Lys	Pro	Gly	Thr	Asp	Tyr	Asp	Gln	Leu	Gln	Asn	Lys	Pro	Asp	
				85					90					95		
15	Leu	Gly	Ala	Phe	Ala	Gln	Lys	Glu	Glu	Thr	Asn	Ser	Lys	Ile	Thr	Lys
			100					105						110		
	Leu	Glu	Ser	Lys	Ala	Asp	Lys	Ser	Ala	Val	Tyr	Ser	Lys	Ala	Glu	
		115				120						125				
	Ser	Lys	Ile	Glu	Leu	Asp	Lys	Leu	Ser	Leu	Thr	Gly	Gly	Ile	Val	
20		130				135					140					
	Thr	Gly	Gln	Leu	Gln	Phe	Lys	Pro	Asn	Lys	Ser	Gly	Ile	Lys	Pro	Ser
	145				150					155					160	
	Ser	Ser	Val	Gly	Gly	Ala	Ile	Asn	Ile	Asp	Met	Ser	Lys	Ser	Glu	Gly
25					165				170						175	
	Ala	Ala	Met	Val	Met	Tyr	Thr	Asn	Lys	Asp	Thr	Thr	Asp	Gly	Pro	Leu
				180					185					190		
	Met	Ile	Leu	Arg	Ser	Asp	Lys	Asp	Thr	Phe	Asp	Gln	Ser	Ala	Gln	Phe
30			195					200					205			
	Val	Asp	Tyr	Ser	Gly	Lys	Thr	Asn	Ala	Val	Asn	Ile	Val	Met	Arg	Gln
		210				215						220				
	Pro	Ser	Ala	Pro	Asn	Phe	Ser	Ser	Ala	Leu	Asn	Ile	Thr	Ser	Ala	Asn
					230						235				240	
35	Glu	Gly	Gly	Ser	Ala	Met	Gln	Ile	Arg	Gly	Val	Glu	Lys	Ala	Leu	Gly
				245						250					255	
	Thr	Leu	Lys	Ile	Thr	His	Glu	Asn	Pro	Asn	Val	Glu	Ala	Lys	Tyr	Asp
			260					265						270		
40	Glu	Asn	Ala	Ala	Ala	Leu	Ser	Ile	Asp	Ile	Val	Lys	Lys	Gln	Lys	Gly
		275						280					285			
	Gly	Lys	Gly	Thr	Ala	Ala	Gln	Gly	Ile	Tyr	Ile	Asn	Ser	Thr	Ser	Gly
		290				295						300				
	Thr	Ala	Gly	Lys	Met	Leu	Arg	Ile	Arg	Asn	Lys	Asn	Glu	Asp	Lys	Phe
45		305				310					315				320	
	Tyr	Val	Gly	Pro	Asp	Gly	Gly	Phe	His	Ser	Gly	Ala	Asn	Ser	Thr	Val
				325						330					335	
	Ala	Gly	Asn	Leu	Thr	Val	Lys	Asp	Pro	Thr	Ser	Gly	Lys	His	Ala	Ala
			340					345					350			
50	Thr	Lys	Asp	Tyr	Val	Asp	Glu	Lys	Ile	Ala	Glu	Leu	Lys	Lys	Leu	Ile
			355				360						365			
	Leu	Lys	Lys													
			370													

55 <210> 33
 <211> 1628
 <212> PRT
 <213> Clostridium perfringens

<220>

<223> hyaluronidase

<400> 33

5

10

15

20

25

30

35

40

45

50

55

```

Met Asn Lys Asn Ile Arg Lys Ile Ile Thr Ser Thr Val Leu Ala Ala
 1      5      10
Met Thr Ile Ser Val Leu Pro Ser Asn Leu Val Val Phe Ala Thr Asp
      20      25      30
Gly Ile Thr Glu Asn Phe Tyr Glu Ile Tyr Pro Lys Pro Gln Glu Ile
      35      40      45
Ser Tyr Ser Gly Gly Glu Phe Gln Ile Ser Asp Glu Ile Asn Ile Val
      50      55      60
Tyr Asp Asp Gly Ile Asp Thr Tyr Thr Lys Lys Arg Val Asp Glu Val
      65      70      75      80
Leu Glu Ala Ser Asn Leu Glu Ala Thr Val Ser Asn Glu Ile Val Pro
      85      90      95
Gly Lys Thr Asn Phe Leu Val Gly Ile Asn Glu Ser Gly Gly Val Val
      100     105     110
Asp Asn Tyr Phe Asn Lys Asn Ile Pro His Asp Glu Ser Phe Phe Asp
      115     120     125
Glu Lys Met Asp Ala Asn Ile Val Ser Val Lys Asp Gly Val Ile Gly
      130     135     140

```

EP 4 269 578 B1

	Val	Ile	Gly	Glu	Asp	Thr	Asp	Ser	Ala	Phe	Tyr	Gly	Val	Thr	Thr	Leu
	145					150					155					160
	Lys	His	Val	Phe	Asn	Gln	Leu	Glu	Glu	Gly	Asn	Lys	Ile	Gln	Ser	Phe
				165						170					175	
5	Arg	Ala	Asp	Asp	Tyr	Ala	Glu	Val	Ala	His	Arg	Gly	Phe	Ile	Glu	Gly
			180						185					190		
	Tyr	Tyr	Gly	Asn	Pro	Trp	Ser	Asn	Glu	Asp	Arg	Ala	Glu	Leu	Met	Lys
			195					200					205			
10	Phe	Gly	Gly	Asp	Tyr	Lys	Leu	Asn	Gln	Tyr	Val	Phe	Ala	Pro	Lys	Asp
	210						215					220				
	Asp	Pro	Tyr	His	Asn	Ser	Lys	Trp	Arg	Asp	Leu	Tyr	Pro	Glu	Glu	Lys
	225					230				235						240
	Leu	Ser	Glu	Ile	Lys	Lys	Leu	Ala	Gln	Val	Gly	Asn	Glu	Thr	Lys	Asn
				245						250					255	
15	Arg	Tyr	Val	Tyr	Ala	Leu	His	Pro	Phe	Met	Asn	Asn	Pro	Val	Arg	Phe
			260						265					270		
	Asp	Thr	Glu	Glu	Asn	Tyr	Gln	Asn	Asp	Leu	Gly	Val	Ile	Lys	Ala	Lys
			275				280						285			
20	Phe	Thr	Gln	Leu	Leu	Glu	Asn	Asp	Val	Arg	Gln	Phe	Ala	Ile	Leu	Ala
	290						295					300				
	Asp	Asp	Ala	Ser	Ala	Pro	Ala	Gln	Gly	Ala	Ser	Met	Tyr	Val	Lys	Leu
	305					310					315					320
	Leu	Thr	Asp	Leu	Thr	Arg	Trp	Leu	Glu	Glu	Gln	Gln	Ser	Thr	Tyr	Pro
				325						330					335	
25	Asp	Leu	Lys	Thr	Asp	Leu	Met	Phe	Cys	Pro	Ser	Asp	Tyr	Tyr	Gly	Asn
			340					345					350			
	Gly	Ser	Ser	Ala	Gln	Leu	Lys	Glu	Leu	Asn	Lys	Ala	Glu	Asp	Asn	Val
		355					360					365				
30	Ser	Ile	Val	Met	Thr	Gly	Gly	Arg	Ile	Trp	Gly	Glu	Val	Asp	Glu	Asn
	370					375						380				
	Phe	Ala	Asn	Asn	Phe	Met	Asn	Asn	Ile	Ser	Thr	Glu	Gly	His	Pro	Gly
	385					390					395					400
	Arg	Ala	Pro	Phe	Phe	Trp	Ile	Asn	Trp	Pro	Cys	Ser	Asp	Asn	Ser	Lys
				405						410				415		
35	Gln	His	Leu	Ile	Met	Gly	Gly	Asn	Asp	Thr	Phe	Leu	His	Pro	Gly	Val
			420						425					430		
	Asp	Pro	Ser	Lys	Ile	Asp	Gly	Ile	Val	Leu	Asn	Pro	Met	Gln	Gln	Ala
		435					440					445				
40	Glu	Ala	Asn	Lys	Ser	Ala	Leu	Phe	Ala	Ile	Ala	Asp	Tyr	Ala	Trp	Asn
	450						455				460					
	Ile	Trp	Asp	Asn	Lys	Glu	Glu	Ala	Asp	Glu	Asn	Trp	Asn	Asp	Ser	Phe
	465					470				475						480
	Lys	Tyr	Met	Asp	His	Gly	Thr	Ala	Glu	Glu	Thr	Asn	Ser	Ser	Leu	Ala
				485					490						495	
45	Leu	Arg	Glu	Ile	Ser	Lys	His	Met	Ile	Asn	Gln	Asn	Met	Asp	Gly	Arg
			500						505					510		
	Val	Arg	Pro	Leu	Gln	Glu	Ser	Val	Glu	Leu	Ala	Pro	Lys	Leu	Glu	Ala
		515						520					525			
50	Phe	Lys	Gln	Lys	Tyr	Asp	Ser	Gly	Ala	Ser	Ile	Lys	Glu	Asp	Ala	Leu
	530						535					540				
	Glu	Leu	Ile	Ala	Glu	Phe	Thr	Asn	Leu	Gln	Lys	Ala	Ala	Asp	Tyr	Tyr
	545					550					555					560
	Lys	Asn	Asn	Pro	Gly	Asn	Glu	Arg	Thr	Arg	Asp	Gln	Ile	Ile	Tyr	Trp
				565						570					575	
55	Leu	Asn	Cys	Trp	Glu	Asp	Thr	Met	Asp	Ala	Ala	Ile	Gly	Tyr	Leu	Lys
			580						585					590		

EP 4 269 578 B1

	Ser	Ala	Ile	Ala	Ile	Glu	Glu	Gly	Asp	Asp	Glu	Ala	Ala	Trp	Ala	Asn
			595					600				605				
	Tyr	Ser	Glu	Ala	Gln	Gly	Ala	Phe	Glu	Lys	Ser	Lys	Thr	Tyr	Gly	Phe
		610				615					620					
5	His	Tyr	Val	Asp	His	Thr	Glu	Tyr	Ala	Glu	Val	Gly	Val	Gln	His	Ile
	625					630					635				640	
	Val	Pro	Phe	Ile	Lys	Ser	Met	Gly	Gln	Asn	Leu	Ser	Val	Val	Ile	Gly
					645					650					655	
10	Ser	Ile	Val	Asp	Pro	Asn	Arg	Ile	Ile	Ala	Thr	Tyr	Ile	Ser	Asn	Arg
				660					665					670		
	Gln	Asp	Ala	Pro	Thr	Gly	Asn	Pro	Asp	Asn	Ile	Phe	Asp	Asn	Asn	Ala
		675						680				685				
	Ser	Thr	Glu	Leu	Val	Tyr	Lys	Asn	Pro	Asn	Arg	Ile	Asp	Val	Gly	Thr
		690					695					700				
15	Tyr	Val	Gly	Val	Lys	Tyr	Ser	Asn	Pro	Ile	Thr	Leu	Asn	Asn	Val	Glu
	705					710					715				720	
	Phe	Leu	Met	Gly	Ala	Asn	Ser	Asn	Pro	Asn	Asp	Thr	Met	Gln	Lys	Ala
				725						730					735	
20	Lys	Ile	Gln	Tyr	Thr	Val	Asp	Gly	Arg	Glu	Trp	Ile	Asp	Leu	Glu	Glu
				740					745					750		
	Gly	Val	Glu	Tyr	Thr	Met	Pro	Gly	Ala	Ile	Lys	Val	Glu	Asn	Leu	Asp
			755					760				765				
	Leu	Lys	Val	Arg	Gly	Val	Arg	Leu	Ile	Ala	Thr	Glu	Ala	Arg	Glu	Asn
		770					775					780				
25	Thr	Trp	Leu	Gly	Val	Arg	Asp	Ile	Asn	Val	Asn	Lys	Lys	Glu	Asp	Ser
	785					790					795				800	
	Asn	Ser	Gly	Val	Glu	Phe	Asn	Pro	Ser	Leu	Ile	Arg	Ser	Glu	Ser	Trp
					805					810					815	
30	Gln	Val	Tyr	Glu	Gly	Asn	Glu	Ala	Asn	Leu	Leu	Asp	Gly	Asp	Asp	Asn
				820					825					830		
	Thr	Gly	Val	Trp	Tyr	Lys	Thr	Leu	Asn	Gly	Asp	Thr	Ser	Leu	Ala	Gly
			835					840					845			
	Glu	Phe	Ile	Gly	Leu	Asp	Leu	Gly	Lys	Glu	Ile	Lys	Leu	Asp	Gly	Ile
		850					855					860				
35	Arg	Phe	Val	Ile	Gly	Lys	Asn	Gly	Gly	Gly	Ser	Ser	Asp	Lys	Trp	Asn
	865					870					875					880
	Lys	Phe	Lys	Leu	Glu	Tyr	Ser	Leu	Asp	Asn	Glu	Ser	Trp	Thr	Thr	Ile
					885					890					895	
40	Lys	Glu	Tyr	Asp	Lys	Thr	Gly	Ala	Pro	Ala	Gly	Lys	Asp	Val	Ile	Glu
				900					905					910		
	Glu	Ser	Phe	Glu	Thr	Pro	Ile	Ser	Ala	Lys	Tyr	Ile	Arg	Leu	Thr	Asn
			915					920					925			
	Met	Glu	Asn	Ile	Asn	Lys	Trp	Leu	Thr	Phe	Ser	Glu	Phe	Ala	Ile	Ile
		930					935					940				
45	Ser	Asp	Glu	Leu	Glu	Asn	Ala	Gly	Asn	Lys	Glu	Asn	Val	Tyr	Thr	Asn
	945					950					955					960
	Thr	Glu	Leu	Asp	Leu	Ser	Leu	Ala	Lys	Glu	Asp	Val	Thr	Lys	Leu	
				965						970					975	
50	Ile	Pro	Thr	Asp	Ile	Ser	Leu	Asn	His	Gly	Glu	Tyr	Ile	Gly	Val	
				980				985					990			
	Lys	Leu	Asn	Arg	Ile	Lys	Asp	Leu	Ser	Asn	Ile	Asn	Leu	Glu	Ile	Ser
			995					1000					1005			
	Asn	Asp	Thr	Gly	Leu	Lys	Leu	Gln	Ser	Ser	Met	Asn	Gly	Val	Glu	Trp
		1010					1015					1020				
55	Thr	Glu	Ile	Thr	Asp	Lys	Asn	Thr	Leu	Glu	Asp	Gly	Arg	Tyr	Val	Arg
	1025					1030					1035					1040

	Leu	Ile	Asn	Thr	Ser	Asn	Glu	Ala	Val	Asn	Phe	Asn	Leu	Thr	Lys	Phe
						1045					1050					1055
	Glu	Val	Asn	Ser	Asn	Glu	Val	Tyr	Glu	Pro	Ser	Leu	Val	Asp	Ala	Tyr
						1060				1065					1070	
5	Val	Gly	Asp	Asp	Gly	Ala	Lys	Lys	Ala	Val	Asp	Gly	Asp	Leu	Lys	Thr
						1075			1080					1085		
	Arg	Val	Lys	Phe	Leu	Gly	Ala	Pro	Ser	Thr	Gly	Asp	Thr	Ile	Val	Tyr
						1090			1095				1100			
10	Asp	Leu	Gly	Gln	Glu	Ile	Leu	Val	Asp	Asn	Leu	Lys	Tyr	Val	Val	Leu
						1105			1110				1115			1120
	Asp	Thr	Glu	Val	Asp	His	Val	Arg	Asp	Gly	Lys	Ile	Gln	Leu	Ser	Leu
						1125				1130						1135
	Asp	Gly	Glu	Thr	Trp	Thr	Asp	Ala	Ile	Thr	Ile	Gly	Asp	Gly	Val	Glu
						1140				1145					1150	
15	Asn	Gly	Val	Asp	Asp	Met	Phe	Ser	Thr	Pro	Leu	Lys	Asn	Gly	Tyr	Lys
						1155			1160					1165		
	His	Gly	Asn	Gln	Ser	Gly	Gly	Ile	Val	Pro	Ile	Asp	Ser	Ala	Tyr	Val
						1170			1175				1180			
20	Glu	Gly	Asp	Asn	Leu	Asn	Gln	Lys	Ala	Arg	Tyr	Val	Arg	Ile	Leu	Phe
						1185			1190			1195				1200
	Thr	Ala	Pro	Tyr	Arg	His	Arg	Trp	Thr	Val	Ile	Asn	Glu	Leu	Met	Ile
						1205				1210						1215
	Asn	Asn	Gly	Glu	Tyr	Ile	Ser	Thr	Val	Asn	Asp	Pro	Thr	Tyr	Ile	Ser
						1220				1225					1230	
25	Asn	Pro	Ile	Glu	Glu	Arg	Gly	Phe	Ala	Pro	Ser	Asn	Leu	Arg	Asp	Gly
						1235			1240					1245		
	Asn	Leu	Thr	Thr	Ser	Tyr	Lys	Pro	Asn	Thr	Asn	Asn	Gly	Glu	Ile	Ser
						1250			1255				1260			
30	Glu	Gly	Ser	Ile	Thr	Tyr	Arg	Leu	Ser	Glu	Lys	Thr	Asp	Val	Arg	Lys
						1265			1270			1275				1280
	Val	Thr	Ile	Val	Gln	Ser	Gly	Ser	Ser	Ile	Ser	Asn	Ala	Lys	Val	Met
						1285				1290						1295
	Ala	Arg	Val	Gly	Asp	Gly	Ser	Glu	Asn	Val	Thr	Asp	Gln	Trp	Val	Gln
						1300			1305					1310		
35	Leu	Gly	Thr	Leu	Ser	Asn	Ser	Leu	Asn	Glu	Phe	Ile	Asn	Arg	Asp	Tyr
						1315			1320					1325		
	Asn	Asn	Ile	Tyr	Glu	Ile	Lys	Ile	Glu	Trp	Thr	Asp	Val	Ala	Pro	Asn
						1330			1335				1340			
40	Ile	Tyr	Glu	Ile	Ile	Thr	Leu	Asn	Gln	Glu	Phe	Glu	Phe	Pro	Val	Asn
						1345			1350			1355				1360
	Asp	Ser	Leu	Lys	Ala	Lys	Tyr	Asp	Glu	Leu	Ile	Asn	Leu	Ser	Gly	Asp
						1365				1370					1375	
	Glu	Tyr	Thr	Leu	Ser	Ser	Phe	Glu	Thr	Leu	Lys	Glu	Ala	Leu	Asn	Glu
						1380				1385				1390		
45	Ala	Lys	Ser	Ile	Leu	Asp	Asp	Ser	Asn	Ser	Ser	Gln	Lys	Lys	Ile	Asp
						1395			1400				1405			
	Lys	Ala	Leu	Glu	Lys	Leu	Asn	Lys	Ala	Glu	Glu	Arg	Leu	Asp	Leu	Arg
						1410			1415			1420				
50	Ala	Thr	Asp	Phe	Glu	Asp	Phe	Asn	Lys	Val	Leu	Thr	Leu	Gly	Asn	Ser
						1425			1430			1435				1440
	Leu	Val	Glu	Glu	Glu	Tyr	Thr	Ala	Glu	Ser	Trp	Ala	Leu	Phe	Ser	Glu
						1445				1450					1455	
	Val	Leu	Glu	Ala	Ala	Asn	Glu	Ala	Asn	Lys	Asn	Lys	Ala	Asp	Tyr	Thr
						1460			1465					1470		
55	Gln	Asp	Gln	Ile	Asn	Gln	Ile	Val	Ile	Asp	Leu	Asp	Ala	Ser	Ile	Lys
						1475			1480					1485		

	Ala	Leu	Val	Lys	Glu	Thr	Pro	Glu	Val	Asp	Lys	Thr	Asn	Leu	Gly	Glu
	1490						1495					1500				
5	Leu	Ile	Asn	Gln	Gly	Lys	Ser	Leu	Leu	Asp	Glu	Ser	Val	Glu	Gly	Phe
	1505					1510					1515				1520	
	Asn	Val	Gly	Glu	Tyr	His	Lys	Gly	Ala	Lys	Asp	Gly	Leu	Thr	Val	Glu
					1525					1530					1535	
	Ile	Asn	Lys	Ala	Glu	Glu	Val	Phe	Asn	Lys	Glu	Asp	Ala	Thr	Glu	Glu
				1540					1545					1550		
10	Glu	Ile	Asn	Leu	Ala	Lys	Glu	Ser	Leu	Glu	Gly	Ala	Ile	Ala	Arg	Phe
			1555					1560					1565			
	Asn	Ser	Leu	Leu	Ile	Glu	Glu	Ser	Thr	Gly	Asp	Phe	Asn	Gly	Asn	Gly
			1570				1575					1580				
15	Lys	Ile	Asp	Ile	Gly	Asp	Leu	Ala	Met	Val	Ser	Lys	Asn	Ile	Gly	Ser
	1585					1590					1595				1600	
	Thr	Thr	Asn	Thr	Ser	Leu	Asp	Leu	Asn	Lys	Asp	Gly	Ser	Ile	Asp	Glu
					1605					1610					1615	
	Tyr	Glu	Ile	Ser	Phe	Ile	Asn	His	Arg	Ile	Leu	Asn				
20				1620					1625							

<210> 34

<211> 435

<212> PRT

25 <213> Homo sapiens

<220>

<223> Hyaluronidase-1 [Precursor]

30 <400> 34

35

40

45

50

55

EP 4 269 578 B1

[illegible]

EP 4 269 578 B1

[illegible]

35

<210> 35
<211> 473
<212> PRT
<213> Homo sapiens

40

<220>
<223> Hyaluronidase-2 [Precursor]
<400> 35

	Met	Arg	Ala	Gly	Pro	Gly	Pro	Thr	Val	Thr	Leu	Ala	Leu	Val	Leu	Ala
	1				5					10					15	
5	Val	Ala	Trp	Ala	Met	Glu	Leu	Lys	Pro	Thr	Ala	Pro	Pro	Ile	Phe	Thr
				20					25					30		
	Gly	Arg	Pro	Phe	Val	Val	Ala	Trp	Asp	Val	Pro	Thr	Gln	Asp	Cys	Gly
			35					40					45			
	Pro	Arg	Leu	Lys	Val	Pro	Leu	Asp	Leu	Asn	Ala	Phe	Asp	Val	Gln	Ala
		50					55					60				
10	Ser	Pro	Asn	Glu	Gly	Phe	Val	Asn	Gln	Asn	Ile	Thr	Ile	Phe	Tyr	Arg
	65					70					75					80
	Asp	Arg	Leu	Gly	Leu	Tyr	Pro	Arg	Phe	Asp	Ser	Ala	Gly	Arg	Ser	Val
				85					90						95	
15	His	Gly	Gly	Val	Pro	Gln	Asn	Val	Ser	Leu	Trp	Ala	His	Arg	Lys	Met
				100					105					110		
	Leu	Gln	Lys	Arg	Val	Glu	His	Tyr	Ile	Arg	Thr	Gln	Glu	Ser	Ala	Gly
			115					120					125			

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Leu	Ala	Val	Ile	Asp	Trp	Glu	Asp	Trp	Arg	Pro	Val	Trp	Val	Arg	Asn
	130						135					140				
5	Trp	Gln	Asp	Lys	Asp	Val	Tyr	Arg	Arg	Leu	Ser	Arg	Gln	Leu	Val	Ala
	145					150					155					160
	Ser	Arg	His	Pro	Asp	Trp	Pro	Pro	Asp	Arg	Ile	Val	Lys	Gln	Ala	Gln
					165					170						175
	Tyr	Glu	Phe	Glu	Phe	Ala	Ala	Gln	Gln	Phe	Met	Leu	Glu	Thr	Leu	Arg
				180						185					190	
10	Tyr	Val	Lys	Ala	Val	Arg	Pro	Arg	His	Leu	Trp	Gly	Phe	Tyr	Leu	Phe
			195					200					205			
	Pro	Asp	Cys	Tyr	Asn	His	Asp	Tyr	Val	Gln	Asn	Trp	Glu	Ser	Tyr	Thr
		210					215					220				
15	Gly	Arg	Cys	Pro	Asp	Val	Glu	Val	Ala	Arg	Asn	Asp	Gln	Leu	Ala	Trp
	225					230					235					240
	Leu	Trp	Ala	Glu	Ser	Thr	Ala	Leu	Phe	Pro	Ser	Val	Tyr	Leu	Asp	Glu
					245					250						255
	Thr	Leu	Ala	Ser	Ser	Arg	His	Gly	Arg	Asn	Phe	Val	Ser	Phe	Arg	Val
				260					265						270	
20	Gln	Glu	Ala	Leu	Arg	Val	Ala	Arg	Thr	His	His	Ala	Asn	His	Ala	Leu
			275					280					285			
	Pro	Val	Tyr	Val	Phe	Thr	Arg	Pro	Thr	Tyr	Ser	Arg	Arg	Leu	Thr	Gly
		290					295					300				
25	Leu	Ser	Glu	Met	Asp	Leu	Ile	Ser	Thr	Ile	Gly	Glu	Ser	Ala	Ala	Leu
	305					310					315					320
	Gly	Ala	Ala	Gly	Val	Ile	Leu	Trp	Gly	Asp	Ala	Gly	Tyr	Thr	Thr	Ser
					325					330						335
30	Thr	Glu	Thr	Cys	Gln	Tyr	Leu	Lys	Asp	Tyr	Leu	Thr	Arg	Leu	Leu	Val
				340					345					350		
	Pro	Tyr	Val	Val	Asn	Val	Ser	Trp	Ala	Thr	Gln	Tyr	Cys	Ser	Arg	Ala
			355					360					365			
	Gln	Cys	His	Gly	His	Gly	Arg	Cys	Val	Arg	Arg	Asn	Pro	Ser	Ala	Ser
		370					375					380				
35	Thr	Phe	Leu	His	Leu	Ser	Thr	Asn	Ser	Phe	Arg	Leu	Val	Pro	Gly	His
	385					390					395					400
	Ala	Pro	Gly	Glu	Pro	Gln	Leu	Arg	Pro	Val	Gly	Glu	Leu	Ser	Trp	Ala
					405					410						415
40	Asp	Ile	Asp	His	Leu	Gln	Thr	His	Phe	Arg	Cys	Gln	Cys	Tyr	Leu	Gly
				420					425					430		
	Trp	Ser	Gly	Glu	Gln	Cys	Gln	Trp	Asp	His	Arg	Gln	Ala	Ala	Gly	Gly
			435					440					445			
45	Ala	Ser	Glu	Ala	Trp	Ala	Gly	Ser	His	Leu	Thr	Ser	Leu	Leu	Ala	Leu
		450					455					460				
	Ala	Ala	Leu	Ala	Phe	Thr	Trp	Thr	Leu							
	465					470										

<210> 36
 <211> 417
 <212> PRT
 <213> Homo sapiens

 <220>
 <223> Hyaluronidase-3 [Precursor]

 <400> 36

Met Thr Thr Gln Leu Gly Pro Ala Leu Val Leu Gly Val Ala Leu Cys

5

10

15

20

25

30

35

40

45

50

55

	1						5						10						15
	Leu	Gly	Cys	Gly	Gln	Pro	Leu	Pro	Gln	Val	Pro	Glu	Arg	Pro	Phe	Ser			
				20					25					30					
5	Val	Leu	Trp	Asn	Val	Pro	Ser	Ala	His	Cys	Glu	Ala	Arg	Phe	Gly	Val			
			35					40					45						
	His	Leu	Pro	Leu	Asn	Ala	Leu	Gly	Ile	Ile	Ala	Asn	Arg	Gly	Gln	His			
		50					55					60							
10	Phe	His	Gly	Gln	Asn	Met	Thr	Ile	Phe	Tyr	Lys	Asn	Gln	Leu	Gly	Leu			
	65					70					75					80			
	Tyr	Pro	Tyr	Phe	Gly	Pro	Arg	Gly	Thr	Ala	His	Asn	Gly	Gly	Ile	Pro			
				85						90					95				
	Gln	Ala	Leu	Pro	Leu	Asp	Arg	His	Leu	Ala	Leu	Ala	Ala	Tyr	Gln	Ile			
				100					105					110					
15	His	His	Ser	Leu	Arg	Pro	Gly	Phe	Ala	Gly	Pro	Ala	Val	Leu	Asp	Trp			
			115					120					125						
	Glu	Glu	Trp	Cys	Pro	Leu	Trp	Ala	Gly	Asn	Trp	Gly	Arg	Arg	Arg	Ala			
		130					135					140							
20	Tyr	Gln	Ala	Ala	Ser	Trp	Ala	Trp	Ala	Gln	Gln	Val	Phe	Pro	Asp	Leu			
	145					150					155					160			
	Asp	Pro	Gln	Glu	Gln	Leu	Tyr	Lys	Ala	Tyr	Thr	Gly	Phe	Glu	Gln	Ala			
				165						170					175				
25	Ala	Arg	Ala	Leu	Met	Glu	Asp	Thr	Leu	Arg	Val	Ala	Gln	Ala	Leu	Arg			
				180					185					190					
	Pro	His	Gly	Leu	Trp	Gly	Phe	Tyr	His	Tyr	Pro	Ala	Cys	Gly	Asn	Gly			
			195					200					205						
	Trp	His	Ser	Met	Ala	Ser	Asn	Tyr	Thr	Gly	Arg	Cys	His	Ala	Ala	Thr			
		210					215					220							
30	Leu	Ala	Arg	Asn	Thr	Gln	Leu	His	Trp	Leu	Trp	Ala	Ala	Ser	Ser	Ala			
	225					230					235					240			
	Leu	Phe	Pro	Ser	Ile	Tyr	Leu	Pro	Pro	Arg	Leu	Pro	Pro	Ala	His	His			
				245						250					255				
35	Gln	Ala	Phe	Val	Arg	His	Arg	Leu	Glu	Glu	Ala	Phe	Arg	Val	Ala	Leu			
				260					265					270					
	Val	Gly	His	Arg	His	Pro	Leu	Pro	Val	Leu	Ala	Tyr	Val	Arg	Leu	Thr			
			275					280					285						
40	His	Arg	Arg	Ser	Gly	Arg	Phe	Leu	Ser	Gln	Asp	Asp	Leu	Val	Gln	Ser			
		290					295					300							
	Ile	Gly	Val	Ser	Ala	Ala	Leu	Gly	Ala	Ala	Gly	Val	Val	Leu	Trp	Gly			
	305					310					315					320			
	Asp	Leu	Ser	Leu	Ser	Ser	Ser	Glu	Glu	Glu	Cys	Trp	His	Leu	His	Asp			
				325						330					335				
45	Tyr	Leu	Val	Asp	Thr	Leu	Gly	Pro	Tyr	Val	Ile	Asn	Val	Thr	Arg	Ala			
				340					345					350					
	Ala	Met	Ala	Cys	Ser	His	Gln	Arg	Cys	His	Gly	His	Gly	Arg	Cys	Ala			
			355					360					365						
50	Arg	Arg	Asp	Pro	Gly	Gln	Met	Glu	Ala	Phe	Leu	His	Leu	Trp	Pro	Asp			
		370					375					380							
	Gly	Ser	Leu	Gly	Asp	Trp	Lys	Ser	Phe	Ser	Cys	His	Cys	Tyr	Trp	Gly			
	385					390					395					400			
	Trp	Ala	Gly	Pro	Thr	Cys	Gln	Glu	Pro	Arg	Pro	Gly	Pro	Lys	Glu	Ala			
				405						410					415				
55	Val																		

<210> 37

<211> 481
<212> PRT
<213> Homo sapiens

5 <220>
 <223> Hyaluronidase-4

 <400> 37

10

15

20

25

30

35

40

45

50

55

EP 4 269 578 B1

	Met	Lys	Val	Leu	Ser	Glu	Gly	Gln	Leu	Lys	Leu	Cys	Val	Val	Gln	Pro
	1				5					10					15	
	Val	His	Leu	Thr	Ser	Trp	Leu	Leu	Ile	Phe	Phe	Ile	Leu	Lys	Ser	Ile
5				20					25					30		
	Ser	Cys	Leu	Lys	Pro	Ala	Arg	Leu	Pro	Ile	Tyr	Gln	Arg	Lys	Pro	Phe
			35					40					45			
	Ile	Ala	Ala	Trp	Asn	Ala	Pro	Thr	Asp	Gln	Cys	Leu	Ile	Lys	Tyr	Asn
		50					55					60				
10	Leu	Arg	Leu	Asn	Leu	Lys	Met	Phe	Pro	Val	Ile	Gly	Ser	Pro	Leu	Ala
	65					70					75					80
	Lys	Ala	Arg	Gly	Gln	Asn	Val	Thr	Ile	Phe	Tyr	Val	Asn	Arg	Leu	Gly
					85					90					95	
	Tyr	Tyr	Pro	Trp	Tyr	Thr	Ser	Gln	Gly	Val	Pro	Ile	Asn	Gly	Gly	Leu
15				100					105					110		
	Pro	Gln	Asn	Ile	Ser	Leu	Gln	Val	His	Leu	Glu	Lys	Ala	Asp	Gln	Asp
			115					120					125			
	Ile	Asn	Tyr	Tyr	Ile	Pro	Ala	Glu	Asp	Phe	Ser	Gly	Leu	Ala	Val	Ile
		130					135					140				
20	Asp	Trp	Glu	Tyr	Trp	Arg	Pro	Gln	Trp	Ala	Arg	Asn	Trp	Asn	Ser	Lys
	145					150					155					160
	Asp	Val	Tyr	Arg	Gln	Lys	Ser	Arg	Lys	Leu	Ile	Ser	Asp	Met	Gly	Lys
					165					170					175	
25	Asn	Val	Ser	Ala	Thr	Asp	Ile	Glu	Tyr	Leu	Ala	Lys	Val	Thr	Phe	Glu
				180					185					190		
	Glu	Ser	Ala	Lys	Ala	Phe	Met	Lys	Glu	Thr	Ile	Lys	Leu	Gly	Ile	Lys
			195					200					205			
	Ser	Arg	Pro	Lys	Gly	Leu	Trp	Gly	Tyr	Tyr	Leu	Tyr	Pro	Asp	Cys	His
30		210					215					220				
	Asn	Tyr	Asn	Val	Tyr	Ala	Pro	Asn	Tyr	Ser	Gly	Ser	Cys	Pro	Glu	Asp
	225					230					235					240
	Glu	Val	Leu	Arg	Asn	Asn	Glu	Leu	Ser	Trp	Leu	Trp	Asn	Ser	Ser	Ala
					245					250					255	
35	Ala	Leu	Tyr	Pro	Ser	Ile	Gly	Val	Trp	Lys	Ser	Leu	Gly	Asp	Ser	Glu
				260					265					270		
	Asn	Ile	Leu	Arg	Phe	Ser	Lys	Phe	Arg	Val	His	Glu	Ser	Met	Arg	Ile
			275					280					285			
40	Ser	Thr	Met	Thr	Ser	His	Asp	Tyr	Ala	Leu	Pro	Val	Phe	Val	Tyr	Thr
		290					295					300				
	Arg	Leu	Gly	Tyr	Arg	Asp	Glu	Pro	Leu	Phe	Phe	Leu	Ser	Lys	Gln	Asp
	305					310					315					320
	Leu	Val	Ser	Thr	Ile	Gly	Glu	Ser	Ala	Ala	Leu	Gly	Ala	Ala	Gly	Ile
45					325					330					335	
	Val	Ile	Trp	Gly	Asp	Met	Asn	Leu	Thr	Ala	Ser	Lys	Ala	Asn	Cys	Thr
				340					345					350		
	Lys	Val	Lys	Gln	Phe	Val	Ser	Ser	Asp	Leu	Gly	Ser	Tyr	Ile	Ala	Asn
			355					360					365			
50	Val	Thr	Arg	Ala	Ala	Glu	Val	Cys	Ser	Leu	His	Leu	Cys	Arg	Asn	Asn
						370		375					380			

55

EP 4 269 578 B1

	Gly	Arg	Cys	Ile	Arg	Lys	Met	Trp	Asn	Ala	Pro	Ser	Tyr	Leu	His	Leu
	385					390					395					400
	Asn	Pro	Ala	Ser	Tyr	His	Ile	Glu	Ala	Ser	Glu	Asp	Gly	Glu	Phe	Thr
5					405					410					415	
	Val	Lys	Gly	Lys	Ala	Ser	Asp	Thr	Asp	Leu	Ala	Val	Met	Ala	Asp	Thr
				420					425					430		
	Phe	Ser	Cys	His	Cys	Tyr	Gln	Gly	Tyr	Glu	Gly	Ala	Asp	Cys	Arg	Glu
			435					440					445			
10	Ile	Lys	Thr	Ala	Asp	Gly	Cys	Ser	Gly	Val	Ser	Pro	Ser	Pro	Gly	Ser
		450					455					460				
	Leu	Met	Thr	Leu	Cys	Leu	Leu	Leu	Ala	Ser	Tyr	Arg	Ser	Ile	Gln	
	465					470				475					480	
15	Leu															

<210> 38
 <211> 467
 <212> PRT
 <213> Homo sapiens
 <220>
 <223> sHuPH20 precursor 1-467
 <400> 38

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
5	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
				20					25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
			35					40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
		50				55						60				
10	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65				70						75				80	
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
				85					90					95		
15	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
				100					105					110		
	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
			115					120					125			
20	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
		130					135					140				
	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155				160	
	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
				165						170				175		
25	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
				180					185					190		
	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
			195					200					205			
30	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
		210					215					220				
	Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
	225				230						235				240	
	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser

35

40

45

50

55

					245					250				255		
	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
					260					265				270		
5	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
			275				280						285			
	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
		290					295					300				
10	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
	305					310					315				320	
	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
					325					330					335	
	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
				340					345					350		
15	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
			355					360					365			
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
		370					375					380				
20	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
	385					390					395				400	
	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
					405					410					415	
	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
25				420					425					430		
	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
			435					440					445			
	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
		450					455					460				
30	Ile	Asp	Ala													
	465															

<210> 39

<211> 477

35 <212> PRT

<213> Homo sapiens

<220>

<223> sHuPH20 precursor 1-477

40

<400> 39

45

50

55

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
5	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
				20					25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
			35					40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
		50				55						60				
10	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65					70					75				80	
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
				85					90					95		
15	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
				100					105					110		
	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys

20

25

30

35

40

45

50

55

EP 4 269 578 B1

		Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
		130						135					140				
5		Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
		145					150					155					160
		Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
						165					170					175	
10		Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
					180					185					190		
		Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
				195					200					205			
		Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
		210						215					220				
15		Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
		225				230						235					240
		Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
						245					250					255	
20		Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
					260					265					270		
		Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
				275				280					285				
		Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
25			290					295					300				
		Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
		305					310					315					320
		Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
						325					330					335	
30		Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
					340					345					350		
		Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
				355				360					365				
35		Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
			370					375					380				
		Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
		385					390					395					400
		Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
40						405					410					415	
		Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
					420					425					430		
		Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
				435				440					445				
45		Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
			450					455					460				
		Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu			
		465					470						475				
50		<210> 40															
		<211> 478															
		<212> PRT															
		<213> Homo sapiens															
55		<220>															
		<223> sHuPH20 precursor 1-478															
		<400> 40															

EP 4 269 578 B1

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
			20						25					30		
5	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
		35						40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
	50					55						60				
10	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65					70					75					80
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
				85						90					95	
	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
				100					105					110		
15	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
		115						120					125			
	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
	130						135					140				
20	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155					160
	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
					165					170					175	
	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
				180					185					190		
25	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
		195						200					205			
	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
	210					215						220				
30	Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
	225					230					235					240
	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
					245					250					255	
	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
				260					265					270		
35	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
		275						280					285			
	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
	290						295					300				
40	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
	305					310					315					320
	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
					325					330					335	
	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
			340						345					350		
45	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
		355						360					365			
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
		370					375					380				
50	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
	385					390					395					400
	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
					405					410					415	
	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
				420					425					430		
55	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp

EP 4 269 578 B1

[illegible]

10 <210> 41
<211> 479
<212> PRT
<213> Homo sapiens

15 <220>
<223> sHuPH20 precursor 1-479

<400> 41

20

25

30

35

40

45

50

55

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
5				20					25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
			35					40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
		50					55					60				
10	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65					70					75					80
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
					85					90					95	
	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
15				100					105					110		
	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
			115					120					125			
	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
20		130					135					140				
	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155					160
	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
					165					170					175	
25	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
				180					185					190		
	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
			195					200					205			
	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
30		210					215					220				
	Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
	225					230					235					240
	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
					245					250					255	
35	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
				260					265					270		
	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
			275					280					285			
40	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
		290					295					300				
	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu

45

50

55

	305				310				315			320
	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly
					325				330			335
5	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met
				340					345			350
	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro
			355						360			365
10	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu
			370						375			380
	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp
	385					390					395	400
	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly
				405					410			415
15	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln
			420						425			430
	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys
			435						440			445
20	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala
		450					455					460
	Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu
	465					470						475

25 <210> 42
 <211> 480
 <212> PRT
 <213> Homo sapiens

 30 <220>
 <223> sHuPH20 precursor 1-480

 <400> 42

35

40

45

50

55

EP 4 269 578 B1

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
5			20						25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
			35					40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
		50					55					60				
10	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65					70					75					80
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
					85					90				95		
	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
15				100					105					110		
	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
			115					120					125			
	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
20		130					135					140				
	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155					160
	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
					165					170				175		
25	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe

30

35

40

45

50

55

EP 4 269 578 B1

5	Glu 205	Lys 210	Ala 195	Gly 180	Lys	Asp	Phe	Leu 200	Val 185	Glu	Thr	Ile	Lys 205	Leu 190	Gly	Lys	
	Leu 225	Leu 210	Arg	Pro	Asn	His	Leu 215	Trp	Gly	Tyr	Tyr	Leu 220	Phe	Pro	Asp	Cys	
	Tyr 225	Asn	His	His	Tyr	Lys 230	Lys	Pro	Gly	Tyr	Asn 235	Gly	Ser	Cys	Phe	Asn 240	
10	Val	Glu	Ile	Lys	Arg 245	Asn	Asp	Asp	Leu	Ser 250	Trp	Leu	Trp	Asn	Glu 255	Ser	
	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu 265	Asn	Thr	Gln	Gln	Ser	Pro	Val	
	Ala	Ala	Thr 275	Leu	Tyr	Val	Arg	Asn 280	Arg	Val	Arg	Glu	Ala 285	Ile	Arg	Val	
15	Ser	Lys 290	Ile	Pro	Asp	Ala	Lys 295	Ser	Pro	Leu	Pro	Val 300	Phe	Ala	Tyr	Thr	
	Arg 305	Ile	Val	Phe	Thr	Asp 310	Gln	Val	Leu	Lys	Phe 315	Leu	Ser	Gln	Asp	Glu 320	
20	Leu	Val	Tyr	Thr	Phe 325	Gly	Glu	Thr	Val	Ala 330	Leu	Gly	Ala	Ser	Gly 335	Ile	
	Val	Ile	Trp	Gly 340	Thr	Leu	Ser	Ile	Met 345	Arg	Ser	Met	Lys	Ser	Cys	Leu	
	Leu	Leu	Asp 355	Asn	Tyr	Met	Glu	Thr 360	Ile	Leu	Asn	Pro	Tyr 365	Ile	Ile	Asn	
25	Val	Thr 370	Leu	Ala	Ala	Lys	Met 375	Cys	Ser	Gln	Val	Leu 380	Cys	Gln	Glu	Gln	
	Gly 385	Val	Cys	Ile	Arg	Lys 390	Asn	Trp	Asn	Ser	Ser	Asp 395	Tyr	Leu	His	Leu 400	
30	Asn	Pro	Asp	Asn	Phe 405	Ala	Ile	Gln	Leu	Glu 410	Lys	Gly	Gly	Lys	Phe 415	Thr	
	Val	Arg	Gly	Lys 420	Pro	Thr	Leu	Glu	Asp 425	Leu	Glu	Gln	Phe	Ser	Glu	Lys	
	Phe	Tyr	Cys 435	Ser	Cys	Tyr	Ser	Thr 440	Leu	Ser	Cys	Lys	Glu 445	Lys	Ala	Asp	
35	Val	Lys 450	Asp	Thr	Asp	Ala	Val 455	Asp	Val	Cys	Ile	Ala 460	Asp	Gly	Val	Cys	
	Ile 465	Asp	Ala	Phe	Leu	Lys 470	Pro	Pro	Met	Glu	Thr 475	Glu	Glu	Pro	Gln	Ile 480	

<210> 43

<211> 481

<212> PRT

<213> Homo sapiens

 $\langle 220 \rangle$

<223> sHuPH20 precursor 1-481

<400> 43

EP 4 269 578 B1

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
5			20						25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
			35					40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
10																
15																
20																
25																
30																
35																
40																
45																
50																
55																

EP 4 269 578 B1

		50				55				60							
		Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
		65					70					75					80
5		Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
						85					90					95	
		Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
					100					105					110		
		Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
10				115					120					125			
		Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
			130					135					140				
		Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
		145					150					155					160
15		Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
						165					170					175	
		Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
					180					185					190		
		Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
20				195					200					205			
		Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
			210					215					220				
		Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
		225				230						235					240
25		Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
					245						250					255	
		Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
					260					265					270		
30		Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
				275					280					285			
		Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
			290					295					300				
		Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
35		305				310						315					320
		Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
					325						330					335	
		Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
					340					345					350		
40		Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
				355				360						365			
		Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
			370					375					380				
		Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
45		385				390						395					400
		Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
					405						410					415	
		Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
					420					425					430		
50		Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
				435					440					445			
		Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
			450					455					460				
55		Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile
		465					470					475					480
		Phe															

<210> 44
 <211> 483
 <212> PRT
 <213> Homo sapiens

5

<220>
 <223> sHuPH20 precursor 1-483

10

<400> 44

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
				20					25					30		
15	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
			35					40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
		50				55						60				
20	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65				70						75				80	
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
				85					90					95		
	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
				100					105					110		
25	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
			115					120					125			
	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
		130					135					140				
30	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155				160	
	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
				165					170					175		
35	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
				180					185					190		
	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
			195					200					205			
	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
		210					215					220				
40	Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
	225				230						235				240	
	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
				245					250					255		
45	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
				260					265					270		
	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
			275					280					285			
50	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
		290					295					300				
	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
	305					310					315				320	
	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
				325					330					335		
55	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
				340					345				350			
	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn

			355				360				365						
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	
		370					375					380					
5	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	
	385					390					395					400	
	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	
				405						410					415		
10	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	
			420						425					430			
	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	
		435					440					445					
	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	
15		450				455					460						
	Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile	
	465					470					475					480	
	Phe	Tyr	Asn														
20	<210> 45																
	<211> 432																
	<212> PRT																
	<213> Homo sapiens																
25	<220>																
	<223> sHuPH20 mature 36-467																
	<400> 45																
30	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp	
	1				5					10					15		
	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro	
			20						25				30				
35	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala	
		35					40					45					
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr	
	50					55					60						
	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro	
40	65				70						75				80		
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile	
				85						90					95		
	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp	
			100						105					110			
45	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val	
		115					120					125					
	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu	
	130					135					140						
	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala	
50	145				150						155					160	
	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg	
				165						170					175		
	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His	
			180						185					190			
55	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile	
		195				200						205					
	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu	

EP 4 269 578 B1

	210		215		220											
	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
	225					230					235					240
5	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
					245						250					255
	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
				260					265							270
	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
10			275					280					285			
	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
		290				295						300				
	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
	305					310						315				320
15	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
					325					330						335
	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
			340						345							350
	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
20			355					360					365			
	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly
		370				375						380				
	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys
25		385			390						395					400
	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp
				405						410						415
	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala
			420						425							430

30

<210> 46
 <211> 448
 <212> PRT
 <213> Homo sapiens

35

<220>
 <223> sHuPH20 mature 36-483

40

<400> 46

45

50

55

	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro	Phe	Leu	Trp
	1				5					10					15	
5	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe	Asp	Glu	Pro
			20						25					30		
	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg	Ile	Asn	Ala
		35						40					45			
	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu	Gly	Tyr	Tyr
		50					55					60				
10	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly	Gly	Ile	Pro
	65					70					75					80
	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys	Lys	Asp	Ile
					85					90					95	
15	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val	Ile	Asp	Trp
				100					105					110		
	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro	Lys	Asp	Val
			115					120					125			
	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn	Val	Gln	Leu

20

25

30

35

40

45

50

55

		130			135			140									
		Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe	Glu	Lys	Ala
		145					150					155					160
5		Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys	Leu	Leu	Arg
					165						170						175
		Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys	Tyr	Asn	His
					180					185					190		
10		His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn	Val	Glu	Ile
			195						200					205			
		Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser	Thr	Ala	Leu
			210					215					220				
		Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val	Ala	Ala	Thr
		225					230					235					240
15		Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val	Ser	Lys	Ile
					245						250						255
		Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr	Arg	Ile	Val
					260					265					270		
20		Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu	Leu	Val	Tyr
			275						280					285			
		Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile	Val	Ile	Trp
			290				295						300				
		Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu	Leu	Leu	Asp
25		305					310					315					320
		Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn	Val	Thr	Leu
					325						330						335
		Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln	Gly	Val	Cys
				340						345					350		
30		Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu	Asn	Pro	Asp
			355						360					365			
		Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr	Val	Arg	Gly
			370					375					380				
35		Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys	Phe	Tyr	Cys
		385					390					395					400
		Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp	Val	Lys	Asp
					405						410						415
		Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys	Ile	Asp	Ala
40					420					425					430		
		Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile	Phe	Tyr	Asn
				435					440					445			

<210> 47

45 <211> 1446

<212> DNA

<213> Homo sapiens

<220>

50 <223> DNA encoding soluble rHuPH20 "precursor"

<400> 47

55

atgggagtgc taaaattcaa gcacatcttt ttcagaagct ttgttaaadc aagtggagta 60
 tcccagatag ttttcacctt ctttctgatt ccatgttgct tgactctgaa tttcagagca 120
 cctcctgtta ttccaaatgt gcctttcctc tgggcctgga atgccccaaag tgaattttgt 180
 cttggaaaat ttgatgagcc actagatatg agcctcttct ctttcatagg aagccccga 240
 ataaacgccca ccgggcaagg tggtacaata ttttatgttg atagacttgg ctactatcct 300

tacatagatt caatcacagg agtaactgtg aatggaggaa tccccagaa gatttcctta 360
 caagaccatc tggacaaaagc taagaaagac attacatttt atatgccagt agacaatttg 420
 ggaatggctg ttattgactg ggaagaatgg agaccactt gggcaagaaa ctggaaacct 480
 aaagatgttt acaagaatag gtctattgaa ttggttcagc aacaaaatgt acaacttagt 540
 ctacagagg cactgagaa agcaaaacaa gaatttgaaa aggcagggaa ggatttcctg 600
 gtagagacta taaaattggg aaaattactt cggccaaatc acttggtggg ttattatctt 660
 tttccggatt gttacaacca tcactataag aaaccgggtt acaatggaag ttgcttcaat 720
 gtagaaataa aaagaaatga tgatctcagc tggttgtgga atgaaagcac tgctctttac 780
 ccatccattt atttgaacac tcagcagtct cctgtagctg ctacactcta tgtgcgcaat 840
 cgagttcggg aagccatcag agtttccaaa atacctgatg caaaaagtcc acttccgggtt 900
 tttgcatata cccgcatagt ttttactgat caagttttga aattcctttc tcaagatgaa 960
 cttgtgtata catttggcga aactgttgct ctgggtgctt ctggaattgt aatatgggga 1020
 accctcagta taatgcgaag tatgaaatct tgcttgctcc tagacaatta catggagact 1080
 atactgaatc cttacataat caacgtcaca ctagcagcca aaatgtgtag ccaagtgcct 1140
 tgccaggagc aaggagtgtg tataaggaaa aactggaatt caagtgacta tcttcacctc 1200
 aaccagata attttgctat tcaacttgag aaagggtgga agttcacagt acgtggaaaa 1260
 ccgacacttg aagacctgga gcaattttct gaaaaatttt attgcagctg ttatagcacc 1320
 ttgagttgta aggagaaagc tgatgtaaaa gacactgatg ctgttgatgt gtgtattgct 1380
 gatggtgtct gtatagatgc ttttctaaaa cctcccatgg agacagaaga acctcaaatt 1440
 ttctac 1446

<210> 48

<211> 509

<212> PRT

<213> Homo sapiens

<220>

<223> PH20 variant P48A

<400> 48

EP 4 269 578 B1

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
5				20					25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Ala
			35					40					45			
	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
		50					55					60				
10	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65					70					75					80
	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
					85					90					95	
	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
15				100					105					110		
	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
			115					120					125			
	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
20		130					135					140				
	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155					160
	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
					165					170					175	
25	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
				180					185					190		
	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
			195					200					205			

30

35

40

45

50

55

	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
	210						215					220				
5	Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
	225				230						235					240
	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
				245						250					255	
	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
				260					265					270		
10	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
			275				280						285			
	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
	290					295						300				
15	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
	305				310						315					320
	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
				325						330					335	
	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
			340						345					350		
20	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
			355				360						365			
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
		370				375						380				
25	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
	385				390					395						400
	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
				405						410					415	
30	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
			420						425				430			
	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
		435				440						445				
	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
		450				455						460				
35	Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile
	465				470					475						480
	Phe	Tyr	Asn	Ala	Ser	Pro	Ser	Thr	Leu	Ser	Ala	Thr	Met	Phe	Ile	Val
				485						490					495	
40	Ser	Ile	Leu	Phe	Leu	Ile	Ile	Ser	Ser	Val	Ala	Ser	Leu			
			500						505							

<210> 49

<211> 509

45 <212> PRT

<213> Homo sapiens

<220>

<223> precursor PH20 variant L499W

50

<400> 49

55

EP 4 269 578 B1

	Met	Gly	Val	Leu	Lys	Phe	Lys	His	Ile	Phe	Phe	Arg	Ser	Phe	Val	Lys
	1				5					10					15	
	Ser	Ser	Gly	Val	Ser	Gln	Ile	Val	Phe	Thr	Phe	Leu	Leu	Ile	Pro	Cys
5			20						25					30		
	Cys	Leu	Thr	Leu	Asn	Phe	Arg	Ala	Pro	Pro	Val	Ile	Pro	Asn	Val	Pro
			35					40					45			
10																
15																
20																
25																
30																
35																
40																
45																
50																
55																

EP 4 269 578 B1

	Phe	Leu	Trp	Ala	Trp	Asn	Ala	Pro	Ser	Glu	Phe	Cys	Leu	Gly	Lys	Phe
	50						55					60				
	Asp	Glu	Pro	Leu	Asp	Met	Ser	Leu	Phe	Ser	Phe	Ile	Gly	Ser	Pro	Arg
	65					70					75					80
5	Ile	Asn	Ala	Thr	Gly	Gln	Gly	Val	Thr	Ile	Phe	Tyr	Val	Asp	Arg	Leu
					85					90					95	
	Gly	Tyr	Tyr	Pro	Tyr	Ile	Asp	Ser	Ile	Thr	Gly	Val	Thr	Val	Asn	Gly
				100					105					110		
10	Gly	Ile	Pro	Gln	Lys	Ile	Ser	Leu	Gln	Asp	His	Leu	Asp	Lys	Ala	Lys
			115					120					125			
	Lys	Asp	Ile	Thr	Phe	Tyr	Met	Pro	Val	Asp	Asn	Leu	Gly	Met	Ala	Val
	130						135					140				
	Ile	Asp	Trp	Glu	Glu	Trp	Arg	Pro	Thr	Trp	Ala	Arg	Asn	Trp	Lys	Pro
	145					150					155					160
15	Lys	Asp	Val	Tyr	Lys	Asn	Arg	Ser	Ile	Glu	Leu	Val	Gln	Gln	Gln	Asn
					165					170					175	
	Val	Gln	Leu	Ser	Leu	Thr	Glu	Ala	Thr	Glu	Lys	Ala	Lys	Gln	Glu	Phe
				180					185					190		
20	Glu	Lys	Ala	Gly	Lys	Asp	Phe	Leu	Val	Glu	Thr	Ile	Lys	Leu	Gly	Lys
			195					200					205			
	Leu	Leu	Arg	Pro	Asn	His	Leu	Trp	Gly	Tyr	Tyr	Leu	Phe	Pro	Asp	Cys
	210						215					220				
	Tyr	Asn	His	His	Tyr	Lys	Lys	Pro	Gly	Tyr	Asn	Gly	Ser	Cys	Phe	Asn
	225					230					235					240
25	Val	Glu	Ile	Lys	Arg	Asn	Asp	Asp	Leu	Ser	Trp	Leu	Trp	Asn	Glu	Ser
					245					250					255	
	Thr	Ala	Leu	Tyr	Pro	Ser	Ile	Tyr	Leu	Asn	Thr	Gln	Gln	Ser	Pro	Val
				260					265					270		
30	Ala	Ala	Thr	Leu	Tyr	Val	Arg	Asn	Arg	Val	Arg	Glu	Ala	Ile	Arg	Val
			275					280					285			
	Ser	Lys	Ile	Pro	Asp	Ala	Lys	Ser	Pro	Leu	Pro	Val	Phe	Ala	Tyr	Thr
	290						295					300				
	Arg	Ile	Val	Phe	Thr	Asp	Gln	Val	Leu	Lys	Phe	Leu	Ser	Gln	Asp	Glu
	305					310					315					320
35	Leu	Val	Tyr	Thr	Phe	Gly	Glu	Thr	Val	Ala	Leu	Gly	Ala	Ser	Gly	Ile
					325					330					335	
	Val	Ile	Trp	Gly	Thr	Leu	Ser	Ile	Met	Arg	Ser	Met	Lys	Ser	Cys	Leu
				340					345					350		
40	Leu	Leu	Asp	Asn	Tyr	Met	Glu	Thr	Ile	Leu	Asn	Pro	Tyr	Ile	Ile	Asn
			355					360					365			
	Val	Thr	Leu	Ala	Ala	Lys	Met	Cys	Ser	Gln	Val	Leu	Cys	Gln	Glu	Gln
						375						380				
	Gly	Val	Cys	Ile	Arg	Lys	Asn	Trp	Asn	Ser	Ser	Asp	Tyr	Leu	His	Leu
	385					390					395					400
45	Asn	Pro	Asp	Asn	Phe	Ala	Ile	Gln	Leu	Glu	Lys	Gly	Gly	Lys	Phe	Thr
					405					410					415	
	Val	Arg	Gly	Lys	Pro	Thr	Leu	Glu	Asp	Leu	Glu	Gln	Phe	Ser	Glu	Lys
				420					425					430		
50	Phe	Tyr	Cys	Ser	Cys	Tyr	Ser	Thr	Leu	Ser	Cys	Lys	Glu	Lys	Ala	Asp
			435					440					445			
	Val	Lys	Asp	Thr	Asp	Ala	Val	Asp	Val	Cys	Ile	Ala	Asp	Gly	Val	Cys
						455						460				
	Ile	Asp	Ala	Phe	Leu	Lys	Pro	Pro	Met	Glu	Thr	Glu	Glu	Pro	Gln	Ile
	465					470					475					480
55	Phe	Tyr	Asn	Ala	Ser	Pro	Ser	Thr	Leu	Ser	Ala	Thr	Met	Phe	Ile	Val
					485					490					495	

Ser Ile Trp Phe Leu Ile Ile Ser Ser Val Ala Ser Leu
500 505

5 <210> 50
<211> 6630
<212> DNA
<213> Artificial Sequence

10 <220>
<223> HZ24 vector

<400> 50

15

20

25

30

35

40

45

50

55

	tcaatattgg	ccattagcca	tattattcat	tggttatata	gcataaatca	atattggcta	60
	ttggccattg	catacgttgt	atctatatca	taatattgtac	atattatattg	gctcatgtcc	120
	aatatgaccg	ccatgttggc	attgattatt	gactagtatt	taatagtaat	caattacggg	180
5	gtcattagtt	catagcccat	atatggagtt	ccgcgttaca	taacttacgg	taaatggccc	240
	gcctggctga	ccgccaacg	acccccgcc	attgacgtca	ataatgacgt	atgttcccat	300
	agtaacgcca	atagggactt	tccattgacg	tcaatgggtg	gagtatttac	ggtaaaactgc	360
	ccacttggca	gtacatcaag	tgtatcatat	gccaaagtccg	ccccctattg	acgtcaatga	420
	cggtaaatgg	cccgcctggc	attatgccc	gtacatgacc	ttacgggact	ttcctacttg	480
10	gcagtacatc	tacgtattag	tcacgcgtat	taccatgggtg	atgcggtttt	ggcagtacac	540
	caatgggctg	ggatagcggg	ttgactcacg	gggatttcca	agtctccacc	ccattgacgt	600
	caatgggagt	ttgttttggc	acaaaaatca	acgggacttt	ccaaaatgtc	gtaataaccc	660
	cgccccgttg	acgcaaatgg	gcggtaggcg	tgtacgggtg	gaggctctata	taagcagagc	720
	tcgttttagtg	aaccgtcaga	tcactagaag	ctttattgcg	gtagtttatc	acagttaaat	780
15	tgctaacgca	gtcagtgctt	ctgacacaac	agtctcgaac	ttaagctgca	gaagttggct	840
	gtgaggcatt	gggcaggtaa	gtatcaaggt	tacaagacag	gtttaaggag	accaatagaa	900
	actgggcttg	tcgagacaga	gaagactctt	gcgtttctga	taggcaccta	ttggtcttac	960
	tgacatccac	tttgcctttc	tctccacagg	tgtccactcc	cagttcaatt	acagctctta	1020
20	aggctagagt	acttaatacg	actcactata	ggctagcatg	ggagtgcctaa	aattcaagca	1080
	catctttttc	agaagctttg	ttaaatcaag	tggagtatcc	cagatagttt	tcaccttcct	1140
	tctgattcca	tgttgcttga	ctctgaattt	cagagcacct	cctgttattc	caaatgtgcc	1200
	tttctcttgg	gctggaatg	ccccaaagtga	attttgtctt	ggaaaatttg	atgagccact	1260
	agatatgagc	ctcttctctt	tcataggaag	ccccgaata	aacgccaccg	ggcaagggtg	1320
25	tacaatattt	tatgttgata	gacttggcta	ctatccttac	atagattcaa	tcacaggagt	1380
	aactgtgaat	ggagggaatcc	cccagaagat	ttccttacaa	gaccatctgg	acaaagctaa	1440
	gaaagacatt	acattttata	tgccagtaga	caatttggga	atggctgtta	ttgactggga	1500
	agaatggaga	cccacttggg	caagaaactg	gaaaccta	gatgtttaca	agaatagggtc	1560
	tattgaattg	gttcagcaac	aaaatgtaca	acttagtctc	acagaggcca	ctgagaaagc	1620
30	aaaacaagaa	tttgaaaaagg	caggggaagga	tttcttggtg	gagactataa	aattgggaaa	1680
	attacttcgg	ccaaatcact	tgtgggggtta	ttatcttttt	ccggattgtt	acaaccatca	1740
	ctataagaaa	cccgggttaca	atggaagtgt	cttcaatgta	gaaataaaaa	gaaatgatga	1800
	tctcagctgg	ttgtggaatg	aaagcactgc	tctttaccca	tccattttatt	tgaacactca	1860
35	gcagtctcct	gtagctgcta	cactctatgt	gcgcaatcga	gttcgggaag	ccatcagagt	1920
	ttccaaaata	cctgatgcaa	aaagtccact	tccggttttt	gcatataccc	gcatagtttt	1980
	tactgatcaa	gttttgaaat	tcctttctca	agatgaactt	gtgtatacat	ttggcgaaac	2040
	tgttgctctg	ggtgcttctg	gaattgtaat	atggggaacc	ctcagtataa	tgcgaaagtat	2100
	gaaatcttgc	ttgctcctag	acaattacat	ggagactata	ctgaatcctt	acataatcaa	2160
40	cgtcacacta	gcagccaaaa	tgtgtagcca	agtgttttgc	caggagcaag	gagtgtgtat	2220
	aaggaaaaac	tgggaattcaa	gtgactatct	tcacctcaac	ccagataatt	ttgtatttca	2280
	acttgagaaa	ggtggaaagt	tcacagtacg	tggaaaaccg	acacttgaag	acctggagca	2340
	atcttctgaa	aaatcttatt	gcagctgtta	tagcaccttg	agttgtgaag	agaaagctga	2400
	tgtaaaagac	actgatgctg	ttgatgtgtg	tattgtctgat	ggtgtctgta	tagatgcttt	2460
45	tctaaaacct	cccatggaga	cagaagaacc	tcaaattttc	tactgaggat	ccatagctaa	2520
	cgccccctctc	cctccccccc	ccctaacgtt	actggccgaa	gccgcttgga	ataaggccgg	2580

50

55

	tgtgcgtttg	tctatatgtt	atthttccacc	atattgcccgt	cttttggcaa	tgtgagggcc	2640
	cggaaacctg	gccctgtctt	cttgacgagc	attcctaggg	gtctttcccc	tctcgccaaa	2700
	ggaatgcaag	gtctgttgaa	tgtcgtgaag	gaagcagttc	ctctggaagc	ttcttgaaaga	2760
5	caaacaacgt	ctgtagcgac	cctttgcagg	cagcgggaacc	ccccacctgg	cgacaggtgc	2820
	ctctgcggcc	aaaagccacg	tgtataagat	acacctgcaa	aggcggcaca	accccagtg	2880
	cacgttgatg	gttggatagt	tgtggaaaga	gtcaaatggc	tctcctcaag	cgtattcaac	2940
	aaggggctga	aggatgcccc	gaaggtaccc	cattgtatgg	gatctgatct	ggggcctcgg	3000
	tgcacatgct	ttacatgtgt	ttagtcgagg	ttaaaaaaac	gtctaggccc	cccgaaccac	3060
10	ggggacgtgg	ttttcctttg	aaaaacacga	tgataagcct	gccacaaccc	acagcggccg	3120
	ctgccatcat	ggttcgacca	ttgaactgca	tcgtcgccgt	gtcccaaaat	atggggattg	3180
	gcaagaacgg	agacctaccc	tggcctccgc	tcaggaacga	gttcaagtac	ttccaaagaa	3240
	tgaccacaac	ctcttcagtg	gaaggtaaac	agaatctggt	gattatgggt	aggaaaacct	3300
	ggttctccat	tcctgagaag	aatcgacctt	taaaggacag	aattaatata	gttctcagta	3360
15	gagaactcaa	agaaccacca	cgaggagctc	atthttcttg	caaaagtttg	gatgatgcct	3420
	taagacttat	tgaacaaccg	gaattggcaa	gtaaaagtaga	catggtttgg	atagtcggag	3480
	gcagttctgt	ttaccaggaa	gccatgaatc	aaccaggcca	cctcagactc	tttgtgacaa	3540
	ggatcatgca	ggaatttgaa	agtgacacgt	ttttcccaga	aattgatttg	gggaaatata	3600
	aacttctccc	agaataccca	ggcgtcctct	ctgaggtcca	ggaggaaaaa	ggcatcaagt	3660
20	ataagtttga	agtctacgag	aagaaagact	aaacgcgtgg	tacctctaga	gtcgacccgg	3720
	gcggccgctt	cgagcagaca	tgataagata	cattgatgag	tttggacaaa	ccacaactag	3780
	aatgcagtga	aaaaaatgct	ttatttgtga	aatttgtgat	gctattgctt	tatttgaac	3840
	cattataagc	tgcaataaac	aagttaacaa	caacaattgc	attcatttta	tgtttcaggt	3900
	tcagggggag	atgtgggagg	ttttttaaag	caagtaaaac	ctctacaaat	gtggtaaaat	3960
25	cgataaggat	ccgggctggc	gtaatagcga	agaggccgc	accgatcgcc	cttccaaca	4020
	gttgccgagc	ctgaatggcg	aatggacgcg	ccctgtagcg	gcgcattaag	cgcgccgggt	4080
	gtgggtggta	cgccgagcgt	gaccgctaca	cttgccagcg	ccctagcgcc	cgctcctttc	4140
	gctttcttcc	cttcttttct	cgccacgttc	gccggctttc	cccgtcaagc	tctaaatcgg	4200
	gggctccctt	tagggttccg	atttagtgct	ttacggcacc	tcgaccccaa	aaaacttgat	4260
30	tagggatgat	gttcacgtag	tgggccatcg	ccctgataga	cggtttttcg	ccctttgacg	4320
	ttggagtcca	cgthctttta	tagtggactc	ttgttccaaa	ctggaacaac	actcaacctt	4380
	atctcggctc	attcttttga	tttataaggg	atthttgccga	tttcggccta	ttggttaaaa	4440
	aatgagctga	tttaacaaaa	atttaacgcg	aaatttaaca	aaatattaac	gcttacaatt	4500
	tcctgatgag	gtatttttct	cttacgcac	tgtgcggat	ttcacaccgc	atatgggtga	4560
35	ctctcagtag	aatctgctct	gatgccgcat	agtttaagcca	gccccgacac	ccgccaacac	4620
	ccgctgacgc	gccctgacgg	gcttgtctgc	tcccggcatc	cgcttacaga	caagctgtga	4680
	ccgtctccgg	gagctgcatg	tgtcagagg	tttcaccgtc	atcaccgaaa	cgcgcgagac	4740
	gaaagggcct	cgtgatacgc	ctatttttat	agggttaagt	catgataata	atggtttctt	4800
	agacgtcagg	tggcactttt	cggggaaatg	tgcgcggaac	ccctatttgt	ttatttttct	4860
40	aaatacattc	aaatatgtat	ccgctcatga	gacaataacc	ctgataaatg	cttcaataat	4920
	attgaaaaag	gaagagtatg	agtattcaac	atthttcggt	cgcccttatt	cccttttttg	4980
	cggcattttg	ccttcctgtt	tttgctcacc	cagaaacgct	gggtgaaagta	aaagatgctg	5040
	aagatcagtt	gggtgcacga	gtgggttaca	tcgaactgga	tctcaacagc	ggtaagatcc	5100
	ttgagagttt	tcgccccgaa	gaacgttttc	caatgatgag	cacttttaaa	gttctgctat	5160
45	gtggcgcggt	attatcccgt	attgacgccg	ggcaagagca	actcggctgc	cgcatacact	5220
	attctcagaa	tgacttggtt	gagtactcac	cagtcacaga	aaagcatctt	acggatggca	5280
	tgacagtaag	agaattatgc	agtgtgccca	taaccatgag	tgataaacct	cgggccaact	5340
	tacttctgac	aacgatcgga	ggaccgaagg	agctaaccgc	ttttttgcac	aacatggggg	5400
	atcatgtaac	tcgccttgat	cgttgggaac	cggagctgaa	tgaagccata	ccaaacgacg	5460
50	agcgtgacac	cacgatgcct	gtagcaatgg	caacaacgtt	gcgcaacta	tttaactggcg	5520
	aactacttac	tctagcttcc	cggcaacaat	taatagactg	gatggaggcg	gataaagttg	5580
	caggaccact	tctgcgctcg	gcccttccgg	ctggctgggt	tattgctgat	aaatctggag	5640
	ccggtgagcg	tgggtctcgc	ggtatcattg	cagcactggg	gccagatgg	aagccctccc	5700
	gtatcgtagt	tatctacacg	acggggagtc	aggcaactat	ggatgaacga	aatagacaga	5760
55	tcgctgagat	aggtgcctca	ctgattaagc	attggttaact	gtcagaccaa	gtttactcat	5820
	atatacttta	gattgattta	aaacttcatt	tttaatttaa	aaggatctag	gtgaagatcc	5880
	tttttgataa	tctcatgacc	aaaatccctt	aacgtgagtt	ttcgtttcac	tgagcgtcag	5940

EP 4 269 578 B1

```

        accccgtaga aaagatcaaa ggatcttctt gagatccttt ttttctgcgc gtaatctgct 6000
        gcttgcaaac aaaaaaacca ccgctaccag cggtgggttg tttgccggat caagagctac 6060
        caactctttt tccgaaggta actggcttca gcagagcgca gataccaaat actgttcttc 6120
5      tagtgtagcc gtagttaggc caccacttca agaactctgt agcaccgcct acatacctcg 6180
        ctctgctaata cctgttacca gtggctgctg ccagtggcga taagtcgtgt cttaccgggt 6240
        tggactcaag acgatagtta ccggataagg cgcagcggtc gggctgaacg ggggggttcgt 6300
        gcacacagcc cagcttggag cgaacgacct acaccgaact gagataccta cagcgtgagc 6360
        tatgagaaag cgccacgctt cccgaaggga gaaaggcggga caggtatccg gtaagcggca 6420
10     gggtcggaac aggagagcgc acgaggggagc ttccagggggg aaacgcctgg tatctttata 6480
        gtcctgtcgg gtttcgccac ctctgacttg agcgtcgtatt tttgtgatgc tcgtcagggg 6540
        ggaggagcct atggaaaaac gccagcaacg cggccttttt acggttcctg gccttttgct 6600
        ggccttttgc tcacatggct cgacagatct                                     6630

```

15 <210> 51
 <211> 186
 <212> PRT
 <213> Mus musculus

20 <220>
 <223> dihydrofolate reductase

<400> 51

```

25      Val Arg Pro Leu Asn Cys Ile Val Ala Val Ser Gln Asn Met Gly Ile
        1          5          10          15
        Gly Lys Asn Gly Asp Leu Pro Trp Pro Pro Leu Arg Asn Glu Phe Lys
        20          25          30
30      Tyr Phe Gln Arg Met Thr Thr Thr Ser Ser Val Glu Gly Lys Gln Asn
        35          40          45
        Leu Val Ile Met Gly Arg Lys Thr Trp Phe Ser Ile Pro Glu Lys Asn
        50          55          60
        Arg Pro Leu Lys Asp Arg Ile Asn Ile Val Leu Ser Arg Glu Leu Lys
35      65          70          75          80
        Glu Pro Pro Arg Gly Ala His Phe Leu Ala Lys Ser Leu Asp Asp Ala
        85          90          95
        Leu Arg Leu Ile Glu Gln Pro Glu Leu Ala Ser Lys Val Asp Met Val
        100         105         110
40      Trp Ile Val Gly Gly Ser Ser Val Tyr Gln Glu Ala Met Asn Gln Pro
        115         120         125
        Gly His Leu Arg Leu Phe Val Thr Arg Ile Met Gln Glu Phe Glu Ser
        130         135         140
45      Asp Thr Phe Phe Pro Glu Ile Asp Leu Gly Lys Tyr Lys Leu Leu Pro
        145         150         155         160
        Glu Tyr Pro Gly Val Leu Ser Glu Val Gln Glu Glu Lys Gly Ile Lys
        165         170         175
        Tyr Lys Phe Glu Val Tyr Glu Lys Lys Asp
50      180         185

```

<210> 52
 <211> 23
 <212> DNA
55 <213> Artificial Sequence

<220>
 <223> HZM3.P1 forward primer

<400> 52
 ttggaacct cagcagtctc ctg 23

5
 <210> 53
 <211> 20
 <212> DNA
 <213> Artificial Sequence

10
 <220>
 <223> HZM3.P2 reverse primer

15
 <400> 53
 aactctgatg gcttcccgaa 20

<210> 54
 <211> 28
 <212> DNA
 <213> Artificial Sequence

20
 <220>
 <223> HZM3 probe

25
 <400> 54
 agctgctaca ctctatgtgc gcaatcga 28

30
 <210> 55
 <211> 1449
 <212> DNA
 <213> Homo sapiens

<220>
 <223> PH20 mRNA sequence from 3D35M cells

35
 <400> 55

atgggagtg	taaaattcaa	gcacatcttt	ttcagaagct	ttgttaaadc	aagtggagta	60
tcccagatag	ttttcacctt	ccttctgatt	ccatgttgct	tgactctgaa	tttcagagca	120
cctcctgtta	ttccaaatgt	gcctttcttc	tgggcctgga	atgccccaa	tgaattttgt	180
cttggaataa	ttgatgagcc	actagatatg	agcctcttct	ctttcatagg	aagccccga	240
ataaacgcca	ccgggcaagg	tgttacaata	ttttatgttg	atagacttgg	ctactatcct	300
tacatagatt	caatcacagg	agtaactgtg	aatggaggaa	tccccagaa	gatttcctta	360
caagaccatc	tggacaaagc	taagaaagac	attacatttt	atatgccagt	agacaatttg	420
ggaatggctg	ttattgactg	ggaagaatgg	agaccacttt	gggcaagaaa	ctggaaacct	480
aaagatgttt	acaagaatag	gtctattgaa	ttggttcagc	aacaaaatgt	acaacttagt	540
ctcacagagg	ccactgagaa	agcaaaaaca	gaatttgaaa	aggcagggaa	ggatttcctg	600
gtagagacta	taaaattggg	aaaattactt	cggccaaatc	acttgtgggg	ttattatctt	660
tttccggatt	gttacaacca	tcactataag	aaacccggtt	acaatggaag	ttgcttcaat	720
gtagaaataa	aaagaaatga	tgatctcagc	tggttggtga	atgaaagcac	tgctctttac	780
ccatccattt	atttgaacac	tcagcagtc	cctgtagctg	ctacactcta	tgtgcgcaat	840
cgagttcggg	aagccatcag	agtttccaaa	atacctgatg	caaaaagtcc	acttccggtt	900
tttgcatata	cccgcatagt	ttttactgat	caagttttga	aattcctttc	tcaagatgaa	960
cttgtgtata	catttggcga	aactgttgct	ctgggtgctt	ctggaattgt	aatatgggga	1020

55

```

accctcagta taatgcgaag tatgaaatct tgcttgctcc tagacaatta catggagact 1080
atactgaatc cttacataat caacgtcaca ctagcagcca aaatgtgtag tcaagtgcctt 1140
tgccaggagc aaggagtgtg tataaggaaa aactggaatt caagtgacta tcttcacctc 1200
5 aacccagata attttgctat tcaacttgag aaaggtggaa agttcacagt acgtggaaaa 1260
ccgacacttg aagacctgga gcaattttct gaaaaatttt attgcagctg ttatagcacc 1320
ttgagttgta aggagaaagc tgatgtaaaa gacactgatg ctgttgatgt gtgtattgct 1380
gatggtgtct gtatagatgc ttttctaaaa cctcccatgg agacagaaga acctcaaatt 1440
ttctactga 1449

10
<210> 56
<211> 17
<212> DNA
<213> Artificial Sequence

15
<220>
<223> AP01

<400> 56
20 ttctctccac aggtgtc 17

<210> 57
<211> 18
<212> DNA
25 <213> Artificial Sequence

<220>
<223> AP02

30 <400> 57
aagatttct tacaagac 18

<210> 58
<211> 18
35 <212> DNA
<213> Artificial Sequence

<220>
<223> AP03

40 <400> 58
tggcgagagg ggaaagac 18

<210> 59
<211> 18
45 <212> DNA
<213> Artificial Sequence

<220>
50 <223> AP04

<400> 59
ccatttattt gaacactc 18

55 <210> 60
<211> 18
<212> DNA
<213> Artificial Sequence

```

<220>
 <223> AP06

 <400> 60
 5 ccgaactcga ttgcgcac 18

 <210> 61
 <211> 18
 <212> DNA
 10 <213> Artificial Sequence

 <220>
 <223> AP07

 15 <400> 61
 agccattccc aaattgtc 18

 <210> 62
 <211> 18
 20 <212> DNA
 <213> Artificial Sequence

 <220>
 <223> AP08
 25
 <400> 62
 ctcccagttc aattacag 18

 <210> 63
 30 <211> 18
 <212> DNA
 <213> Artificial Sequence

 <220>
 35 <223> AP09

 <400> 63
 cgtagctat ggatcctc 18

 40 <210> 64
 <211> 22
 <212> DNA
 <213> Artificial Sequence

 45 <220>
 <223> AP10

 <400> 64
 50 cgagacagag aagactcttg cg 22

 <210> 65
 <211> 22
 <212> DNA
 <213> Artificial Sequence
 55
 <220>
 <223> AP12

<400> 65
cattcaacag accttgcat cc 22

<210> 66
5 <211> 17
<212> PRT
<213> Homo sapiens

<220>
10 <223> C-terminal peptide cleaved from soluble rHuPH20 aa 431-447

<400> 66

15 Asp Ala Phe Lys Leu Pro Pro Met Glu Thr Glu Glu Pro Gln Ile Phe
1 5 10 15
Tyr

<210> 67
20 <211> 16
<212> PRT
<213> Homo sapiens

<220>
25 <223> C-terminal peptide cleaved from soluble rHuPH20 aa 431-446

<400> 67

30 Asp Ala Phe Lys Leu Pro Pro Met Glu Thr Glu Glu Pro Gln Ile Phe
1 5 10 15

<210> 68
35 <211> 15
<212> PRT
<213> Homo sapiens

<220>
40 <223> C-terminal peptide cleaved from soluble rHuPH20 aa 431-445

<400> 68

45 Asp Ala Phe Lys Leu Pro Pro Met Glu Thr Glu Glu Pro Gln Ile
1 5 10 15

<210> 69
50 <211> 14
<212> PRT
<213> Homo sapiens

<220>
55 <223> C-terminal peptide cleaved from soluble rHuPH20 aa 431-444

<400> 69

Asp Ala Phe Lys Leu Pro Pro Met Glu Thr Glu Glu Pro Gln
 1 5 10

5 <210> 70
 <211> 13
 <212> PRT
 <213> Homo sapiens

10 <220>
 <223> C-terminal peptide cleaved from soluble rHuPH20 aa 431-443
 <400> 70

15 Asp Ala Phe Lys Leu Pro Pro Met Glu Thr Glu Glu Pro
 1 5 10

20 <210> 71
 <211> 12
 <212> PRT
 <213> Homo sapiens

25 <220>
 <223> C-terminal peptide cleaved from soluble rHuPH20 aa 431-442
 <400> 71

30 Asp Ala Phe Lys Leu Pro Pro Met Glu Thr Glu Glu
 1 5 10

Claims

35 1. Composition comprising a mixture of polypeptides, said mixture comprising the polypeptides set forth in SEQ ID NOS: 4-8, wherein the composition is obtainable by the following method:

40 a) inoculating cell medium in a bioreactor with an inoculum of cells that encode soluble recombinant human PH20 (rHuPH20) to produce a cell culture, wherein:

the cells comprise between 150 and 300 copies of nucleic acid encoding soluble rHuPH20;
 the bioreactor contains at least 100 liters of cell culture;
 45 10^{10} - 10^{11} cells are inoculated per 100 liters cell culture; and
 the cells are cultured at a set temperature;

b) feeding the cells with a first feed medium containing glucose, L-alanyl-L-glutamine, human insulin and yeast extract in amounts sufficient to increase cell growth and peak cell density, and to increase soluble rHuPH20 synthesis, wherein the feed medium is added to the culture at a volume of 0.5 % to 20% of the cell culture volume;
 50 c) feeding the cells with a second feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and induce cell cycle arrest; and lowering the temperature compared to the temperature in step a) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; wherein:

55 the amount of L-alanyl-L-glutamine is decreased compared to the amount of L-alanyl-L-glutamine in step b);
 the amount of yeast extract is increased compared to the amount of yeast extract in step b); and
 the feed medium is added to the culture at a volume of 0.5 % to 20% of the cell culture volume;

d) feeding the cells with a third feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and increase cell cycle arrest, and lowering the temperature compared to the temperature in step c) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; wherein:

the amount of L-alanyl-L-glutamine is decreased compared to the amount of L-alanyl-L-glutamine in step c); the amounts of yeast extract, glucose and sodium butyrate are increased compared to the amounts of yeast extract, glucose and sodium butyrate in step c); and the feed medium is added to the culture at a volume of 0.5 % to 20 % of the cell culture volume;

e) feeding the cells with a fourth feed medium containing glucose, L-alanyl-L-glutamine, yeast extract and sodium butyrate in amounts sufficient to increase soluble rHuPH20 synthesis and increase cell cycle arrest, and lowering the temperature compared to the temperature in step d) to a temperature sufficient to increase cell cycle arrest, increase cell viability and stabilize the soluble hyaluronidase; wherein:

the amount of L-alanyl-L-glutamine and glucose is decreased compared to the amount of L-alanyl-L-glutamine and glucose in step d); the amount of sodium butyrate is decreased compared to the amount of sodium butyrate in step d); and the feed medium is added to the culture at a volume of 0.5 % to 20% of the cell culture volume;

f) continuing to culture the cells until viability drops below at least 50%;

g) obtaining the harvest cell culture fluid; and

h) purifying the rHuPH20 from the harvest cell culture fluid,

wherein soluble rHuPH20 is a soluble form of human PH20 that is recombinantly expressed in Chinese Hamster Ovary (CHO) cells.

2. The composition of claim 1, wherein the temperature in step a) is 37 °C.

3. The composition of claim 1 or 2, wherein the temperature in step c) is 36.5 °C.

4. The composition of any of claims 1-3, wherein the temperature in step d) is 36 °C.

5. The composition of any of claims 1-4, wherein the temperature in step e) is 35.5 °C.

6. The composition of any of claims 1-5, wherein the harvest cell culture fluid is filtered prior to purification.

7. The composition of any of claims 1-6, wherein the soluble rHuPH20 purification is effected by column chromatography.

8. The composition of claim 7, wherein the column chromatography comprises beaded crosslinked agarose column chromatography, beaded crosslinked phenyl-substituted agarose column chromatography, amino phenyl boronate column chromatography and hydroxyapatite column chromatography.

9. The composition of any of claims 1-8, wherein the feed medium is added to the culture at a volume of 4 % of the cell culture volume.

10. The composition of claim 1, wherein:

in step a) the cells are cultured at 37 °C;

in step b) the first feed medium contains 33 g/L glucose, 32 mM L-alanyl-L-glutamine, 16.6 g/L yeast extract and 33 mg/L insulin, and the feed medium is added to the culture at a volume of 4 % of the cell culture volume; in step c) the second feed medium contains 33 g/L glucose, 16 mM L-alanyl-L-glutamine, 33.4 g/L yeast extract and 0.92 g/L sodium butyrate, and the feed medium is added to the culture at a volume of 4 % of the cell culture volume; and

the temperature is lowered to 36.5 °C;

in step d) the third feed medium contains 50 g/L glucose, 10 mM L-alanyl-L-glutamine, 50 g/L yeast extract and 1.8 g/L sodium butyrate, and the feed medium is added to the culture at a volume of 4 % of the cell culture volume; and

the temperature is lowered to 36 °C;

in step e) the fourth feed medium contains 33 g/L glucose, 6.6 mM L-alanyl-L-glutamine, 50 g/L yeast extract and 0.92 g/L sodium butyrate, and the feed medium is added to the culture at a volume of 4 % of the cell culture volume; and

the temperature is lowered to 35.5 °C; further comprising filtering the harvest cell culture fluid obtained in step g) and

in step h) purifying the rHuPH20 from the harvest culture fluid using beaded crosslinked agarose column chromatography, beaded crosslinked phenyl-substituted agarose column chromatography, amino phenyl boronate column chromatography or hydroxyapatite column chromatography.

11. The composition of claim 1, wherein:

in step a) the cells are inoculated at a cell density of 4×10^5 cells/mL; and

the cells are cultured at 37 °C;

in step b) the first feed medium contains 33 g/L glucose, 32 mM L-alanyl-L-glutamine, 83.3 g/L yeast extract and 33 mg/L insulin, and the feed medium is added to the culture at a volume of 4% of the cell culture volume;

in step c) the second feed medium contains 33 g/L glucose, 13 mM L-alanyl-L-glutamine, 166.7 g/L yeast extract and 0.92 g/L sodium butyrate, and the feed medium is added to the culture at a volume of 4% of the cell culture volume; and

the temperature is lowered to 36.5 °C;

in step d) the third feed medium contains 50 g/L glucose, 10 mM L-alanyl-L-glutamine, 250 g/L yeast extract and 1.8 g/L sodium butyrate, and the feed medium is added to the culture at a volume of 4 % of the cell culture volume; and

the temperature is lowered to 36 °C;

in step e) the fourth feed medium contains 33 g/L glucose, 6.7 mM L-alanyl-L-glutamine, 250 g/L yeast extract and 0.92 g/L sodium butyrate, and the feed medium is added to the culture at a volume of 4% of the cell culture volume; and

the temperature is lowered to 35.5 °C; further comprising filtering the harvest cell culture fluid obtained in step g) and

in step h) purifying the rHuPH20 from the harvest culture fluid using beaded crosslinked agarose column chromatography, beaded crosslinked phenyl-substituted agarose column chromatography, amino phenyl boronate column chromatography or hydroxyapatite column chromatography.

12. The composition of any of claims 1-11, wherein at least 0.5, 1, 5, 10, 15, 20, 25, 30, 35 or 40 grams of soluble rHuPH20 is produced per 100 L of cell culture.

13. The composition of any of claims 1-12, wherein the specific activity of the soluble rHuPH20 is at least 80000, 100000, 120000, 140000, 160000 or 180,000 units/mg.

14. The composition of any of claims 1-13, wherein the volume of cell culture in the bioreactor is 200, 300, 400, 500, 1000, 1500, 2000, 2500, 3000 or 3500 liters.

15. The composition of any of claims 1-14, wherein the cells that encode soluble rHuPH20 are DG44 CHO cells.

16. The composition of any of claims 1-15, wherein the rHuPH20 is encoded by nucleic acid set forth in SEQ ID NO:47.

Patentansprüche

1. Zusammensetzung, umfassend eine Mischung von Polypeptiden, wobei die Mischung die in SEQ ID NOs: 4-8 dargelegten Polypeptide umfasst, wobei die Zusammensetzung durch das folgende Verfahren erhältlich ist:

a) Inokulieren von Zellmedium in einem Bioreaktor mit einem Inokulum aus Zellen, welche für lösliches rekombinantes menschliches PH20 (rHuPH20) kodieren, um eine Zellkultur herzustellen, wobei:

die Zellen zwischen 150 und 300 Kopien von Nukleinsäure, welche für lösliches rHuPH20 kodiert, umfassen; der Bioreaktor mindestens 100 Liter Zellkultur enthält; 10^{10} - 10^{11} Zellen pro 100 Liter Zellkultur inokuliert werden; und

die Zellen bei einer Solltemperatur kultiviert werden;

b) Füttern der Zellen mit einem ersten Nährmedium ("feed medium"), welches Glucose, L-Alanyl-L-Glutamin, menschliches Insulin und Hefeextrakt in Mengen enthält, welche ausreichend sind, Zellwachstum und Spitzen-Zelldichte zu erhöhen, und Synthese von löslichem rHuPH20 zu erhöhen, wobei das Nährmedium zu der Kultur in einem Volumen von 0,5% bis 20% des Zellkulturvolumens zugegeben wird;

c) Füttern der Zellen mit einem zweiten Nährmedium, welches Glucose, L-Alanyl-L-Glutamin, Hefeextrakt und Natriumbutyrat in Mengen enthält, welche ausreichend sind, Synthese von löslichem rHuPH20 zu erhöhen und Stillstand des Zellzykluses ("cell cycle arrest") zu induzieren; und

Erniedrigen der Temperatur verglichen mit der Temperatur in Schritt a) auf eine Temperatur, welche ausreichend ist, Stillstand des Zellzykluses zu erhöhen, Zellviabilität zu erhöhen und die lösliche Hyaluronidase zu stabilisieren; wobei:

die Menge von L-Alanyl-L-Glutamin verringert wird, verglichen mit der Menge von L-Alanyl-L-Glutamin in Schritt b);

die Menge von Hefeextrakt erhöht wird, verglichen mit der Menge von Hefeextrakt in Schritt b); und das Nährmedium zu der Kultur in einem Volumen von 0,5% bis 20% des Zellkulturvolumens zugegeben wird;

d) Füttern der Zellen mit einem dritten Nährmedium, welches Glucose, L-Alanyl-L-Glutamin, Hefeextrakt und Natriumbutyrat in Mengen enthält, welche ausreichend sind, Synthese von löslichem rHuPH20 zu erhöhen und Stillstand des Zellzykluses zu erhöhen, und

Erniedrigen der Temperatur verglichen mit der Temperatur in Schritt c) auf eine Temperatur, welche ausreichend ist, Stillstand des Zellzykluses zu erhöhen, Zellviabilität zu erhöhen und die lösliche Hyaluronidase zu stabilisieren; wobei:

die Menge von L-Alanyl-L-Glutamin verringert wird, verglichen mit der Menge von L-Alanyl-L-Glutamin in Schritt c);

die Mengen von Hefeextrakt, Glucose und Natriumbutyrat erhöht werden, verglichen mit den Mengen von Hefeextrakt, Glucose und Natriumbutyrat in Schritt c); und

das Nährmedium zu der Kultur in einem Volumen von 0,5% bis 20% des Zellkulturvolumens zugegeben wird;

e) Füttern der Zellen mit einem vierten Nährmedium, welches Glucose, L-Alanyl-L-Glutamin, Hefeextrakt und Natriumbutyrat in Mengen enthält, welche ausreichend sind, Synthese von löslichem rHuPH20 zu erhöhen und Stillstand des Zellzykluses zu erhöhen, und

Erniedrigen der Temperatur verglichen mit der Temperatur in Schritt d) auf eine Temperatur, welche ausreichend ist, Stillstand des Zellzykluses zu erhöhen, Zellviabilität zu erhöhen und die lösliche Hyaluronidase zu stabilisieren; wobei:

die Menge von L-Alanyl-L-Glutamin und Glucose verringert wird, verglichen mit der Menge von L-Alanyl-L-Glutamin und Glucose in Schritt d);

die Menge von Natriumbutyrat verringert wird, verglichen mit der Menge von Natriumbutyrat in Schritt d); und das Nährmedium zu der Kultur in einem Volumen von 0,5% bis 20% des Zellkulturvolumens zugegeben wird;

f) Fortfahren die Zellen zu kultivieren, bis die Viabilität unter mindestens 50% fällt;

g) Erhalten der Ernte-Zellkulturflüssigkeit; und

h) Reinigen des rHuPH20 von der Ernte-Zellkulturflüssigkeit, wobei lösliches rHuPH20 eine lösliche Form von menschlichem PH20 ist, das rekombinant in chinesischen Hamster-Eierstockzellen ("Chinese Hamster Ovary cells"; CHO cells) exprimiert wird.

2. Zusammensetzung gemäß Anspruch 1, wobei die Temperatur in Schritt a) 37°C ist.

3. Zusammensetzung gemäß Anspruch 1 oder Anspruch 2, wobei die Temperatur in Schritt c) 36,5 °C ist.

4. Zusammensetzung gemäß einem der Ansprüche 1-3, wobei die Temperatur in Schritt d) 36 °C ist.

5. Zusammensetzung gemäß einem der Ansprüche 1-4, wobei die Temperatur in Schritt e) 35,5 °C ist.

6. Zusammensetzung gemäß einem der Ansprüche 1-5, wobei die Ernte-Zellkulturflüssigkeit vor der Reinigung filtriert

wird.

7. Zusammensetzung gemäß einem der Ansprüche 1-6, wobei die Reinigung von löslichem rHuPH20 durch Säulenchromatographie bewirkt wird.

8. Zusammensetzung gemäß Anspruch 7, wobei die Säulenchromatographie vernetzte Agarose in Form von Beads-Säulenchromatographie ("beaded crosslinked agarose column chromatography"), vernetzte phenylsubstituierte Agarose in Form von Beads-Säulenchromatographie ("beaded crosslinked phenyl-substituted agarose column chromatography"), Aminophenylboronat-Säulenchromatographie ("amino phenyl boronate column chromatography") oder Hydroxylapatit-Säulenchromatographie umfasst.

9. Zusammensetzung gemäß einem der Ansprüche 1-8, wobei das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird.

10. Zusammensetzung gemäß Anspruch 1, wobei:

in Schritt a) die Zellen bei 37 °C kultiviert werden;

in Schritt b) das erste Nährmedium 33 g/L Glucose, 32 mM L-Alanyl-L-Glutamin, 16,6 g/L Hefeextrakt und 33 mg/L Insulin enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird;

in Schritt c) das zweite Nährmedium 33 g/L Glucose, 16 mM L-Alanyl-L-Glutamin, 33,4 g/L Hefeextrakt und 0,92 g/L Natriumbutyrat enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird; und

die Temperatur auf 36,5 °C verringert wird;

in Schritt d) das dritte Nährmedium 50 g/L Glucose, 10 mM L-Alanyl-L-Glutamin, 50 g/L Hefeextrakt und 1,8 g/L Natriumbutyrat enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird; und

die Temperatur auf 36 °C verringert wird;

in Schritt e) das vierte Nährmedium 33 g/L Glucose, 6,6 mM L-Alanyl-L-Glutamin, 50 g/L Hefeextrakt und 0,92 g/L Natriumbutyrat enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird; und

die Temperatur auf 35,5 °C verringert wird; weiter umfassend Filtrieren der Ernte-Zellkulturflüssigkeit, welche in Schritt g) erhalten wird und

in Schritt h) Reinigen des rHuPH20 von der Ernte-Kulturflüssigkeit durch vernetzte Agarose in Form von Beads-Säulenchromatographie, vernetzte phenylsubstituierte Agarose in Form von Beads-Säulenchromatographie, Aminophenylboronat-Säulenchromatographie oder Hydroxylapatit-Säulenchromatographie.

11. Zusammensetzung gemäß Anspruch 1, wobei:

in Schritt a) die Zellen bei einer Zelldichte von 4×10^5 Zellen/mL inokuliert werden; und

die Zellen bei 37°C kultiviert werden;

in Schritt b) das erste Nährmedium 33 g/L Glucose, 32 mM L-Alanyl-L-Glutamin, 83,3 g/L Hefeextrakt und 33 mg/L Insulin enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird;

in Schritt c) das zweite Nährmedium 33 g/L Glucose, 13 mM L-Alanyl-L-Glutamin, 166,7 g/L Hefeextrakt und 0,92 g/L Natriumbutyrat enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird; und

die Temperatur auf 36,5 °C verringert wird;

in Schritt d) das dritte Nährmedium 50 g/L Glucose, 10 mM L-Alanyl-L-Glutamin, 250 g/L Hefeextrakt und 1,8 g/L Natriumbutyrat enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird; und

die Temperatur auf 36 °C verringert wird;

in Schritt e) das vierte Nährmedium 33 g/L Glucose, 6,7 mM L-Alanyl-L-Glutamin, 250 g/L Hefeextrakt und 0,92 g/L Natriumbutyrat enthält und das Nährmedium zu der Kultur in einem Volumen von 4% des Zellkulturvolumens zugegeben wird; und

die Temperatur auf 35,5 °C verringert wird; weiter umfassend

Filtrieren der Ernte-Zellkulturflüssigkeit, welche in Schritt g) erhalten wird und

in Schritt h) Reinigen des rHuPH20 von der Ernte-Kulturflüssigkeit durch vernetzte Agarose in Form von Beads-

Säulenchromatographie, vernetzte phenylsubstituierte Agarose in Form von Beads-Säulenchromatographie, Aminophenylboronat-Säulenchromatographie oder Hydroxylapatit-Säulenchromatographie.

12. Zusammensetzung gemäß einem der Ansprüche 1-11, wobei mindestens 0,5, 1, 5, 10, 15, 20, 25, 30, 35 oder 40 Gramm lösliches rHuPH20 pro 100 L Zellkultur hergestellt wird.

13. Zusammensetzung gemäß einem der Ansprüche 1-12, wobei die spezifische Aktivität des löslichen rHuPH20 mindestens 80000, 100000, 120000, 140000, 160000 oder 180000 Einheiten/mg ist.

14. Zusammensetzung gemäß einem der Ansprüche 1-13, wobei das Volumen der Zellkultur in dem Bioreaktor 200, 300, 400, 500, 1000, 1500, 2000, 2500, 3000 oder 3500 Liter ist.

15. Zusammensetzung gemäß einem der Ansprüche 1-14, wobei die Zellen, die für lösliches rHuPH20 kodieren, DG44 CHO Zellen sind.

16. Zusammensetzung gemäß einem der Ansprüche 1-15, wobei das rHuPH20 durch Nukleinsäure, welche in SEQ ID NO:47 dargelegt ist, kodiert wird.

Revendications

1. Composition comprenant un mélange de polypeptides, ledit mélange comprenant les polypeptides présentés dans SEQ ID NOS: 4-8, dans laquelle la composition peut être obtenue par la méthode suivante :

a) inoculation d'un milieu cellulaire dans un bioréacteur avec un inoculum de cellules qui codent pour le PH20 humain recombinant soluble (rHuPH20) pour produire une culture cellulaire, dans laquelle :

les cellules comprennent entre 150 et 300 copies d'acide nucléique codant pour la rHuPH20 soluble ;

le bioréacteur contient au moins 100 litres de culture cellulaire ;

10^{10} - 10^{11} cellules sont inoculées par 100 litres de culture cellulaire ; et

les cellules sont cultivées à une température déterminée ;

b) alimentation des cellules avec un premier milieu d'alimentation contenant du glucose, de la L-alanyl-L-glutamine, de l'insuline humaine et de l'extrait de levure en quantités suffisantes pour augmenter la croissance cellulaire et la densité cellulaire maximale, et pour augmenter la synthèse de rHuPH20 soluble, le milieu d'alimentation étant ajouté à la culture à un volume de 0,5 % à 20 % du volume de la culture cellulaire ;

c) alimentation des cellules avec un second milieu d'alimentation contenant du glucose, de la L-alanyl-L-glutamine, de l'extrait de levure et du butyrate de sodium en quantités suffisantes pour augmenter la synthèse de rHuPH20 soluble et induire l'arrêt du cycle cellulaire ; et

abaissement de la température par rapport à la température de l'étape a) à une température suffisante pour augmenter l'arrêt du cycle cellulaire, augmenter la viabilité cellulaire et stabiliser la hyaluronidase soluble ; dans laquelle :

la quantité de L-alanyl-L-glutamine est diminuée par rapport à la quantité de L-alanyl-L-glutamine dans l'étape b) ;

la quantité d'extrait de levure est augmentée par rapport à la quantité d'extrait de levure dans l'étape b) ; et

le milieu d'alimentation est ajouté à la culture à un volume de 0,5 % à 20 % du volume de la culture cellulaire ;

d) alimentation des cellules avec un troisième milieu d'alimentation contenant du glucose, de la L-alanyl-L-glutamine, de l'extrait de levure et du butyrate de sodium en quantités suffisantes pour augmenter la synthèse de rHuPH20 soluble et augmenter l'arrêt du cycle cellulaire, et

abaissement de la température par rapport à la température de l'étape c) à une température suffisante pour augmenter l'arrêt du cycle cellulaire, augmenter la viabilité cellulaire et stabiliser la hyaluronidase soluble ; dans lequel :

la quantité de L-alanyl-L-glutamine est diminuée par rapport à la quantité de L-alanyl-L-glutamine dans l'étape c) ;

les quantités d'extrait de levure, de glucose et de butyrate de sodium sont augmentées par rapport aux

quantités d'extrait de levure, de glucose et de butyrate de sodium dans l'étape c) ; et
le milieu d'alimentation est ajouté à la culture à un volume de 0,5 % à 20 % du volume de la culture cellulaire ;

e) alimentation des cellules avec un quatrième milieu d'alimentation contenant du glucose, de la L-alanyl-L-glutamine, de l'extrait de levure et du butyrate de sodium en quantités suffisantes pour augmenter la synthèse de la rHuPH20 soluble et augmenter l'arrêt du cycle cellulaire, et
abaissement de la température par rapport à la température de l'étape d) à une température suffisante pour augmenter l'arrêt du cycle cellulaire, augmenter la viabilité cellulaire et stabiliser la hyaluronidase soluble ; dans lequel :

la quantité de L-alanyl-L-glutamine et de glucose est diminuée par rapport à la quantité de L-alanyl-L-glutamine et de glucose dans l'étape d) ;
la quantité de butyrate de sodium est diminuée par rapport à la quantité de butyrate de sodium dans l'étape d) ; et

le milieu d'alimentation est ajouté à la culture à un volume de 0,5 % à 20 % du volume de la culture cellulaire ;

f) continuer à cultiver les cellules jusqu'à ce que la viabilité tombe en dessous d'au moins 50 % ;

g) obtention du liquide de culture cellulaire de récolte ; et

h) purification de la rHuPH20 à partir du liquide de culture cellulaire de récolte,
dans laquelle la rHuPH20 soluble est une forme soluble de PH20 humaine exprimée de manière recombinante dans des cellules d'Ovaire de Hamster Chinois (CHO).

2. La composition de la revendication 1, dans laquelle la température dans l'étape a) est de 37 °C.

3. La composition de la revendication 1 ou 2, dans laquelle la température dans l'étape c) est de 36,5 °C.

4. La composition de l'une des revendications 1 à 3, dans laquelle la température dans l'étape d) est de 36 °C.

5. La composition de l'une des revendications 1 à 4, dans laquelle la température dans l'étape e) est de 35,5 °C.

6. La composition de l'une des revendications 1 à 5, dans laquelle le liquide de culture cellulaire de récolte est filtré avant la purification.

7. La composition de l'une des revendications 1 à 6, dans laquelle la purification de la rHuPH20 soluble est effectuée par chromatographie sur colonne.

8. La composition de la revendication 7, dans laquelle la chromatographie sur colonne comprend la chromatographie sur colonne d'agarose réticulé perlé, la chromatographie sur colonne d'agarose phényl-substitué réticulé perlé, la chromatographie sur colonne d'acide aminé phényl boronate et la chromatographie sur colonne d'hydroxyapatite.

9. La composition de l'une des revendications 1 à 8, dans laquelle le milieu d'alimentation est ajouté à la culture à un volume de 4 % du volume de la culture cellulaire.

10. La composition de la revendication 1, dans laquelle :

dans l'étape a), les cellules sont cultivées à 37 °C ;

dans l'étape b), le premier milieu d'alimentation contient 33 g/L de glucose, 32 mM de L-alanyl-L-glutamine, 16,6 g/L d'extrait de levure et 33 mg/L d'insuline, et le milieu d'alimentation est ajouté à la culture à un volume correspondant à 4 % du volume de la culture cellulaire ;

dans l'étape c), le second milieu d'alimentation contient 33 g/L de glucose, 16 mM de L-alanyl-L-glutamine, 33,4 g/L d'extrait de levure et 0,92 g/L de butyrate de sodium, et le milieu d'alimentation est ajouté à la culture à un volume correspondant à 4 % du volume de la culture cellulaire ; et

la température est abaissée à 36,5 °C ;

dans l'étape d), le troisième milieu d'alimentation contient 50 g/L de glucose, 10 mM de L-alanyl-L-glutamine, 50 g/L d'extrait de levure et 1,8 g/L de butyrate de sodium, et le milieu d'alimentation est ajouté à la culture à un volume correspondant à 4 % du volume de la culture cellulaire ; et

la température est abaissée à 36 °C ;

dans l'étape e), le quatrième milieu d'alimentation contient 33 g/L de glucose, 6,6 mM de L-alanyl-L-glutamine,

50 g/L d'extrait de levure et 0,92 g/L de butyrate de sodium, et le milieu d'alimentation est ajouté à la culture à un volume de 4 % du volume de la culture cellulaire ; et la température est abaissée à 35,5 °C ; comprenant en outre la filtration du liquide de culture cellulaire de récolte obtenu dans l'étape g) et

5 dans l'étape h), la purification de la rHuPH20 à partir du liquide de culture de récolte par chromatographie sur colonne d'agarose réticulé perlé, par chromatographie sur colonne d'agarose phényl-substitué réticulé perlé, par chromatographie sur colonne d'acide aminé phényl boronate ou par chromatographie sur colonne d'hydroxyapatite.

10 **11.** La composition de la revendication 1, dans laquelle :

dans l'étape a), les cellules sont inoculées à une densité cellulaire de 4×10^5 cellules/mL ; et les cellules sont cultivées à 37 °C ;

15 dans l'étape b), le premier milieu d'alimentation contient 33 g/L de glucose, 32 mM de L-alanyl-L-glutamine, 83,3 g/L d'extrait de levure et 33 mg/L d'insuline, et le milieu d'alimentation est ajouté à la culture à un volume de 4 % du volume de la culture cellulaire ;

dans l'étape c), le second milieu d'alimentation contient 33 g/L de glucose, 13 mM de L-alanyl-L-glutamine, 166,7 g/L d'extrait de levure et 0,92 g/L de butyrate de sodium, et le milieu d'alimentation est ajouté à la culture à un volume de 4 % du volume de la culture cellulaire ; et

20 la température est abaissée à 36,5 °C ;

dans l'étape d), le troisième milieu d'alimentation contient 50 g/L de glucose, 10 mM de L-alanyl-L-glutamine, 250 g/L d'extrait de levure et 1,8 g/L de butyrate de sodium, et le milieu d'alimentation est ajouté à la culture à un volume de 4 % du volume de la culture cellulaire ; et la température est abaissée à 36 °C ;

25 dans l'étape e), le quatrième milieu d'alimentation contient 33 g/L de glucose, 6,7 mM de L-alanyl-L-glutamine, 250 g/L d'extrait de levure et 0,92 g/L de butyrate de sodium, et le milieu d'alimentation est ajouté à la culture à un volume de 4 % du volume de la culture cellulaire ; et la température est abaissée à 35,5 °C ; comprenant en outre la filtration du liquide de culture cellulaire de récolte obtenu à l'étape g) et

30 dans l'étape h), la purification de la rHuPH20 à partir du liquide de culture de récolte par chromatographie sur colonne d'agarose réticulé en billes, par chromatographie sur colonne d'agarose phényl-substitué réticulé en billes, par chromatographie sur colonne d'acide aminé phényl boronate ou par chromatographie sur colonne d'hydroxyapatite.

35 **12.** La composition de l'une des revendications 1 à 11, dans laquelle au moins 0,5, 1, 5, 10, 15, 20, 25, 30, 35 ou 40 grammes de rHuPH20 soluble sont produits par 100 L de culture cellulaire.

13. La composition de l'une des revendications 1 à 12, dans laquelle l'activité spécifique de la rHuPH20 soluble est d'au moins 80000, 100000, 120000, 140000, 160000 ou 180 000 unités/mg.

40 **14.** La composition de l'une des revendications 1 à 13, dans laquelle le volume de la culture cellulaire dans le bioréacteur est de 200, 300, 400, 500, 1000, 1500, 2000, 2500, 3000 ou 3500 litres.

45 **15.** La composition de l'une des revendications 1 à 14, dans laquelle les cellules qui codent pour la rHuPH20 soluble sont des cellules CHO DG44.

16. La composition de l'une des revendications 1 à 15, dans laquelle la rHuPH20 est codée par l'acide nucléique présenté dans SEQ ID NO:47.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 2004268425 A1 [0003]
- US 795095 [0090] [0094]
- US 11065716 B [0090] [0094]
- US 11238171 B [0090] [0094]
- US 5292509 A [0098]
- US 6745776 B [0100]
- WO 198802261 A [0107]
- US 20040268425 A [0120]
- US 20050260186 A [0120] [0186]
- US 20060104968 A [0120]
- WO 10795095 A [0189]
- WO 11065716 A [0189]
- WO 11238171 A [0189]
- US 61068622 B [0305]

Non-patent literature cited in the description

- *J. Biol. Chem.*, 1969, vol. 243, 3552-3559 [0040]
- Computational Molecular Biology. Oxford University Press, 1988 [0046] [0047]
- Biocomputing: Informatics and Genome Projects. Academic Press, 1993 [0046] [0047]
- Computer Analysis of Sequence Data. Humana Press, 1994 [0046]
- VON HEINJE, G. Sequence Analysis in Molecular Biology. Academic Press, 1987 [0046] [0047]
- Sequence Analysis Primer. M Stockton Press, 1991 [0046] [0047]
- CARILLO, H ; LIPTON, D. *SIAM J Applied Math*, 1988, vol. 48, 1073 [0046]
- Computer Analysis of Sequence Data, Part I. Humana Press, 1994 [0047]
- CARILLO et al. *SIAM J Applied Math*, 1988, vol. 48, 1073 [0047] [0048]
- PEARSON et al. *Proc. Natl. Acad. Sci. USA*, 1988, vol. 85, 2444 [0048]
- DEVEREUX, J. et al. *Nucleic Acids Research*, 1984, vol. 12 (I), 387 [0048]
- ATSCHUL, S.F. et al. *J Molec Biol*, 1990, vol. 275, 403 [0048]
- Guide to Huge Computers. Academic Press, 1994 [0048]
- NEEDLEMAN et al. *J. Mol. Biol.*, 1970, vol. 48, 443 [0048]
- SMITH ; WATERMAN. *Adv. Appl. Math.*, 1981, vol. 2, 482 [0048]
- GRIBSKOV et al. *Nucl. Acids Res.*, 1986, vol. 14, 6745 [0048]
- ATLAS OF PROTEIN SEQUENCE AND STRUCTURE. National Biomedical Research Foundation, 1979, 353-358 [0048]
- *Biochem.*, 1972, vol. 11, 1726 [0076]
- FROST et al. *Anal Biochemistry*, 1997, vol. 251, 263-269 [0084]
- DANILKOVITCH-MIAGKOVA et al. *Proc Natl Acad Sci USA.*, 2003, vol. 100 (8), 4580-5 [0084]
- FROST et al. *Biochem Biophys Res Commun.*, 1997, vol. 236 (1), 10-5 [0084]
- DUBENSKY et al. *Proc Natl Acad Sci USA*, 1984, vol. 81 (23), 7529-33 [0102]
- FAVRE et al. *Gene Therapy*, 2000, vol. 7 (16), 1417-20 [0102]
- NETTELBLADT et al. *Am. Rev. Resp. Dis.*, 1991, vol. 139, 759-762 [0106]
- WALDENSTROM et al. *J. Clin. Invest.*, 1991, vol. 88 (5), 1622-1628 [0106]
- HA'LLGREN et al. *J. Exp. Med.*, 1990, vol. 171, 2063-2076 [0106]
- WELLS et al. *Transplantation*, 1990, vol. 50, 240-243 [0106]
- WALLANDER et al. *Transplant. Int.*, 1993, vol. 6, 133-137 [0106]
- HAELLGREN et al. *J Clin Invest*, 1990, vol. 185, 668-673 [0106]
- WALDENSTRDM et al. *Eur. J. Clin. Invest.*, 1993, vol. 23, 277-282 [0106]
- DE MAEYER et al. *Int. J. Cancer*, 1992, vol. 51, 657-660 [0107]
- PAWLOWSKI et al. *Int. J. Cancer*, 1979, vol. 23, 105-109 [0107]
- ST CROIX et al. *Cancer Lett*, September 1998, vol. 131 (1), 35-44 [0107]
- BAUMGARTNER et al. *Reg. Cancer Treat.*, 1988, vol. 1, 55-58 [0108]
- ZANKER et al. *Proc. Amer. Assoc. Cancer Res.*, 1986, vol. 27, 390 [0108]
- LAURENT et al. *Acta Neurol Scand*, September 1996, vol. 94 (3), 194-206 [0110]
- MACLEAN et al. *Science*, 1976, vol. 194 (4261), 199-200 [0113]
- KOLODIE et al. *Arterioscler Thromb Vase Biol.*, 2002, vol. 22 (10), 1642-8 [0114]

- **BJERMER et al.** *Br Med J (Clin Res Ed)*, 1987, vol. 295 (6602), 803-6 [0115]
- **FEW et al.** *Amer. J. Matern. Child Nurs.*, 1987, vol. 12, 23-26 [0116]
- **PAUL et al.** *J Hand Surg.*, 1997, vol. 22 (2), 219-21 [0116]
- **ELDER et al.** *Lancet*, 1980, 648-649 [0116]
- **PRZYSIECKI et al.** *PNAS*, 1987, vol. 84, 7856-7860 [0185]
- **HARVEY et al.** *J Biol Chem*, vol. 278, 8363-8369 [0185]
- **MAUM et al.** *Prot Sci*, vol. 14, 1171-1180 [0185]
- **FROST ; STEM.** *Anal. Biochem.*, 1997, vol. 251, 263-269 [0186]
- **DELPECH et al.** *Anal. Biochem.*, 1995, vol. 229, 35-41 [0186]
- **TAKAHASHI et al.** *Anal. Biochem.*, 2003, vol. 322, 257-263 [0186]